Mohammad Anwar Hossain
Sergi Munné-Bosch · David J. Burritt
Pedro Diaz-Vivancos · Masayuki Fujita
Argelia Lorence *Editors*

Ascorbic Acid in Plant Growth, Development and Stress Tolerance

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Editors
Mohammad Anwar Hossain
Department of Genetics and Plant Breeding
Bangladesh Agricultural University
Mymensingh, Bangladesh

David J. Burritt
Department of Botany
University of Otago
Dunedin, New Zealand

Masayuki Fujita
Department of Applied Biological Science
Kagawa University
Kagawa, Japan

Sergi Munné-Bosch
Department of Evolutionary Biology
Ecology and Environmental Sciences
University of Barcelona
Barcelona, Spain

Pedro Diaz-Vivancos
Department of Plant Biology
University of Murcia
Espinardo, Murcia, Spain

Department of Plant Breeding
CEBAS-CSIC
Espinardo, Murcia, Spain

Argelia Lorence
Arkansas Biosciences Institute
Arkansas State University
Jonesboro, AR, USA

Department of Chemistry and Physics
Arkansas State University
Jonesboro, AR, USA

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Preface

L-Ascorbic acid (AsA), vitamin C, is one of the most abundant water-soluble antioxidants in plants and animals. In plants, AsA serves as a major redox buffer and regulates various physiological processes controlling growth, development, signal transduction and stress tolerance. Recent studies on AsA biosynthesis, oxidation, recycling, transport and redox regulation have provided a wealth of information of the mechanisms associated with AsA-mediated plant growth and development, as well as plant stress tolerance. As plant-based foods constitute the principal source of AsA in the human diet, the possibility of increasing the AsA content of plants to improve their nutritional value has also received considerable attention in recent years. The importance of the relationship between reactive oxygen species (ROS) and AsA for regulation of cell signalling and metabolic processes has been well established. In contrast to the single pathway responsible for AsA biosynthesis in animals, plants use multiple pathways to synthesize AsA, and this reflects the metabolic diversity of plants and the importance of AsA for plant health. Any changes in the AsA content of plant cells can result in a diverse range of effects on growth, development and stress tolerance, as AsA is involved in redox signalling, cell cycle regulation, enzyme functioning and the expression of defence and stress-related genes. Being a major component of the Foyer-Halliwell-Asada cycle, AsA helps to modulate oxidative stress tolerance by controlling ROS detoxification, both alone and in cooperation with glutathione (GSH) and other antioxidants. While tremendous progress has been made over the last few years regarding the multiple roles of AsA in improving environmental stress tolerance, the manipulation of the AsA contents in plants as a means to improve stress tolerance and the nutritional value of some crop plants is still an evolving area of research.

In this book, *Ascorbate in Plant Growth Development and Stress Tolerance*, we present a collection of 19 chapters written by leading experts on the importance of AsA metabolism for plant growth, development and stress tolerance and for the production of nutritious plant-based foods. Chapter “Chemistry and Metabolism of Ascorbic Acid in Plants” addresses the chemical properties and redox reactions of AsA and its metabolism, including biosynthesis and degradation. Chapter “The Role of Ascorbate in Plant Growth and Development” describes the involvement of AsA

and its interaction with redox regulatory signalling networks during growth and development, at the cellular, tissue and organ levels, including its participation in cell cycle regulation, seed germination, embryogenesis, root and shoot meristem development and flowering. Chapter "Ascorbate as a Key Player in Plant Abiotic Stress Response and Tolerance" deals with the involvement of AsA metabolism in plants under abiotic stress and how to improve resilience to stressors in transgenic plants with altered enzymes involved in AsA biosynthesis and recycling. Chapter "Ascorbate Peroxidases: Crucial Roles of Antioxidant Enzymes in Plant Stress Responses" describes the physiological roles of ascorbate peroxidase isoforms in plants, by reviewing the findings of key biochemical, physiological and genetic studies. Chapter "Molecular and Functional Characterization of Monodehydroascorbate and Dehydroascorbate Reductases" addresses on the molecular properties, structural and enzymatic, of AsA-regenerating enzymes (monodehydroascorbate reductase, MDHAR and dehydroascorbate reductase, DHAR) and their biochemical functions. Chapter "Regulation of Ascorbic Acid Biosynthesis in Plants" focuses on the regulation of AsA biosynthesis at the transcriptional level by internal and environmental factors affecting the AsA contents of plants. Chapter "Ascorbate-Glutathione Cycle and Abiotic Stress Tolerance in Plants" addresses the role of the ascorbate-glutathione (AsA-GSH) cycle in regulating abiotic stress responses and tolerance. Chapter "Ascorbate-Glutathione Cycle and Biotic Stress Tolerance in Plants" describes the involvement of the AsA-GSH cycle in biotic stress tolerance, the role of compartment-specific changes in the AsA-GSH cycle activity in biotic stress signalling and the AsA- and GSH-dependent mechanisms that enable plants to respond to abiotic and biotic stress combinations. Chapter "Exogenous Ascorbic Acid Mediated Abiotic Stress Tolerance in Plants" gives an overview of physiological, biochemical and molecular effects of exogenous applications of AsA to various plant species under abiotic stress. Chapter "The Role of Ascorbic Acid in Plant–Pathogen Interactions" provides a broad picture of the mechanisms by which AsA interacts with key components of a complex network regulating both basal and induced resistance in different pathosystems. Chapter "Ascorbate Oxidase in Plant Growth, Development, and Stress Tolerance" discusses the roles of ascorbate oxidase in plant growth, development and stress tolerance, based on the current research findings, and examines additional roles the enzyme could play at the cellular level. Chapter "AsA/DHA Redox Pair Influencing Plant Growth and Stress Tolerance" gives an overview of the literature with an emphasis on the role of the AsA/DHA redox pair in plant growth and abiotic and biotic stress tolerance. Chapter "The Role of Plant High-Throughput Phenotyping in the Characterization of the Response of High Ascorbate Plants to Abiotic Stresses" describes the genetic engineering of AsA biosynthetic genes in relation to abiotic stress tolerance and the potential of high-throughput plant phenotyping (phenomics) to accelerate the characterization of phenotypes of model plants and crops with high AsA levels. Chapter "Physiological Role of Ascorbic Acid Recycling Enzymes in Plants" focuses on the primary structure and the expression of genes encoding plant MDHAR and DHAR isozymes, as well as their contributions to the regulation of AsA contents in the leaves and fruits of plants. This chapter also provides information about the roles of MDHAR and

DHAR in chloroplasts, the cytosol and guard cells in relation to plant stress tolerance. Chapter "Ascorbic Acid Biofortification in Crops" provides an overview of our current understanding of AsA metabolism in model species and the ever-expanding molecular genetic toolset that modern molecular breeders can use to improve the AsA contents of crop plants. Chapter "Evolution of the Metabolic Network Leading to Ascorbate Synthesis and Degradation Using *Marchantia polymorpha* as a Model System" explores the evolution of the metabolic network leading to AsA synthesis and degradation using *Marchantia polymorpha* as a model system. Chapter "Ascorbic Acid in Processed Plant-Based Foods" focuses on the use of conventional food processing techniques to stabilize/protect AsA levels in plant-based foods and how novel processing techniques, such as pulsed electric fields and high hydrostatic pressure processing, could improve the retention of biologically active AsA in plant-based foods. Chapter "Ascorbate Metabolism and Nitrogen Fixation in Legumes" deals with AsA metabolism in relation to biological nitrogen fixation, with special reference to the production of ROS and reactive nitrogen species (RNS) in nodules, the benefits of exogenous AsA on N_2 fixation and the metabolic pathways of AsA biosynthesis in plants and nodules. Chapter "Importance of Vitamin C in Human Health and Disease" describes the importance of AsA in human health and disease.

This book is intended to provide comprehensive and in-depth information for advanced students, teachers and plant scientists around the globe, who are working on AsA metabolism in relation to plant growth and development, stress tolerance and quality improvement in plant-based foods. The editors gratefully acknowledge the excellent cooperation of all the experienced and well-versed contributors, who cordially accepted our invitation and contributed to this book. We would also like to extend our thanks to Dr. Kenneth Teng and the editorial staff of Springer, New York, who enabled us to initiate this book project. We believe that the information covered in this book will make a sound contribution to this fascinating area of research.

Mymensingh, Bangladesh — Mohammad Anwar Hossain
Barcelona, Spain — Sergi Munné-Bosch
Dunedin, New Zealand — David J. Burritt
Murcia, Spain — Pedro Diaz-Vivancos
Kagawa, Japan — Masayuki Fujita
Jonesboro, AR, USA — Argelia Lorence

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About the Editors

Mohammad Anwar Hossain is a professor in the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh. He received his B.Sc. in agriculture and M.S. in genetics and plant breeding from Bangladesh Agricultural University, Bangladesh. He also received an M.S. in agriculture from Kagawa University, Japan, in 2008 and a Ph.D. in abiotic stress physiology and molecular biology from Ehime University, Japan, in 2011 through Monbukagakusho Scholarship. As a JSPS postdoctoral researcher, he has worked on isolating low-phosphorus stress-tolerant genes from rice at the University of Tokyo, Japan, in 2015–2017. His current research interests include the isolation and characterization of abiotic stress-responsive genes and proteins; physiological and molecular mechanisms of abiotic stress response and tolerance with special reference to oxidative stress, antioxidants and methylglyoxal metabolism and signalling; and generation of stress-tolerant and nutrient-efficient plants through breeding and biotechnology and cross-stress tolerance in plants. He has over 50 peer-reviewed publications and has edited 7 books, including this one, published by CRC Press, Springer, and Elsevier.

Sergi Munné-Bosch is a full professor in the Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Spain. He received his B.Sc. in biology and his Ph.D. in plant biology from the University of Barcelona. His research interests include antioxidants, stress and senescence in plants, including research not only in plant physiology but also in plant ecophysiology and agrifood biotechnology. Main interests include the biosynthesis and function of antioxidant vitamins, plant hormones and secondary metabolites, as well as seed dormancy

and germination, leaf, flower and whole-plant senescence and fruit ripening. He is also interested in plant responses to drought and adaptation to extreme temperatures, as well as the ecophysiology of invasive plants. He has co-authored 180 peer-reviewed SCI-indexed journal articles, 17 book chapters and 3 books. He is editor-in-chief of the journal *Environmental and Experimental Botany* from Elsevier.

David J. Burritt is an associate professor in the Department of Botany, the University of Otago, Dunedin, New Zealand. He received his B.Sc. and M.Sc. (Hons) in botany and his Ph.D. in plant biotechnology from the University of Canterbury, Christchurch, New Zealand. His research interests include oxidative stress and redox biology, plant-based foods and bioactive molecules, plant breeding and biotechnology, cryopreservation of germplasm and the stress biology of plants, animals and algae. He has over 100 peer-reviewed publications and has edited 3 books for Springer and 2 for Elsevier.

Pedro Diaz-Vivancos is a professor in the Department of Plant Biology, Murcia University (Murcia, Spain). He is also linked to the Plant Breeding Department at CEBAS-CSIC (Murcia, Spain). He received his B.Sc. in biological sciences from the University of Murcia and his Ph.D. in 2007 in agricultural and food technology from the University of Cartagena (Spain). He has worked during the period of 2008–2009 at Newcastle and Leeds Universities (UK) as a postdoctoral scientist on the role of glutathione in the regulation of cell proliferation and plant development. His main research interests are the study of the role of the antioxidative metabolism in plant development and in plant stress responses, including the induction of tolerance against environmental stresses in plum plants by increasing their antioxidant capacity by genetic engineering. He has over 40 peer-reviewed publications and 2 book chapters. He has edited two book volumes for Springer including this one.

Masayuki Fujita is a professor in the Department of Plant Science, Faculty of Agriculture, Kagawa University, Kagawa, Japan. He received his B.Sc. in chemistry from Shizuoka University, Shizuoka, and his M.Agr. and Ph.D. in plant biochemistry from Nagoya University, Nagoya, Japan. His research interests include physiological, biochemical and molecular biological responses based on secondary metabolism in plants under biotic (pathogenic fungal infection) and abiotic (salinity, drought, extreme temperatures and

heavy metals) stresses, phytoalexin, cytochrome P-450, glutathione *S*-transferase, phytochelatin and redox reaction and antioxidants. He has over 150 peer-reviewed publications and has edited 2 books for Springer including this one.

Argelia Lorence is the James and Wanda Lee Vaughn endowed professor in the Department of Chemistry and Physics at Arkansas State University (A-State) in Jonesboro, Arkansas, USA, where she also leads the A-State Plant Phenomics Facility. Lorence received a B.Sc. in biochemical engineering from Universidad Autónoma Metropolitana, Iztapalapa (UAM-I), Mexico. She earned M.S. and Ph.D. degrees in biotechnology from the Institute of Biotechnology of Universidad Nacional Autónoma de Mexico (UNAM). Prior to joining A-State, Lorence was a postdoctoral research associate at Texas A&M and at Virginia Tech. The most significant contribution Prof. Lorence has made to plant sciences has been the discovery of a novel biosynthetic pathway for ascorbate that involves *myo*-inositol as main precursor. Her laboratory uses *Arabidopsis* to better understand the role of various subcellular pools of ascorbate in plant physiology. In addition to *Arabidopsis*, her current models of study include rice, soybean, tobacco and maize. Her research interests include ascorbate metabolism, redox biology, plant phenomics and the study of plant's responses to stresses using high-throughput phenotyping approaches to develop crops with enhanced resilience. She has over 50 peer-reviewed publications and 3 patents and has edited special issues of peer-reviewed journals (e.g. *Functional Plant Biology*) and 3 books, one published by Humana Press and two published by Springer including this one.

Contributors

Melanie R. Abt Department of Biology, ETH Zurich, Zurich, Switzerland

Lucia M. Acosta-Gamboa Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

Adolfo Aguilar-Cruz Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico

Javier Alberto Miret Department of Evolutionary Biology, Ecology and Environmental Sciences, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain

Omar Altehua-Lopez Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico

Mario A. Arteaga-Vazquez Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico

Pierre Baldet Fruit Biology and Pathology Research Unit, INRA, Bordeaux, France

Carlos G. Bartoli Instituto de Fisiología Vegetal, CCT CONICET La Plata—UNLP, La Plata, Argentina

Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata, Argentina

Manuel Becana Estación Experimental de Aula Dei, CSIC, Zaragoza, Spain

Hatem Boubakri Laboratory of Leguminous, Centre of Biotechnology of Borj-Cédria, Hammam-Lif, Tunisia

John L. Bowman School of Biological Sciences, Monash University, Melbourne, VIC, Australia

Agustina Buet Instituto de Fisiología Vegetal, CCT CONICET La Plata—UNLP, La Plata, Argentina

Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Argentina

Simon E. Bull Department of Biology, ETH Zurich, Zurich, Switzerland

David J. Burritt Department of Botany, University of Otago, Dunedin, New Zealand

Viankail Cedillo-Castelán Instituto de Biotecnologia (IBT), Universidad Nacional Autonoma de Mexico (UNAM), Cuernavaca, Morelos, Mexico

Matthew Chisnall Department of Biochemistry, University of Otago, Dunedin, New Zealand

Joanna Chojak-Koźniewska Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

Genetically Modified Organisms Controlling Laboratory, Plant Breeding and Acclimatization Institute – National Research Institute, Błonie, Poland

Jarrod Creameans Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

David A. Dalton Biology Department, Reed College, Portland, OR, USA

Ana E. Dorantes-Acosta Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico

Muneharu Esaka Graduate School of Biosphere Sciences, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan

Dulce Flores-Martínez Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico

Yukichi Fujikawa Graduate School of Biosphere Sciences, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan

Tanya Gómez-Díaz Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico

Andrea Galatro Instituto de Fisiología Vegetal, CCT CONICET La Plata—UNLP, La Plata, Argentina

Hélène Gautier Plants and Cropping Systems in Horticulture Research Unit, INRA, Avignon, France

Gavin M. George Department of Biology, ETH Zurich, Zurich, Switzerland

Gustavo Gergoff Grozeff Instituto de Fisiología Vegetal, CCT CONICET La Plata—UNLP, La Plata, Argentina

Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Argentina

Bingru Huang Department of Plant Biology, Rutgers University, New Brunswick, NJ, USA

Takahiro Ishikawa Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane, Japan

Ana Jiménez Department of Stress Biology and Plant Pathology, CEBAS-CSIC, Murcia, Spain

Takayuki Kondo Graduate School of Biosphere Sciences, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan

Tomasz Kopczewski Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

Elżbieta Kuźniak Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

Sze Ying Leong Department of Food Science, University of Otago, Dunedin, New Zealand

Riddet Institute, Palmerston North, New Zealand

Tingting Liu Department of Food Science, University of Otago, Dunedin, New Zealand

Riddet Institute, Palmerston North, New Zealand

Department of Botany, University of Otago, Dunedin, New Zealand

Grecia Lopez-Ramirez Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico

Argelia Lorence Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

Department of Chemistry and Physics, Arkansas State University, Jonesboro, AR, USA

Richard Macknight Department of Biochemistry, University of Otago, Dunedin, New Zealand

New Zealand Institute for Plant and Food Research Ltd, Dunedin, New Zealand

Maren Müller Department of Evolutionary Biology, Ecology and Environmental Sciences, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain

Takanori Maruta Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane, Japan

Manuel A. Matamoros Estación Experimental de Aula Dei, CSIC, Zaragoza, Spain

Karina Medina-Jiménez Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico

Filis Morina Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia

Biology Centre of the Czech Academy of Sciences, Institute of Plant Molecular Biology, České Budejovice, Czechia

Nirman Nepal Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

Indrawati Oey Department of Food Science, University of Otago, Dunedin, New Zealand

Riddet Institute, Palmerston North, New Zealand

Ana Ortiz-Espín Department of Stress Biology and Plant Pathology, CEBAS-CSIC, Murcia, Spain

Michael E. Ruckle Molecular Plant Breeding, Institute of Agricultural Sciences, Zurich, Switzerland

Antonio Sánchez-Guerrero Department of Stress Biology and Plant Pathology, CEBAS-CSIC, Murcia, Spain

Satoshi Sano Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

Francisca Sevilla Department of Stress Biology and Plant Pathology, CEBAS-CSIC, Murcia, Spain

Marcela Simontacchi Instituto de Fisiología Vegetal, CCT CONICET La Plata—UNLP, La Plata, Argentina

Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Argentina

Rebecca Stevens Genetics and Breeding of Fruit and Vegetable Research Unit, INRA, Avignon, France

Marina Suekawa Graduate School of Biosphere Sciences, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan

Vincent Truffault Institute of Applied Research on Fruit and Vegetables, CTIFL, Nantes, France

Sonja Veljović-Jovanović Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia

Marija Vidović Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia

Yi Xu Department of Plant Biology, Rutgers University, New Brunswick, NJ, USA

Jessica P. Yactayo-Chang Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

Kazuya Yoshimura Department of Food and Nutritional Science, College of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi, Japan

Chemistry and Metabolism of Ascorbic Acid in Plants

Kazuya Yoshimura and Takahiro Ishikawa

Abstract Ascorbic acid (ascorbate/AsA/vitamin C) is one of the most abundant and versatile biomolecules in plants and animals. In particular, AsA is present in high concentrations in the chloroplasts and cytosol of plant species. In plants, AsA serves as a major antioxidant and enzyme cofactor and regulates various physiological processes including stress tolerance as well as growth, development, and signal transduction. To accomplish crucial physiological roles adequately, intracellular levels of AsA in plant cells must be tightly regulated. Recent studies have revealed the pathways for AsA biosynthesis and their regulation in plants. In addition, AsA is known to be utilized as a biosynthetic precursor in the formation of several organic acids. This chapter presents up-to-date information on the metabolic processes of AsA, including biosynthesis and degradation, which influence the intracellular concentration of AsA.

Keywords Ascorbic acid · Biosynthesis · Antioxidant · VTC · Redox

1 Introduction

Ascorbic acid (ascorbate/AsA) is a ubiquitous molecule in eukaryotes. AsA is an important antioxidant and has various metabolic functions owing to its redox potential in humans. However, AsA is not synthesized by humans and, therefore, it must be incorporated into the diet as "vitamin C." In plants, which are the main source of vitamin C for humans, AsA is the most abundant and naturally essential compound.

K. Yoshimura (✉)
Department of Food and Nutritional Science, College of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi, Japan
e-mail: k-yosi@isc.chubu.ac.jp

T. Ishikawa
Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane, Japan

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AsA plays an important role in many aspects of control not only of the cellular redox state and anti-oxidative activity in plant cells, but also of cell division and cell expansion, and plant development and growth as an enzyme cofactor (Noctor and Foyer 1998; Loewus 1999; Arrigoni and De Tullio 2000; Davey et al. 2000; Smirnoff 2000a; Smirnoff and Wheeler 2000; Conklin 2001; Smirnoff et al. 2001; Smirnoff and Gatzek 2004; Shigeoka and Maruta 2014). In addition, AsA has recently been recognized as having specific functions in redox signaling, such as the response to pathogen insults, the determination of flowering time, and the regulation of the expression of various genes involved in plant growth, hormonal signaling pathways, and stress defense networks (Pastori et al. 2003; Conklin and Barth 2004; Barth et al. 2006; Noctor 2006; Gao et al. 2011).

AsA is present in plant species in concentrations that range from an estimated 300 mM in the chloroplast stroma to less than 20 mM in other organelles (Smirnoff 2000b). Therefore, AsA mostly accumulates in photosynthetic organs but can also reach high concentrations in non-photosynthetic tissues. However, the subcellular concentration of AsA remains controversial since the concentrations in plant organelles have differed in respective reports owing to different methods utilized (Foyer et al. 1983; Rautenkranz et al. 1994; Zechmann et al. 2011). The levels of AsA varies between not only plant species but also different cultivars of the same plant species (Gest et al. 2013; Bulley and Laing 2016). In addition, AsA levels are different between the respective plant tissues and are influenced with a wide range of values by the growth environment. For example, AsA content is generally high in meristematic tissues, flowers or young fruits, root tips, and apices of stolons or tubers (Gest et al. 2013). Light is the most important environmental cue for regulating the biosynthesis of AsA and the accumulation of AsA in plant leaves is enhanced depending on light intensity and is suppressed in the shade (Bartoli et al. 2006; Dowdle et al. 2007; Yabuta et al. 2007). To accomplish its crucial physiological roles adequately and to prevent waste of the carbon source required for AsA synthesis, the levels of AsA in plant cells must be rapidly and tightly regulated. This is achieved at various levels, such as the regulation of gene expression and modulation of enzyme activities involved in the biosynthesis, degradation, and recycling of AsA depending on types of tissues and/or in response to developmental and environmental cues. In addition, subcellular compartmentation and intercellular transport of AsA maintain AsA concentrations in each organelle and tissue. This chapter focuses on the processes of biosynthesis and degradation of AsA that vastly influence the intracellular concentrations of AsA.

2 Chemical Properties and Redox Reaction of Ascorbic Acid

Structurally, AsA is related to hexose sugars and has the conjugated structure of the five-carbon lactone ring, containing an enediol group on C2 and C3. Delocalization of the π-electrons over the C2-C3 enediol group stabilizes the molecule and causes dissociation of the hydrogen of the C3 hydroxyl to become a monovalent anion at physiological pH values since pKa_1 is 4.13 ($pKa_2 = 11.6$). AsA can donate electrons

to a wide range of electron receptors (Gest et al. 2013). There are two experimental values for the redox potential of AsA, +0.06 V (Loach 1976) and +0.35 V (Zhang and Dong 2004). Matsui et al. (2015) computed that the redox potential for AsA ranges from +0.40 to +0.50 V, thus supporting the experimental value of +0.35 V. The redox potential of AsA means it interacts with hydroxyl radicals, singlet oxygen, superoxide, and also with oxidized glutathione and tocopherol radicals (Noctor and Foyer 1998; Gest et al. 2013). AsA can act directly to neutralize such reactive oxygen species (ROS) and also repair oxidized organic molecules, in certain cases more efficiently than glutathione (Tsuchiya et al. 1985; Niki 1991; Sturgeon et al. 1998). The first oxidation product, monodehydroascorbate (MDA), is very stable arising from the way that MDA can delocalize spare electrons around a central carbon ring and its three carbonyl groups (Bielski 1982). A MDA radical also undergoes spontaneous disproportionation with another MDA radical to create an AsA molecule and a non-radical dehydroascorbate (DHA) molecule (Smirnoff 2000b). AsA is regenerated from its oxidized forms by the action of specific reductases and electrons from glutathione, NADH, or NADPH in the AsA–glutathione cycle (Foyer and Noctor 2011) (Molecular structure of DHAR and MDHAR and their roles in modulating abiotic stress tolerance in plants).

3 Metabolism of Ascorbic Acid

3.1 Biosynthetic Pathways of Ascorbic Acid

All animals except for humans, non-human primates, and a few other mammals generate AsA. The committed step in the biosynthesis of AsA consists of a branch of the D-glucuronate pathway via the pentose phosphate pathway in which the stereochemistry of the carbon skeleton of the primary substrate glucose is inverted for AsA via uronate, D-glucuronate, L-gulonate, and L-gulono-1,4-lactone (Burns 1967; Nishikimi and Yagi 1996) (Fig. 1). In this pathway, the microsome-localized enzyme, L-gulono-1,4-lactone oxidase (L-GulLO), catalyzes the final step to produce AsA (Nishikimi and Yagi 1996). Compared to animal cells, plant cells can accumulate much higher levels of AsA (Foyer and Shigeoka 2011). Specifically, the AsA levels in leaf cells often exceed those in chlorophyll and represent over 10% of soluble carbohydrates (Noctor and Foyer 1998). After the first investigations of AsA biosynthesis in plants during the 1950s, the biosynthetic pathway of AsA has created a notable gap in our understanding of carbon metabolism in plants (Smirnoff and Wheeler 2000). However, since 1999 the AsA biosynthetic pathways in plants have been expeditiously uncovered using a combination of genetic and biochemical approaches (Conklin et al. 1999). Until now, three different pathways for AsA biosynthesis via D-mannose/L-galactose, D-glucuronate, and D-galacturonate in plant cells have been proposed (Wheeler et al. 1998; Valpuesta and Botella 2004; Hancock and Viola 2005a, b; Ishikawa et al. 2006a; Ishikawa and Shigeoka 2008) (Fig. 1), which implies that the biosynthetic pathway for AsA differs between animals, plants, and possibly certain species of algae.

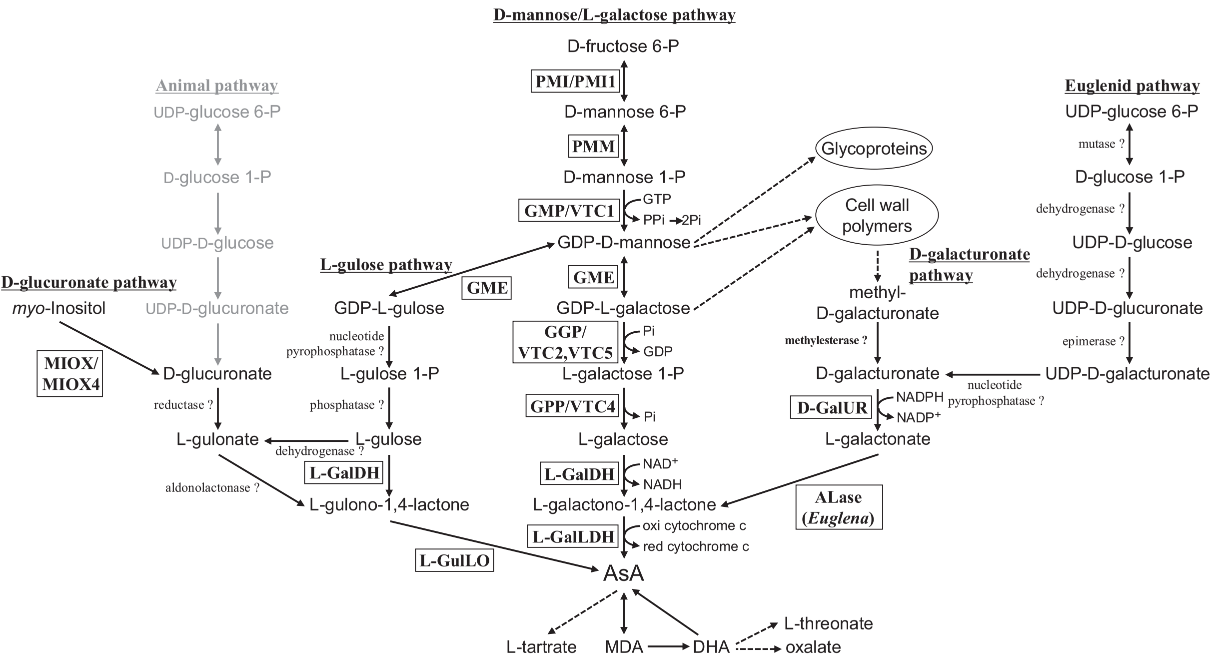

Fig. 1 The metabolisms of AsA in photosynthetic organisms. The D-mannose/L-galactose, D-galacturonate, euglenid, and D-glucuronate pathways, and the degradation pathways are shown. Animal pathway is shown by gray color. The names of enzymes/genes in the AsA biosynthetic pathways are indicated in rectangular boxes. ALase has been identified only from *Euglena*. Question marks indicate possible reactions where gene and specific enzyme have not yet been identified. Dotted line with arrowhead indicates that this reaction is composed of multistep. Among the enzymes involved in the AsA biosynthesis, L-GalLDH is localized to mitochondrial inner membrane, while others are in the cytosol. The conversion of AsA to oxalate via the formation of DHA occurs in the apoplast of cultured *Rosa* sp. cells. *PMI* phosphomannose isomerase; *PMM* phosphomannose mutase; *GMP* GDP-D-mannose pyrophosphorylase; *GME* GDP-D-mannose-3′,5′-epimerase; *GGP* GDP-L-galactose phosphorylase; *GPP* L-galactose-1-P phosphatase; L-*GalDH* L-galactose dehydrogenase; L-*GalLDH* L-L-galactono-1,4-lactone dehydrogenase; L-*GulLO* L-gulono-1,4-lactone oxidase; *MIOX myo*-inositol oxygenase; D-*GalUR* D-galacturonic acid reductase; *ALase* aldonolactonase

3.2 D-Mannose/L-Galactose Pathway

Since the sensitivity of plants to the oxidant ozone are correlated with levels of AsA, in previous studies, vitamin C-deficient (*vtc*) mutants were isolated from ethyl methanesulfonate (EMS)-mutagenized Arabidopsis seedlings that had ozone-sensitive phenotypes (Conklin et al. 2000; Dowdle et al. 2007; Kerchev et al. 2011). When several *vtc* mutants were identified, information on the properties and functions of the enzymes for the D-mannose/L-galactose pathway in a model plant, Arabidopsis, were uncovered (Conklin et al. 2000). The D-mannose/L-galactose pathway has been recognized as the predominant pathway in leaves of higher plants. In addition, the existence of D-mannose/L-galactose pathway has been reported for many fruit-bearing plants such as kiwifruits, acerola, apple, peach, citrus, and tomato (Badejo et al. 2009; Bulley et al. 2009; Imai et al. 2009; Ioannidi et al. 2009; Li et al. 2009) although the contribution of this pathway to the control of AsA levels during fruit ripening is still under debate. Therefore, it is most likely that the other pathways contribute to the tissue- and/or species-specific alternative AsA biosynthesis.

The homologs of all genes involved in the D-mannose/L-galactose pathway are also found in other plant species such as kiwifruit, acerola, tomato, rice, and maize (Badejo et al. 2008; Bulley et al. 2009; Ioannidi et al. 2009; Wheeler et al. 2015). Although AsA biosynthesis in non-vascular plants is poorly understood, it is known that bryophytes and green algae may contain 100-fold less AsA than higher plants contain (Gest et al. 2013; Wheeler et al. 2015), and green algae, bryophytes, and pteridophytes also probably use the D-mannose/L-galactose pathway (Running et al. 2003; Urzica et al. 2012; Wheeler et al. 2015). In this pathway, D-mannose is synthesized from hexose phosphates, such as D-glucose 1- or 6-phosphate and D-fructose 6-phosphate, produced by hexokinase and phosphoglucose isomerase in glycolysis, and proceeds via GDP-D-mannose and L-galactose, with the final aldonolactone precursor of AsA being L-galactono-1,4-lactone. This process does not involve inversion of the carbon chain of the primary substrate for AsA. The D-mannose/L-galactose pathway is composed of eight reaction steps catalyzed by phosphomannose isomerase (PMI), phosphomannose mutase (PMM), GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose-3′,5′-epimerase (GME), GDP-L-galactose phosphorylase/L-galactose guanylyltransferase (GGP), L-galactose-1-phosphate phosphatase (GPP), L-galactose dehydrogenase (L-GalDH), and L-galactono-1,4-lactone dehydrogenase (L-GalLDH) (Fig. 1). Except for L-GalLDH, which is localized in the inner membrane of the mitochondria, all remaining enzymes are localized in the cytosol, indicating that almost all predominant AsA biosynthesis steps occur in the cytoplasm of plants. The first four steps of the D-mannose/L-galactose pathway are shared with the synthesis of nucleotide sugars since GDP-D-mannose and GDP-L-galactose are precursors of cell wall polysaccharides and glycoproteins. Therefore, the committed pathway to AsA biosynthesis consists of four subsequent steps, by which GDP-L-galactose is sequentially converted to L-galactose-1-phosphate, L-galactose, L-galactono-1,4-lactone, and

AsA. The molecular and enzymatic properties and functions of these enzymes in higher plants, mainly in a model plant, Arabidopsis, are summarized below.

PMI—For the first step of this pathway in Arabidopsis, there are two enzymes, PMI1 and PMI2, which can catalyze reversible isomerization between D-fructose 6-phosphate and D-mannose 6-phosphate (Dowdle et al. 2007; Maruta et al. 2008). The K_m and V_{max} values for mannose 6-phosphate of the recombinant PMI1 have been shown to be 41.3 μM and 1.89 μmol min^{-1} mg^{-1} protein, respectively, whereas those of the recombinant PMI2 are 372 μM and 22.5 μmol min^{-1} mg^{-1} protein, respectively (Maruta et al. 2008). Both PMI1 and PMI2 are inhibited by EDTA, Zn^{2+}, Cd^{2+}, and AsA. The expression of PMI2 (also named DARK INDUCIBLE 9) has been detected in leaves only 24 h after dark treatment (Fujiki et al. 2001) although the levels of AsA increased under light as described above. On the other hand, the expression levels of PMI1 changed largely in parallel with leaf AsA levels during dark and light periods (Maruta et al. 2008). The analyses of Arabidopsis plants suppressing or depleting their expression have clearly shown that PMI1 is the sole enzyme for the AsA biosynthesis; that is, the approximately 70% decrease in PMI activity by the suppression of *PMI1* expression using the RNAi method caused an approximately 50% decrease in leaf AsA levels although the complete inhibition of *PMI2* expression was not affected (Maruta et al. 2008). However, conditional overexpression of the *PMI1* expression did not result in an increase in AsA levels (Yoshimura et al. 2014), indicating that the reaction catalyzed by this enzyme is not the limiting step in the D-mannose/L-galactose pathway. Conversely, since PMI was inhibited by high concentrations of AsA, feedback inhibition at this step of AsA biosynthesis has been shown to be involved in regulating intracellular AsA levels (Maruta et al. 2008).

PMM—PMM catalyzes reversible isomerization between D-mannose-6-phosphate and D-mannose-1-phosphate. The recombinant Arabidopsis PMM is able to catalyze the conversion of not only mannose 1-phosphate into mannose 6-phosphate but also glucose 1-phosphate into glucose 6-phosphate (Qian et al. 2007). However, the former reaction (V_{max}, 14.4 μmol min^{-1} mg^{-1} protein; K_m, 29.7 μM) was more efficient than the latter reaction (V_{max}, 1.40 μmol min^{-1} mg^{-1} protein; K_m, 65.4 μM). A decrease in the expression levels of *PMM* using a gene silencing method caused a substantial decrease in the AsA levels in the leaves of *Nicotiana benthamiana* (Qian et al. 2007). In contrast, an increase in the *PMM* expression levels led to a 20–50% increase in the AsA levels in *N. benthamiana* and Arabidopsis, suggesting that the step catalyzed by PMM has little effect on the overall rate of AsA biosynthesis. However, the activity of PMM in Arabidopsis leaves was remarkably high even under low light conditions (Dowdle et al. 2007).

GMP—GMP catalyzes the reversible conversion of GTP and D-mannose 1-phosphate into diphosphate and GDP-D-mannose; however, the substrate, diphosphate, is readily degraded into two phosphates and, therefore, this reaction is irreversible. The crystal structure of the recombinant GMP from Arabidopsis has been investigated (Zhao and Liu 2016). The *GMP* gene was first identified as the gene responsible for AsA-deficiency from the Arabidopsis *vtc* mutants and, therefore, was named *VTC1* (Conklin et al. 1996, 1999). This mutant had missense mutation

in the *GMP/VTC1* gene leading to an approximately 40% decrease in GMP activity and thereby accumulated only approximately 25% of the normal AsA levels. Similar to the result in Arabidopsis (Keller et al. 1999), the expression levels of *GMP/VTC1* were correlated with the AsA levels in several plant species (Badejo et al. 2007, 2008). These results suggest that some control of the AsA biosynthesis could occur at this step. However, conditional overexpression of the *GMP/VTC1* gene did not result in an increase in AsA levels (Yoshimura et al. 2014). The photomorphogenic factor, COP9 signalosome subunit 5B, has been shown to promote ubiquitination-dependent GMP/VTC1 degradation via the 26S proteasome pathway, which suppressed the overaccumulation of AsA under both light and dark conditions (Wang et al. 2013). This suggests the importance of degradation of the GMP/VTC1 enzyme in the regulation of AsA biosynthesis.

Since GDP-D-mannose is also an important intermediate in cell wall synthesis, protein *N*-glycosylation, and glycosylphosphatidylinositol-anchoring in plants (Lukowitz et al. 2001), the modulation of intracellular levels of GDP-D-mannose could be important for maintaining various cellular processes. In fact, the Arabidopsis mutant *cyt1*, which is a mutant allele of the *vtc1* mutants, showed not only lower AsA levels, but also more severe phenotypes, such as ectopic accumulation of callose and occurrence of incomplete cell walls, than the *vtc1* mutants (Lukowitz et al. 2001). Therefore, it is clear that the reaction catalyzed by this enzyme results in many downstream consequences. A relationship has been reported between the metabolism of GDP-D-mannose and sensitivity to ammonium (NH_4^+), a major nitrogen source for plants. The root growth of *vtc1* mutants was stunted in the presence of NH_4^+, whereas they developed roots similar to those of the wild-type plants in the absence of NH_4^+ (Barth et al. 2010). The *vtc1* mutants were also found to have *N*-glycosylation defects, enhanced programmed cell death, and some cell cycle defects in the presence of NH_4^+ (Qin et al. 2008; Kempinski et al. 2011). An Arabidopsis GDP-D-mannose pyrophosphohydrolase, AtNUDX9, which hydrolyzes GDP-D-mannose to guanosine monophosphate and mannose 1-phosphate, has been identified ($K_m = 376 \pm 24$ μM; $V_{max} = 1.61$ μmol min^{-1} mg^{-1} protein) (Tanaka et al. 2015). Therefore, it is suggested that AtNUDX9 and GMP/VTC1 constitute a futile cycle and function cooperatively in fine-tuning the GDP-D-mannose levels. Although the kinetic parameters of GMP/VTC1 have not yet been demonstrated, it is likely that the production capacity of GDP-D-mannose by the action of GMP/VTC1 is much higher than its hydrolysis by AtNUDX9, at least under normal conditions. In fact, no difference was observed in the levels of AsA in the wild-type and KO-*nudx9* plants, whereas a marked increase in *N*-glycoprotein levels and enhanced growth were detected in the KO-*nudx9* plants in the presence of NH_4^+ (Tanaka et al. 2015).

GME—GDP-D-mannose and GDP-L-galactose are precursors of cell wall polysaccharides and glycoproteins, which implies the importance of the equilibrium constant of the epimerization by GME. GME catalyzes the 3′ and 5′ epimerization of GDP-D-mannose, by which GDP-L-galactose is produced (Wolucka et al. 2001). However, GDP-L-galactose is not the only epimerization product. The native and recombinant GME from Arabidopsis also produces GDP-L-gulose as an

interconversion compound, which results from 5′-epimerization of the GDP-D-mannose (Wolucka and Van Montagu 2003). By the reaction of GME, GDP-L-gulose was in equilibrium with GDP-D-mannose and GDP-L-galactose (0.2:1.0:0.4). The K_m and V_{max} values for GDP-D-mannose of the native and recombinant GME in Arabidopsis have been shown as 4.5–31 μM and 0.31–1.76 μmol min^{-1} mg^{-1} protein, respectively (Wolucka and Van Montagu 2003). GDP-L-gulose can initiate an alternative branch (the L-gulose pathway) of the D-mannose/L-galactose pathway, suggesting that GME may impact on the flow of intermediates into these pathways. It has been demonstrated that exogenous L-gulose and L-gulono-1,4-lactone serve as direct precursors to AsA in certain plant species (Wolucka and Van Montagu 2003). In addition, the intermediate L-gulonate has been detected in plant cells (Wagner et al. 2003). As described below, L-GalDH can convert L-gulose into L-gulono-1,4-lactone, which might be sequentially oxidized to AsA by the reaction of L-GulLO. However, it remains unclear how the relative contribution of the L-gulose pathway to AsA biosynthesis occurs in vivo. The activity of GME in Arabidopsis leaves was remarkably low even under high light conditions (Dowdle et al. 2007). As described above, the recombinant GME has very low V_{max} compared to the other enzymes involved in the D-mannose/L-galactose pathway (Wolucka and Van Montagu 2003; Dowdle et al. 2007). In addition, a feedback inhibition of this enzyme by AsA has been observed (Wolucka and Van Montagu 2003). Therefore, it is suggested that this step is important in the limitation of subcellular AsA levels. However, conditional overexpression of the *GME* did not result in an increase in AsA levels (Yoshimura et al. 2014). On the other hand, transient co-expression of both the *GGP/VTC2* (known as the rate-limiting enzyme in the D-mannose/L-galactose pathway) and *GME* genes in tobacco leaves have been shown to result in an approximately eightfold increase in AsA levels, while the *GGP/VTC2* gene only resulted in an approximately fourfold increase. This suggests the importance of the step catalyzed by GME as the second rate-limiting step following GGP/VTC2 in the D-mannose/L-galactose pathway (Bulley et al. 2009).

GGP—GGP catalyzes the first committed step in the AsA biosynthesis via the D-mannose/L-galactose pathway. This enzyme converts GDP-L-galactose to L-galactose 1-phosphate. Among the ozone-sensitive AsA-deficient Arabidopsis *vtc* mutants, three mutants had a mutation in the gene encoding GGP, which was named *vtc2* (Conklin et al. 2000). Originally, it had been reported that one of the three mutant alleles, the *vtc2-1* that has complete loss of the GGP activity, had approximately 20–30% wild-type AsA levels and showed decreased growth under normal conditions, suggesting that 70–80% loss of AsA levels may affect plant growth. However, subsequent analyses using the other mutant (KO-*vtc2/vtc2-4*) with T-DNA insertion into the *GGP/VTC2* gene and segregation analysis of F2 progeny from crossing between the wild-type and *vtc2-1* mutants revealed that the *vtc2-1* mutants have an independent cryptic mutation affecting growth, and a 70–80% decrease in AsA levels (at least in Arabidopsis) does not cause growth inhibition (Lim et al. 2016).

There has been some controversy regarding whether the physiological guanylyl acceptor of the reaction by GGP/VTC2 is inorganic phosphate, which leads to a phosphorylase activity that generates GDP, or a hexose 1-phosphate such as D-

mannose 1-phosphate, which leads to a transferase activity that generates the corresponding GDP-hexose (Laing et al. 2007; Linster et al. 2007; Wolucka and Van Montagu 2007; Linster and Clarke 2008). Laing et al. (2007) and Wolucka and Van Montagu (2007) have proposed the VTC2 cycle, whereby L-galactose 1-phosphate is produced without the reactions by PMI, PMM, and GMP. However, suppression of the expression of *PMM* and *GMP* caused a decrease in AsA levels (Conklin et al. 1999; Qian et al. 2007), indicating the involvement of the steps producing GDP-L-galactose from D-fructose 6-phosphate during AsA biosynthesis. A dual radiolabeling experiment in Arabidopsis cell cultures indicated the in vivo involvement of PMI in GDP-D-mannose synthesis (Sharples and Fry 2007). In addition, GGP/VTC2 was discovered to be more than 100-fold more efficient as a GDP-L-galactose phosphorylase than as a GDP-L-galactose-D-glucose 1-phosphate guanylyltransferase (Linster et al. 2008). Therefore, at present, there is general agreement that GGP/VTC2 acts predominantly as a phosphorylase.

Arabidopsis also possesses the other gene (*VTC5*) that encodes the GGP enzyme. Judging from the results that AsA levels in the T-DNA insertion mutants, KO-*vtc2* and KO-*vtc5*, were approximately 20% and 80%, respectively, it is clear that GGP/VTC2 is predominant over GGP/VTC5 for AsA biosynthesis (Dowdle et al. 2007). The double KO mutant of *GGP/VTC2 and GGP/VTC5* genes showed seedling lethal without supplementation with AsA or L-galactose, indicating that the reaction catalyzed by GGP is indispensable for biosynthesis of AsA. The fact that the expression levels of *GGP/VTC2* and *GGP/VTC5* genes were relatively high in leaves and roots, respectively, suggests that GGP/VTC2 and GGP/VTC5 are important for AsA biosynthesis in photosynthetic and non-photosynthetic tissues, respectively (Dowdle et al. 2007).

The step catalyzed by GGP appears to have a major role in the regulation of AsA biosynthesis. The activity of GGP in Arabidopsis leaves was relatively low under both low and high light conditions (Dowdle et al. 2007). The expression of *GGP/VTC2* and *GGP/VTC5* is demonstrated to be in parallel with the subcellular AsA levels. For example, levels of both *GGP/VTC2* gene expression and AsA increased under light irradiation and their degree was facilitated depending on light intensity (Dowdle et al. 2007; Gao et al. 2011). On the other hand, there is feedback suppression of *GGP/VTC2* expression at post-transcriptional levels under high concentrations of AsA (Laing et al. 2015). The GGP/VTC2 mRNA has an upstream open reading frame (uORF) encoding a peptide, which may function in the inhibition of translation. Studies have shown that disruption of the uORF abolished the feedback regulation of GGP/VTC2 translation and increased the subcellular AsA levels, and high levels of ribosomes were colocated with both the uORF and the downstream open reading frame of GGP/VTC2. Therefore, it was proposed that the uORF translated preferentially rather than the downstream open reading frame of GGP/VTC2 under high AsA levels, leading to suppression of *GGP/VTC2* expression (Laing et al. 2015). The uORF is conserved in *GGP/VTC2* genes from mosses to angiosperms, implying its ubiquity. Yoshimura et al. (2014) reported that conditional overexpression of the *GGP/VTC2* gene without uORF markedly increased AsA levels in Arabidopsis seedlings, while that of the other

genes encoding GMP/VTC1, GPP/VTC4, GME, and PMI did not. In addition, the constitutive overexpression of the *GGP/VTC2* gene enhanced AsA levels in the leaves of tomato, strawberry, and potato plants (Bulley et al. 2012). These findings clearly indicate that GGP/VTC2 catalyzes the rate-limiting step in the D-mannose/L-galactose pathway, and the expression of *GGP/VTC2* is regulated to maintain proper levels of AsA in the cell.

As described above, all enzymes except for L-GalLDH are localized in the cytosol. However, Müller-Moulé (2008) demonstrated that a VTC2:YFP fusion protein is found not only in the cytosol where AsA biosynthesis occurs, but also in the nucleus, implying that VTC2 might have a dual function as the GGP enzyme and as a regulatory factor. Orthologs of the *GGP/VTC2* gene are found in invertebrates, vertebrates, and plants, and these genes show a low similarity with members of the histidine triad (HIT) protein superfamily, which are characterized as nucleotide-binding proteins and hydrolases (Linster et al. 2007; Müller-Moulé 2008).

GPP—GPP catalyzes a dephosphorylation reaction, by which L-galactose 1-phosphate is converted into L-galactose and inorganic phosphate. Originally, this gene had been annotated as a *myo*-inositol-1-phosphate phosphatase in GenBank. However, partially purified GPP from young kiwifruit (*Actinidia deliciosa*) berries and its recombinant proteins expressed by *E. coli* were very specific to L-galactose 1-phosphate. The activity was completely dependent on Mg^{2+}. The K_m value for L-galactose 1-phosphate of the partially purified enzyme was demonstrated to be 20–40 μM and a K_a for Mg^{2+} was 0.2 mM (Laing et al. 2004). Similar results were observed in the recombinant GPP from tobacco (*Nicotiana tabacum*) (Sakamoto et al. 2012). The *VTC4* gene, initially identified from the Arabidopsis *vtc* mutants (Conklin et al. 2000), encodes the GPP enzyme (Laing et al. 2004; Conklin et al. 2006). However, the *vtc4* mutants, which have a missense mutation in the *GPP/VTC4* gene causing complete loss of production of the encoded protein, is reported to be only partially decreased in AsA levels (by approximately 60%) as well as GPP activity (by approximately 40%) (Conklin et al. 2006; Torabinejad et al. 2009). In addition, GPP activity did not change under high light conditions although the expression levels of GPP/VTC4 mRNA as well as the AsA levels were correlated with light intensity (Dowdle et al. 2007; Yabuta et al. 2007). These facts suggest that GPP/VTC4 is not the sole enzyme catalyzing the GPP reaction in Arabidopsis. The purple acid phosphatase, AtPAP15, is a candidate for the remaining GPP activity in Arabidopsis since overexpression of the *AtPAP15* gene has been shown to increase AsA levels in the leaves of Arabidopsis (Zhang et al. 2008).

L-*GalDH*—L-GalDH catalyzes the conversion of L-galactose and NAD^+ into L-galactono-1,4-lactone and NADH. Antisense suppression of L-GalDH in Arabidopsis has been reported to result in a 70% decrease in expression levels and thereby the AsA levels decrease by 50% (Gatzek et al. 2002). In addition, the activity of L-GalDH in Arabidopsis leaves was remarkably high even under low light conditions (Dowdle et al. 2007). These results imply that this reaction is not a limiting step *in planta*. On the other hand, L-GalDH from spinach exhibited reversible competitive inhibition by AsA with a K_i of 0.13 mM (Mieda et al. 2004), sug-

gesting a feedback inhibition of AsA biosynthesis at this step. However, substantial accumulation of AsA was observed after L-galactose feeding (Wheeler et al. 1998) and overexpression of Arabidopsis L-GalDH in tobacco did not influence AsA levels (Gatzek et al. 2002). These results suggest that the inhibition of L-GalDH by AsA is absent or attenuated in vivo.

L-*GalLDH*—L-GalLDH is a flavoprotein and catalyzes the last step of the D-mannose/L-galactose pathway where AsA is produced from L-galactono-1,4-lactone (Leferink et al. 2008). The enzymatic properties of this enzyme in various plant species have been studied extensively (Oba et al. 1995; Østergaard et al. 1997; Imai et al. 1998; Yabuta et al. 2000). For example, the K_m values for L-galactono-1,4-lactone of the native and recombinant enzymes from tobacco have been stated as 0.08 mM and 0.06 mM, respectively, while the values for cytochrome *c* as the electron donor are 3.6 μM and 3.7 μM, respectively (Yabuta et al. 2000). The tobacco L-GalLDH has been shown to be extremely inhibited by incubation with *N*-ethylmaleimide, *p*-chloromercuribenzoate, and divalent cations such as Cu^{2+} and Zn^{2+}. Notably, the reaction of L-GalLDH involved electron transfer to cytochrome *c*, suggesting its association with the mitochondrial respiratory chain (Smirnoff et al. 2001; Hancock and Viola 2005a, b; Leferink et al. 2008). In fact, unlike the other enzymes in the D-mannose/L-galactose pathway, L-GalLDH has been reported to localize to the mitochondrial inner membrane as an integral protein (Siendones et al. 1999; Bartoli et al. 2000); however, it has also been suggested to be a peripheral protein (Leferink et al. 2008). Importantly, it has been reported that L-GalLDH is detected in a smaller form of complex I (NADH:ubiquinone oxidoreductase) (Heazlewood et al. 2003; Schertl et al. 2012; Schimmeyer et al. 2016). In addition, L-GalLDH has been demonstrated to be required for the accumulation of complex I since complex I was not detected in the KO mutants of the L-*GalLDH* gene (Pineau et al. 2008). From these results, it has been proposed that L-GalLDH is a dual-function protein that has a second, non-enzymatic function in complex I assembly as a plant-specific assembly factor (Schimmeyer et al. 2016).

The expression level and enzyme activity of L-GalLDH have been observed to positively correlate with AsA levels in various plant tissues (Smirnoff 2000b; Ishikawa et al. 2006a; Dowdle et al. 2007; Yabuta et al. 2007). However, an 80% decrease in L-GalLDH activity using the RNAi method in tomato did not cause any decrease in AsA levels, even though growth rates and fruit development of the RNAi plants were decreased and inhibited, respectively (Alhagdow et al. 2007). These effects on growth rates and fruit development have been demonstrated to be accompanied by significant changes in mitochondrial function and altered redox state of AsA. A combination of transcriptomic and metabolomic approaches have shown that several primary metabolic pathways, such as the tricarboxylic acid cycle, as well as secondary metabolic pathways related to stress response are modified in leaves and fruit of RNAi plants. These results suggest that the role of L-GalLDH in the complex I assembly is linked to plant metabolism rather than to AsA biosynthesis.

3.3 D-Galacturonate Pathway

The D-galacturonate pathway, which has been identified in strawberry fruits (*Fragaria x ananassa*), involves inversion of the carbon skeleton of the primary substrate glucose (Li et al. 2010) (Fig. 1). All the gene encoding enzymes that are involved in this pathway have not been fully identified yet. The strawberry D-galacturonate reductase (D-GalUR), encoded by *AKR2* that shared significant homology to cytosolic NAD(P)H-dependent aldo-keto reductases (AKRs), catalyzes production of L-galactonate from D-galacturonate (Agius et al. 2003). Among diverse cultivars of strawberry and different *Fragaria* species, a correlation between D-GalUR and AsA content was apparent in most cases (Cruz-Rus et al. 2011). The proteins most similar to AKR2 are a codeinone reductase from the opium poppy (*Papaver somniferum*) and chalcone reductases from soybean (*Glycine max*), alfalfa (*Medicago sativa*), and a tropical legume (*Sesbania rostrata*). However, this similarity is mainly restricted to the consensus motif characteristic of AKRs (Agius et al. 2003). Aldonolactonase (ALase) that is required to catalyze the conversion of L-galactonate to L-galactono-1,4-lactone has not been found. L-galactono-1,4-lactone is a substrate of L-GalLDH, which is the enzyme catalyzing the final step of the D-mannose/L-galactose pathway as described above. This pathway was activated during fruit ripening, demonstrating its importance in the accumulation with considerably higher levels of AsA in fruits. The precursor to the D-galacturonate pathway is likely to be either cell wall pectin or UDP-glucuronate epimerization to UDP-galacturonate (Agius et al. 2003; Di Matteo et al. 2010; Badejo et al. 2012). That is, this pathway could constitute a carbon salvage mechanism after the breakdown of cell walls in ripening fruits. However, the importance of the D-galacturonate pathway in the fruits of other plant species is largely unknown. A positive correlation between the AsA levels and the expression levels of genes involved in the D-mannose/L-galactose pathway has been found in the fruits of some plant species. For example, the levels of AsA and expression of *GMP*, *GGP/VTC2*, L-*GalDH*, and L-*GalLDH* in orange (*Citrus sinensis Osb.*) during fruit ripening were higher than those in Satsuma mandarin (*Citrus unshiu Marc.*) (Yang et al. 2011). Similarly, in the fruits of kiwifruit species (*Actinidia eriantha*, *A. chinensis*, and *A. deliciosa*), the rise in AsA levels during ripening occurred close to the peak in expression of *GGP/VTC2* and *A. eriantha* showed highest levels of both AsA and *GGP/VTC2* expression (Bulley et al. 2009). Therefore, it is reasonable that this pathway shares the control of fruit AsA levels with the D-galacturonate pathways depending on ripening stage and/or plant species (Melino et al. 2009; Cruz-Rus et al. 2011; Badejo et al. 2012; Gest et al. 2013). In fact, it has been demonstrated that tomato fruits (Micro-Tom) could switch between different sources for AsA supply depending on their ripening stages (Badejo et al. 2012). L-galactose feeding, but neither D-galacturonate nor L-gulono-1,4-lactone, enhanced the levels of AsA in immature green fruit. In contrast, L-galactose and D-galacturonate, but not L-gulono-1,4-lactone, resulted in an increase in the AsA levels of red ripened fruits. In addition,

crude extract prepared from insoluble fractions of green and red fruits showed the D-GalUR and ALase activities involved in the D-galacturonate pathway. Translocation of the precursor of AsA and/or AsA itself from leaves, in which AsA is synthesized predominantly by the D-mannose/L-galactose pathway, also impacts on the AsA levels of fruits (Badejo et al. 2012) (Ascorbate transporter in plants). Overexpression of *GalUR* in Arabidopsis resulted in a two- to threefold increase in leaf AsA levels, implying the existence of this pathway in plant leaves.

3.4 Euglenid Pathway

In the unicellular alga, *Euglena gracilis*, the biosynthesis of AsA is proceeded via the conversion of UDP-D-glucuronate to UDP-D-galacturonate, with production of D-galacturonate and reduction to L-galactonate (Shigeoka et al. 1979a) (Fig. 1). *Euglena* D-galacturonate reductase (D-GalUR) was demonstrated to catalyze the reduction of D-galacturonate to L-galactonate, which is subsequently converted to L-galactono-1,4-lactone (Shigeoka et al. 1979a; Ishikawa et al. 2006b). In addition, *Euglena* cells have ALase that shows significant sequence identity with rat gluconolactonase, a key enzyme for the production of AsA via D-glucuronate in animals (Ishikawa et al. 2008). *Euglena* ALase catalyzed the reversible reaction of L-galactonate and L-galactono-1,4-lactone. Although D-GalUR and ALase had almost the same catalytic efficiency with uronates, *Euglena* accumulated greater than two-fold amounts of AsA on supplementation with D-galacturonate than with D-glucuronate (Ishikawa et al. 2008) and *Euglena* ALase had higher activity with L-galacturonate than with L-glucuronate (Shigeoka et al. 1979a, b; Ishikawa et al. 2008). These findings indicate that the pathway via D-galacturonate/L-galactonate, which is analogous to both the animal D-glucuronate pathway and the plant D-mannose/L-galactose pathway and a hybrid of them, is predominantly utilized to produce AsA in *Euglena* cells. A similar pathway appears to be functional in some stramenopile algae (Helsper et al. 1982; Grün and Loewus 1984). This pathway involves inversion of the carbon chain of primary substrate. Wheeler et al. (2015) proposed that the host for certain eukaryote lineages, such as diatoms, haptophytes, and euglenids initially synthesized AsA via an animal-type pathway (involving inversion of the chain and L-GulLO) and that the red or green algal symbiont used a plant-type pathway (involving non-inversion of the carbon chain and L-GalLDH). However, neither pathway appears to operate in photosynthetic eukaryotes with secondary plastids, including *Euglena*, which instead uses a euglenid-type D-galacturonate/L-galactonate pathway. In such organisms, the endosymbiotic gene transfer of L-*GalLDH* from the symbiont may have resulted in the functional replacement of L-*GulLO* in the animal-type pathway of the host leading to a hybrid biosynthetic pathway involving inversion of the carbon chain that employed D-galacturonate rather than D-glucuronate as the intermediate to provide L-galactono-1,4-lactone as a substrate for L-GalLDH.

3.5 D-Glucuronate Pathway

The precursor to the D-glucuronate pathway without inversion of the carbon skeleton is likely to be either from UDP-glucose or from *myo*-inositol, which are converted to (UDP-) D-glucuronate (Lorence et al. 2004; Li et al. 2010) (Fig. 1). All enzymes involved in this pathway have not been fully identified yet; however, the Arabidopsis *myo*-inositol oxygenase (MIOX4) that catalyzes the production of D-glucuronate from *myo*-inositol has been characterized (Lorence et al. 2004). By this pathway, D-glucuronate appears to be converted to L-gulono-1,4-lactone, which is subsequently converted to AsA by L-GulLO, a homolog of an animal enzyme (Maruta et al. 2010). However, contribution of this pathway to the AsA biosynthesis *in planta* is controversial although overexpression of the *MIOX4* and L-*GulLO* genes have increased AsA levels of the transgenic plants (Jain and Nessler 2000; Agius et al. 2003; Lorence et al. 2004; Maruta et al. 2010).

3.6 Degradation of Ascorbic Acid

The balance between synthesis and degradation could affect the intracellular levels of AsA. There is evidence of a relatively high turnover rate of AsA in some plant tissues (Pallanca and Smirnoff 2000). AsA is not a stable metabolic end-product nor is limited to oxidation–reduction reactions that alter the balance of AsA to DHA. That is, one of the multifunction of AsA is as a biosynthetic precursor. It has been demonstrated that AsA is catabolized to form oxalate, L-threonate, and L-tartrate in certain plants (Loewus 1999; Bánhegyi and Loewus 2004; Debolt et al. 2007; Melino et al. 2009) (Fig. 1). Cleavage of AsA between carbon atoms 2 and 3 results in the formation of oxalate from carbon atoms 1 and 2, and L-threonate from carbon atoms 3 to 6 via L-idonate (Wagner and Loewus 1973; Debolt et al. 2007). L-Threonate may be further oxidized to form L-tartrate. The cleavage reactions may follow a number of steps involving delactonization, oxidation, and reduction. In the majority of plants, AsA degradation has been demonstrated to occur via DHA, yielding oxalate and L-threonate (Yang and Loewus 1975; Green and Fry 2005; Helsper and Loewus 1982; Saito et al. 1997; Bánhegyi and Loewus 2004; deBolt et al. 2004). It appears that the cleavage pathways of AsA are different depending on plant species; however, the entire cleavage pathways remain unresolved at present.

In Arabidopsis mature leaves, the loss or turnover of AsA has been demonstrated to be only about 2.5% of the pool per hour, while in embryonic axes of pea seedlings, the turnover is about 13% per hour (Conklin et al. 1997; Pallanca and Smirnoff 2000; Melino et al. 2009). In blackcurrant (*Ribes nigrum L.*), AsA turnover of fruits was low at 1.41% of the total AsA pool per hour during the early stage of ripening and was increased to excess of 3% per hour during late ripening stage (Hancock et al. 2007). The rate of AsA turnover in high oxalate or tartrate accumulating plants, such as in grape berries, is yet to be established (Melino et al. 2009).

L-Tartrate does not commonly accumulate in plants and the *in planta* function of accumulation of this compounds remains unclear. However, it is clear that L-tartrate is produced as the dominant organic acid in the vacuoles of grape berries (*Vitis vinifera*) although the berries do not accumulate large amounts of AsA in comparison to other fruits where fruit acidity is conferred by malic acid, citric acid, or AsA. L-Tartrate in grape berries has been found to increase during the initial 4 weeks post-anthesis and remain unchanged until maturation (Iland and Coombe 1988). Economically, L-tartrate plays a critical role in determining the suitability of grapes for use in winemaking; berry L-tartrate is largely responsible for controlling juice pH through L-tartrate addition during vinification, the winemaker can minimize oxidative and microbial spoilage, thereby promoting both organoleptic and aging potentials of the finished wine (Debolt et al. 2007). In grape berries, cleavage of the intermediate 5-keto-D-gluconate between carbon atoms 4 and 5 leads to L-tartrate formation, with the 2-carbon fragment of atoms 5 and 6 putatively recycled into central metabolic pathways (Saito and Kasai 1969, 1982; Saito et al. 1997; DeBolt et al. 2006; Melino et al. 2009). The synthesis of oxalate and L-tartrate has been found to occur within these organs (DeBolt et al. 2004).

While the accumulation of L-tartrate is limited to a handful of plant genera, oxalate is widely distributed and often forms crystals of calcium oxalate (Stafford 1959; Debolt et al. 2007). Oxalate-producing plants accumulate oxalate in the range of 3–80% (w/w) of their dry weight. Members of more than 215 plant families including many crop plants accumulate crystals within their tissues (McNair 1932; Nakata 2003). Crystals have been observed in virtually all the tissues of a plant, and they most often accumulate within the vacuoles of specialized cells called crystal idioblasts (Horner and Wagner 1995). In plants that accumulate significant amounts of calcium oxalate, this compound has been found to form raphides, needle-shaped crystals often clustered into bundles; this has been proposed to allow toxins to be secreted into the wounds produced upon contact with the crystal tips (Franceschi and Nakata 2005). In addition, accumulation of calcium oxalate crystals in plant tissues may be involved in regulation of cellular calcium levels and sequestration of toxic metals, and resistance to herbivory (Nakata 2003; Franceschi and Nakata 2005).

Green and Fry (2005) have demonstrated that the conversion of AsA to oxalate via the formation of DHA occurs in the apoplast of cultured *Rosa* sp. cells (Fig. 1). This pathway produces oxalate and L-threonate via the uncharacterized intermediate 4-*O*-oxalyl-L-threonate, which is formed from DHA and involves at least one new soluble esterase having hydrolysis activity toward the intermediate. The pathway can also operate non-enzymatically, accounting for approximately 50% of total AsA loss. Four steps in this pathway may generate peroxide; this may contribute to the role of AsA as a pro-oxidant, which is potentially capable of loosening the plant cell wall accompanying physiological processes such as fruit ripening and softening, and/or triggering an oxidative burst.

4 Conclusions

This chapter has highlighted recent knowledge of the biosynthetic and degradation pathways of AsA. The control of intracellular AsA levels in cells potentially involves regulation of biosynthesis, degradation, recycling, and transport of this compound. Current knowledge about recycling and transport of AsA, and regulation of its biosynthesis has been reviewed in other chapters.

There is no clear evidence for AsA biosynthesis in prokaryotes, suggesting that the ability to synthesize AsA evolved in eukaryotes. Wheeler et al. (2015) proposed that ancestral eukaryotes developed multiple antioxidant mechanisms to protect themselves from ROS derived from organelles. However, evolutionary events such as the transition to an aerobic environment and to life on land have exposed organisms to new and challenging environments. The requirement of a large amount of energy by aerobic metabolism in eukaryotes might lead to increased requirements for cellular antioxidants to protect the host cell from ROS. In addition, acquisition of a photosynthetic cyanobacterial endosymbiont in photosynthetic eukaryotes has resulted in a greatly increased requirement for cellular antioxidants to protect the host cell from ROS secreted by the plastid. Especially, plants must show particularly good adaptation capacity as they are unable to move and must withstand various environmental conditions. Via evolutionary processes, photosynthetic eukaryotes might have potentiated the capability of AsA biosynthesis to maintain proper redox states in the cells and evolved multiple functions of AsA. In addition, by accumulating in fruits and seeds, AsA can affect a plant's reproductive capacity via roles during the development of these organs and the seed dormancy and germination, in addition to ensuring seedling and plant survival in different environments (Gest et al. 2013).

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The Role of Ascorbate in Plant Growth and Development

Ana Ortiz-Espín, Antonio Sánchez-Guerrero, Francisca Sevilla, and Ana Jiménez

Abstract The participation of redox changes and antioxidants in the control of plant growth and development is important for the normal course of these physiological processes. Ascorbate (AsA) is a multifunctional molecule that is highly necessary for both plants and animals. It performs a variety of functions such as being a major redox buffer and cofactor for enzymes involved in multiple cell processes, including hormone biosynthesis, photosynthesis, and respiration as well as in the regeneration of other antioxidants. In addition to its well-known antioxidant properties, AsA is able to influence normal cell cycle progression and plant growth, and it takes part in signal transduction events. Reactive oxygen and nitrogen species and other antioxidants are also involved in these processes. This chapter focuses on the involvement of AsA and its interaction with redox regulatory signaling networks during growth and development at cellular, tissue, and organ levels, including its participation in cell cycle and proliferation, seed germination and during embryogenesis and meristem development in root and shoots, as well as during flowering. The book chapter seeks to supply valuable information of the current state of research on this multifaceted molecule in these processes at various organization levels.

Keywords Cell cycle · Cell elongation · Embryogenesis · Seed development · Root development · Shoot development · Flowering

1 Introduction

Ascorbate (AsA) is one of the main antioxidants in the cell, but its functions go beyond this in the cell metabolism, acting as a key regulator in plant growth and development. The presence of AsA in plants seems to be indispensable for survival and indeed, plants without ascorbate are inviable (Dowdle et al. 2007). The

A. Ortiz-Espín · A. Sánchez-Guerrero · F. Sevilla · A. Jiménez (✉)
Department of Stress Biology and Plant Pathology, CEBAS-CSIC, Murcia, Spain
e-mail: ajimenez@cebas.csic.es

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isolation of mutants in the biosynthetic AsA pathway containing less AsA or the application of a biosynthesis inhibitor like lycorine are useful tools for the study of plant development in a low AsA content environment. Some of the clearest evidence of the role of AsA in plant growth has been reported in *Arabidopsis thaliana* vitamin C (*vtc*) mutants. These plants have altered the AsA biosynthetic pathway, and hence levels of this antioxidant are lower than in wild-type (WT) plants. Interestingly, these mutants showed a slow-growth phenotype and accelerated senescence (Conklin et al. 1999; Veljovic-Jovanovic et al. 2001a; Pastori et al. 2003; Dowdle et al. 2007). On the other hand, the inhibition of the synthesis of AsA by lycorine reduces the rate of cell division which is complemented with the addition of AsA (Arrigoni et al. 1997b; Stasolla et al. 2008). The correlation between AsA and growth capacity has been reported in different plant tissues (Liso et al. 1988; Córdoba-Pedregosa et al. 2003). AsA pool and redox status together with GSH and other metabolites regulate cell cycle progression and the maturation of several organs, including seeds, roots, shoots, or flowers (Fig. 1).

Plant growth and development are strongly guided by phytohormones. AsA acts as a cofactor of dioxygenases in the biosynthesis of several phytohormones such as ethylene, gibberellins (GA), auxins (IAA), and abscisic acid (ABA). The endoge-

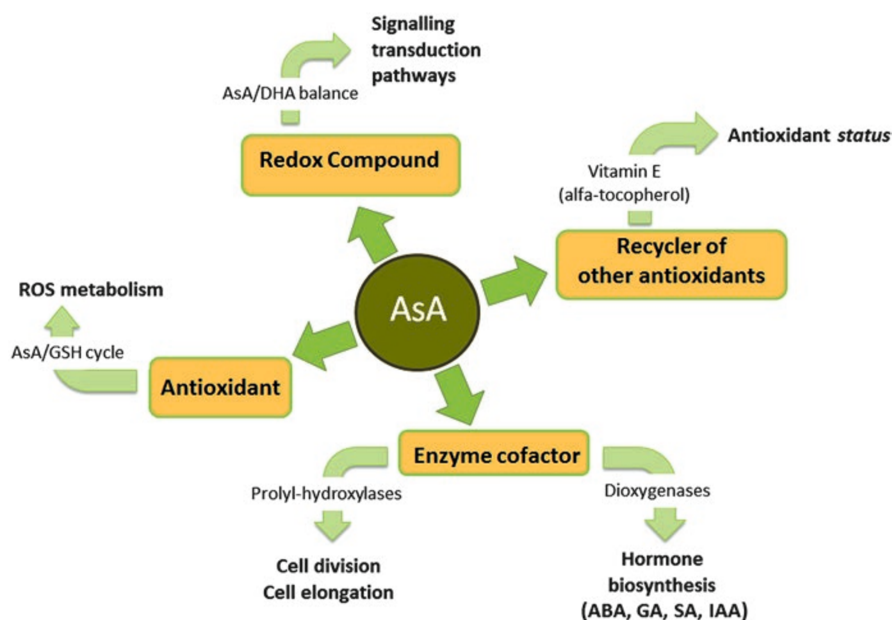

Fig. 1 Main roles of AsA in plant growth and development. AsA participates in different pathways leading to plant growth and development. AsA acts as an antioxidant, removing ROS through AsA-GSH cycle and it also stimulates the antioxidant capacity of the cell by recycling other antioxidants, such as vitamin E. The relative pool of reduced versus oxidized AsA forms perturbs the cell redox status, leading to different signaling transduction pathways. AsA also acts as enzyme cofactor of enzymes involved in cell growth and hormone biosynthesis

nous AsA influences the biosynthesis of phytohormones, as well as their signal transduction pathway. For example, some findings support the view that ABA and ABA signaling participate in the ascorbate-dependent control of growth. Arabidopsis mutants that show low levels of AsA induce high levels of ABA and hence, a restrained growth (Pastori 2003; Kerchev et al. 2011). IAA is also largely involved in the stimulation of plant growth, and it indirectly modifies the AsA redox status and consequently the growth state. These findings suggest that AsA is involved in the metabolic cross talk among redox-regulated pathways and hormones (Fig. 1).

All these aspects encourage us to deepen our knowledge of the role of AsA in the growth and plant development to unravel not only the biological significance of this key antioxidant in plants but also to increase the yields of our harvests (Paciolla et al. 2001).

2 Cell Cycle and Cell Elongation

There is a lot of evidence pointing to the level of AsA being a modulator of progression during the cell cycle. One of the earliest reports dealing with this topic is by Liso et al. (1984, 1988) in onion root cells. Roots are divided into different zones according to cell division and differentiation (see Sect. 5). Particularly studied are the cells from the quiescent center (QC) located in the root tip. These cells suffer a low rate of division and a larger G1 phase. The addition of AsA to the root tips leads to a shortening of the G1 phase and a prompted entry into the S-phase, stimulating the activity of the QC cells but also cell proliferation in the entire root meristem and in the pericycle (Liso et al. 1988; Arrigoni et al. 1989; Innocenti et al. 1990). In contrast, the inhibition of the AsA synthesis by lycoporine blocked the cell cycle in G1 and G2 phases, indicating a role for AsA in the cell cycle progression through the G1/S and G2/M transitions. A similar correlation was also observed during the growth of *Zea mays* (Kerk and Feldman 1995), *Lupinus albus* seedlings (Arrigoni et al. 1997a), pea roots (Citterio et al. 1994), and tobacco BY2 cells (de Pinto et al. 1999).

The state of competence in cells is an important issue when evaluating the effects of AsA in cell division. This state makes cells able to respond to antioxidant and hormones stimuli, which lead to cell cycle progression. Only in competent cells of embryo roots is the quick transition through the G1/S-phase checkpoint possible due to high levels of AsA. In some species, AsA does not promote cell cycle progression if cells are not competent (Arrigoni et al. 1989; Innocenti et al. 1990), suggesting that competence is a state which is necessary but not sufficient for cell cycling; the progression has also to be stimulated.

The influence of AsA redox status and AsA oxidized forms are also important in cell cycle progression. Ascorbate acts as an electron donor and is converted to a free radical intermediate monodehydroascorbate (MDHA) which presents a relatively short lifetime and can be spontaneously deprotonated to the final oxidized form, dehydroascorbate (DHA). The regeneration of these compounds to the reduced state is carried out by two reductases: MDHA reductase (MDHAR) using NADPH as

electron donor and DHA reductase (DHAR) which uses GSH as reductant. This last reaction generates glutathione disulfide (GSSG), which is in turn re-reduced to GSH by NADPH, a reaction catalyzed by glutathione reductase (GR). All these reactions are part of the so-called Foyer-Halliwell-Asada cycle (Foyer and Halliwell 1976), which together with ascorbate peroxidase (APX), is in charge of H_2O_2 scavenging. This cycle constitutes the main redox buffer in the cell (Jiménez et al. 1997; Foyer and Noctor 2011) and also a mechanism for the regeneration of the oxidized forms of ascorbate and glutathione, the main antioxidants in the plant cell (Noctor and Foyer 1998; Sevilla et al. 2015).

Changes in the AsA redox state (ratio AsA/DHA) have been observed during cell division (with a ratio of 6–10) and elongation (ratio of 1–3), indicating that the reduced state of AsA is predominant during cell division (Arrigoni et al. 1992). This correlation between AsA redox status and cell proliferation was later corroborated by (Kerk and Feldman 1995) in QC of maize cells, and by de Pinto et al. (1999) in tobacco BY2 cells. In this sense, sometimes the influence exerted by the oxidized forms of AsA on the cell cycle is not attributable to a rise in ascorbate concentration. As an example, De Cabo et al. (1993) demonstrated the action of MDHA and not AsA, in the shortening of the G1 phase in onion root meristem cells, so stimulating growth. Moreover, a specific inhibitory effect of DHA on the cell cycle have been proved (Cordoba and Gonzalez-Reyes 1994). In this context, Arrigoni et al. (1992) found that levels of DHA were higher in non-meristematic than meristematic zones and also that its contents in different tissues were rather related to its mitotic activity (Foyer and Mullineaux, 1998). A relationship exists between the levels of DHA and expression and activity of the enzymes DHAR (Arrigoni et al. 1997a; de Pinto et al. 1999; Potters et al. 2004) and ascorbate oxidase (AO) (Pignocchi et al. 2003). Levels of DHAR are higher in meristematic zones than expanding cells, allowing lower levels of DHA in these areas and stimulating an elevated rate of cell division (Potters et al. 2000). The DHAR enzyme uses GSH as cofactor, so levels of this antioxidant can also contribute to regulate DHA levels and hence, cell cycle. However, the importance of GSH in the reduction of DHA by Halliwell-Asada mechanism in the cell cycle is under debate. Similarly to AsA, GSH has a positive effect on the progression to cell cycle. Whereas high levels of GSH induce cell proliferation, high levels of GSSG block the cell cycle (Potters et al. 2004). Additionally, high ascorbate and glutathione levels are required for normal progression of the cell cycle in meristematic tissues (Potters et al. 2000; Vernoux et al. 2000; Jiang 2003).

Despite the fact that both AsA and GSH act in the cell cycle, they seem to exert their functions independently. It has been reported that GSH did not affect AsA pools and did not revert the effects of DHA over cell division (de Pinto et al. 1999; Potters et al. 2004). All this suggests that DHA reduction may be occurring in a glutathione-independent pathway and that both antioxidants act independently in the control of plant growth (Potters et al. 2004). In this sense, the enzymes thioredoxin reductase and glutaredoxin have been described as alternative reductive enzymes of DHA (Potters et al. 2002; Meyer et al. 2012).

The redox state in each cellular compartment is highly important in the cell cycle progression (Pignocchi et al. 2003; Martí et al. 2009; Diaz Vivancos et al.

2010; Calderón et al. 2017). It has been demonstrated that the redox state of the nuclei is an important factor, and GSH content is low at early G1 whereas it increases during the G1- and S-phases of the cell cycle (Diaz Vivancos et al. 2010). These redox fluctuations in the nucleus create an oxidative status early in the G1 phase followed by a reductive environment determined by an increase in GSH, necessary for cell cycle progression and to overcome the G1/S checkpoint (De Simone et al. 2017). The redox state of the cell at the G1-S is determined by *de novo* synthesis of GSH (Diaz Vivancos et al. 2010), and the level of GSH may directly modulate the synthesis of DNA after mitogenic stimulation (Suthanthiran et al. 1990; Poot et al. 1995). On the other hand, *vtc* mutants with lower ascorbate showed a higher growth due to a major oxidative stress and a slow growth under the addition of ascorbate (De Simone et al. 2017). Also, the importance of AsA during the logarithmic growth phase in tobacco cultured cells has been emphasized (Kato and Esaka 1999; De Pinto et al. 2000). Transient oxidations have been described in the current model of cell cycle regulation, in which ROS bursts serve to regulate key proteins thiol–disulfide exchange reactions at critical cysteine residues and, in this way, regulate cell cycle progression (Menon and Goswami 2007). In this redox control, GSH and thiol reductases such as thioredoxins have redundant roles in cell proliferation. As an example, TBY-2-overexpressing PsTrxo1 mutants in culture cells increased proliferation rate, particularly in the basal and stationary phase coinciding with an upregulation of proteins involved in DNA synthesis and an increase in nuclear GSH levels, but a decrease in total GSH pool (Ortiz-Espín et al. 2015; Calderón et al. 2017).

Several reports indicate that symplastic and apoplastic AsA play a relevant role in the control of cell division and proliferation (Arrigoni 1994; de Pinto et al. 1999; Horemans et al. 2000). The apoplast constitutes the space between the plasma membrane and the cell wall, and it plays an important role in the transition of cells from division to elongation (Cordoba and Gonzalez-Reyes 1994; Kato and Esaka 1999). Besides, being in contact with external environment, the apoplast constitutes the first line of defense against stressor and environmental clues (Hernández et al. 2001; Pignocchi and Foyer 2003). In the apoplast, AsA is oxidized to MDHA by AO. As we commented above, MDHA is an unstable radical and rapidly deprotonate to yield DHA and AsA. DHA is then transported into the cytosol by a specific carrier that preferentially translocates the oxidized form in exchange for the reduced form. In this way, a continuous flux of reducing power to the cell wall is ensured (Horemans et al. 2000), balancing AsA/MDHA levels in apoplast and symplast according to the needs of the cell. Changes in expression and activity of the apoplastic enzyme AO have been related with changes in DHA along the progression of the cell cycle (de Pinto et al. 1999; Pignocchi and Foyer 2003; De Pinto and De Gara 2004; Fotopoulos et al. 2006). The stimulation of AO activity provokes an increase in ascorbate oxidized forms which stimulates cell elongation without affecting cell division (de Pinto et al. 1999; Pignocchi et al. 2003; Li et al. 2017). The regulation of the ascorbate redox state in the apoplast is one of the mechanisms proposed to explain how ascorbate promotes cell expansion and elongation. An increase of AO is detected in rapidly elongating tissues and higher contents of MDHA and DHA are found in

these tissues. MDHA acts as an electron acceptor in transmembrane electron transport, where cytochrome B, transports electrons from NADH across the plasma membrane onto MDHA, which is reduced to AsA. The intensification of this process results in plasma membrane hyperpolarization which is followed by activation of plasma membrane H^+-ATPase. The activity of this enzyme provokes an acidification of apoplast that promotes cell wall loosening (González-Reyes et al. 1994). Simultaneously, the NADH oxidation enhances acidification in cytoplasm, activating vacuolar H^+-ATPase, which increases cell vacuolization and hence, cell expansion (González-Reyes et al. 1994; Horemans et al. 2000). Moreover, AsA can transform O_2 to H_2O_2 in the presence of $Cu^{2+\cdot}$ and by means of Fenton reaction generates $OH^{\cdot}$ radicals, which intensify the oxidative status in the apoplast. The acidification of apoplast triggers the oxidative degradation of polysaccharides (Fry 1998; Schopfer 2001) and delayed cell wall lignification, reducing precursors of peroxidase-dependent lignin synthesis (Takahama 1992; Padu 1999; Fig. 2).

Another mechanism points to the hydroxylation of proteins as a key factor of cell division and elongation. Ascorbate is a cofactor of the enzyme prolyl hydroxylase. This enzyme provokes the posttranslational hydroxylation of proline residues of cell wall-associated glycoproteins like extensins and arabinogalactan proteins, which are involved in cell wall stiffening, cell proliferation, and signaling (Cooper et al. 1994; De Tullio et al. 1999). The lack of AsA will prevent the hydroxylation of these proteins and hence, may block the cell cycle and in fact, it has been reported that a lower rate of hydroxylation of proline residues induced a cell cycle block during metaphase (De Tullio et al. 1999).

All the above evidence suggests the regulatory role of AsA pool and AsA redox status in the cell growth. In this sense, AsA redox status in the apoplast seems to be critical in the fate of the cell towards division or elongation.

3 Embryogenesis

The process of zygotic embryogenesis (ZE) in higher plants comprises the part of the development which leads to the formation of dry mature seeds and it is divided into three phases. The first stage is a process of morphogenesis, where the polar axis of the plant body is defined with the generation of the shoot and root poles, embryonic axis and cotyledons (West and Harada 1993). The second phase comprises the embryo maturation where storage reserves are accumulated and finally, the embryo becomes desiccated and the formation of the seed comes to an end. During the morphogenesis phase, AsA has been described as regulator of polarity. This process starts with the transverse division of a zygote into an apical, proembryo, and basal cells that gives rise to the suspensor. During this division, a reduced state of AsA promotes the transverse cell division, whereas an increase of DHA provokes a longitudinal division (Chen and Gallie 2012). The elongation of newly formed cells is favored by higher contents of DHA, which reduce AsA redox status (Belmonte and Yeung 2004). A declined in the reduced AsA carries on up to the end of seed

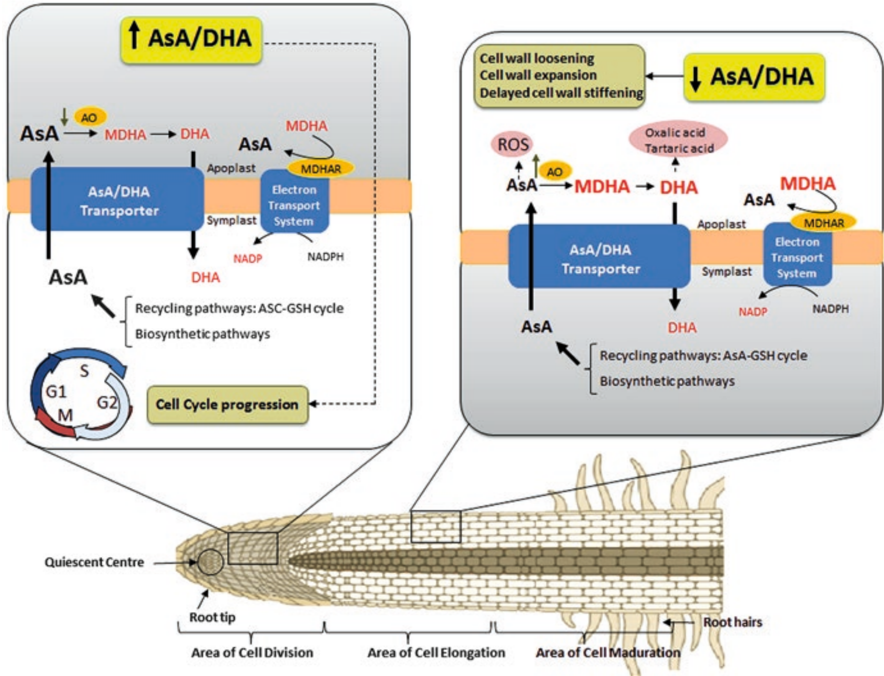

Fig. 2 AsA redox status in apoplast regulates cell cycle progression and cell wall elongation by antagonic mechanism in meristematic versus mature cells in root onion. According to acidic growth hypotheses, pH between apoplast and symplast regulates cell division and elongation. (1) In meristematic cells (area of cell division), high levels of AsA are correlated with low levels and activity of ascorbate oxidase (AO), which leads to a higher AsA/DHA ratio. This AsA redox status in apoplast enhances cell cycle, stimulating G1-S transition and consequently, cell division. (2) In differentiated cells (area of cell elongation), higher levels of ascorbate oxidase lead to an increase in AsA oxidized forms (MDHA) and products of secondary oxidations (DHA, oxalic acid, tartaric acid, ROS). Besides, the hyperpolarization of the plasma membrane induced by the oxidation of NADH triggers the activity of H^{+}ATPase, which further decreases the pH in apoplast. These processes decrease the AsA redox status and enhance oxidative degradation of wall polysaccharides (cell wall loosening), increasing vacuolar volume (cell wall expansion), and decreasing precursors of lignification (delayed cell wall stiffening). Consequently, cell wall elongation occurs

maturation, where almost all AsA is oxidized. Throughout this process, the role of DHAR is prevalent in controlling the AsA redox status. These results are in agreement with the general oxidative status observed in dry seeds. In this condition, it is easy to maintain a lower metabolic state and long-term storage (Tommasi et al. 2001).

Somatic embryogenesis is characterized by the growth of the plant from somatic cells or indirectly through an intermediate callus phase. The development of the new somatic embryos is reminiscent of that of ZE. This process is used in the production of genetically identical plants with desirable traits for agricultural and commercial applications. The role of AsA in the formation of somatic embryos has been

investigated using white spruce (*Picea glauca*) embryogenesis as a model system. Important changes in AsA metabolism have been observed in the maturation and germination processes in this species. During the first days of germination, embryos accumulate high levels of AsA with a high redox state and present a decrease in APX activity. Moreover, the inhibition of AsA synthesis by lycorine resulted in a lower DNA synthesis due to a decrease in the incorporation of purines and pyrimidines in nucleotides (Stasolla et al. 2008). Therefore, an increase in AsA pool and higher AsA/DHA ratio towards the reduced state is required for the progression of cell divisions within developing embryos (Stasolla and Yeung 2001). During the maturation of somatic embryos, AsA pool diminishes followed by a decrease in the ratio of AsA/DHA. The change from a reduced to an oxidized redox environment is favorable for the final development in the embryo. In fact, the application of glutathione biosynthesis inhibitor, buthionine sulfoximine, which turns GSH to GSSG, increased the quality of somatic embryos and their regeneration ability (Stasolla et al. 2008), inducing the expression of meristem formation genes such as *SHOOTMERISTEM/ZWILLE* and embryo maturation genes involved in ABA responsive and storage (*LEA* genes). Consequently, somatic embryos reach an appropriate morphology and physiology maturity, improving their post-embryonic growth (Stasolla and Yeung 2001; Stasolla et al. 2008; Stasolla 2010). On the other hand, a strict control of AsA pool must be exerted during the meristem formation, because an antagonic effect on development may be achieved above a threshold. The stimulating role of AsA during meristem formation in apical shoots involves inactivation of the peroxidase-dependent cell wall stiffening. An increase in AsA content decreases the activities of ferulic acid and guaiacol peroxidases allowing cell division and cell growth, while their activities increase in lycorine-treated embryos resulting in meristem abortion (Stasolla and Yeung 2007; Stasolla 2010). As another example, no apical dominance and early formation of auxiliary buds have been reported in tomato with suppressed AsA synthesis (Zhang 2012).

All these experiments demonstrate that the antioxidant pool and redox state of AsA and GSH are very important in the transition from cell division to maturity state during both zygotic and somatic embryogenesis. The knowledge of the mechanisms underlining the changes in these antioxidants will help us to unravel their specific role in the embryo development.

4 Seed Germination

A cell cycle of a seed involves three phases: development, maturation (see Sect. 3), and germination. Mature seeds are able to germinate under advantageous environmental conditions. The water imbibition of seed reactivates metabolism and the growth of seminal tissues. This process involves an increase of ROS and antioxidants including AsA, as well as redox proteins such as thioredoxin (Trx) or peroxiredoxin (Prx) (Simontacchi et al. 1993; De Gara et al. 1997; De Tullio and Arrigoni 2003; Martí et al. 2011; Ortiz-Espín et al. 2017). Oxidative imbalances can reduce

the potential for proper seed germination (Chen et al. 2014b) and so a tight regulation is required to balance ROS production and scavenging. AsA contents increase in the early stages of germination and this is correlated with an increase in the enzyme L-galactono-1,4-lactone dehydrogenase (GLDH), the last enzyme in AsA synthesis. In agreement with this, the DHA reduction capacity and APX activity decreases gradually (De Gara et al. 1991, 2000, Tommasi et al. 1999, 2001). Therefore, the higher AsA levels seem to be due to an *ex novo* synthesis rather than to AsA recycling (Tommasi et al. 1995, 2001). On the other hand, whereas in dry seed levels of GSSG and GSH are balanced, during germination the increase of GSH is accompanied by a strong decrease in GSSG and an increase in GR, indicating an increased recycling ability in the cell. Therefore, the opposite changes in DHAR and GR during germination indicate that GSH may have other functions not related with AsA-GSH cycle. In this sense, it has been demonstrated that the change in GSH redox status has a role in allowing protein synthesis by changes in the redox state of cysteine residues (Kranner and Grill 1993). In this regard, other thiol-redox proteins such as thioredoxins have been studied during germination (Ortiz-Espín et al. 2017). In this case, expression of promoter Trx::GUS in embryo axes and cotyledons coincided with AsA but not GSH, suggesting its role in redox regulation in these tissues rather than GSH and the existence of a feasible link between AsA and Trx (Ortiz-Espín et al. 2015). In fact, it has been reported in tobacco that DHA inhibits the activity of the enzymes regulated by the thioredoxin-peroxiredoxin system such as ribonuclease, involved in nucleotides synthesis (Morell et al. 1997), suggesting that AsA could stimulate cell proliferation (indirectly) through the Trx activity. Other evidences point to seeds of KO mutants of ascorbate oxidase and Trx under salt stress behaving in a similar way, causing early germination in salt imbibed seeds (Yamamoto et al. 2005; Ortiz-Espín et al. 2015). All these changes suggest that these components of the antioxidant and redox systems participate in the regulation of ROS levels and redox status during germination under stress conditions.

Seed germination is governed by an adequate ratio ABA/GA and during this process levels of ABA decreased in favor of GA which enhanced germination up to the seedling stage (Kucera et al. 2005; Finkelstein et al. 2008). The mechanism that directs this process seems to be related with ROS and AsA. The application of ABA to imbibed seeds provokes a decrease of ROS which leads to an inhibition of AsA production and a repression of GA biosynthesis. Moreover, GA biosynthesis was inhibited by lycorine, an inhibitor of AsA biosynthesis, and by reduced ROS levels, suggesting the role of AsA in this process. Additionally, the application of exogenous AsA can partially rescue seed germination from an ABA treatment (Ye et al. 2012). Therefore, AsA can control ABA/GA biosynthesis during seed germination.

Another interesting point is the influence of AsA in the germinating process by modulating the sensitivity to ABA and IAAs through APX6, which replaces APX1 as the dominant ascorbate peroxidase in dry seeds. The *apx6* defective mutant seeds showed an altered homeostasis of primary metabolites including accumulation of TCA cycle metabolites, ABA and IAAs and a repression of seed germination. These seeds respond to ABA or IAA in germination assays and show a repression of the ABA expression positive regulators, ABI3 and ABI5, but an induction of ABI4.

Therefore, the patterns of expression of these genes could be the reason for the inhibition of germination in *apx6* seeds in an ABI3-independent way (Chen et al. 2014a). These results imply the importance of APX6 activity for the seeds maturation-drying phase since it modulates the ROS signal cross talk with hormone signals to execute the germination program properly.

In short, all this evidence supports the role of AsA as an inductor of seed germination. AsA participates in a complex metabolic network integrated by ROS, hormones, and other antioxidants and redox proteins which regulate the development of the process.

5 Root and Shoot Development

AsA level is linked to root growth, development of root architecture, and root response to gravity (Olmos et al. 2006). The main evidence relating AsA with plant growth and development was obtained in onion root models (*Allium cepa* L.), (Liso et al. 1988). Roots can be divided into three parts according to the stage of differentiation: tip, medium zone, and base. Tip contains meristematic (apical meristem) and non-differentiated cells which include the quiescent center (QC), a group of cells where cell division proceeds very slowly or not at all, but the cells are capable of resuming meristematic activity if surrounding tissue is damaged. In the medium zone, the newly formed cells grow and elongate. Finally, at the base of the root, tissues are fully differentiated and hair roots and lateral roots emerge from this area (Fig. 2).

An analysis of AsA and AsA-GSH cycle partners inside the root provides deeper information about the requirements of cells according to their degree of differentiation. Studies of Córdoba-Pedregosa et al. (2003) showed a close relationship with the age of the tissue, which is related to the degree of differentiation and AsA contents. Whereas older tissues (base of root) have low AsA, younger tissues (tip root) have higher AsA levels, confirming the well-described relationship between AsA and cell proliferation (see Sect. 2). As stated above, AsA promotes cell division by inducing G1 to S progression of cells, and high AsA concentrations are required for normal progression of the cell cycle in meristematic tissues, including elongation of root tips in which AsA pool increases (De Pinto et al. 2000; Potters et al. 2000). In the QC of the root, the AsA content is substantially lower and AO mRNA and activity are found to be higher relative to the adjacent meristematic cells (Kerk and Feldman 1995). This condition enhances the oxidative status in apoplast, which blocks the cell cycle (Pignocchi 2006). As AO activity is auxin regulated (see Sect. 2), an involvement of this enzyme in the maintenance of QC cell identity has been suggested (Pignocchi 2006). AO-overexpressing tobacco plants showed high DHA in apoplast, which leads to an oxidation of the redox state in the apoplast and an insensitive response to IAA. The stimulation of ROS by auxin mediates plant growth reactions (Schopfer and Bechtold, 2002; Liszkay et al. 2004). Due to the high oxi-

dative status of the apoplast, auxin has minimal effect on oxidative signaling in these plants. On the other hand, AO has been shown to participate in the degradation of IAA by oxidative decarboxylation (Kerk et al. 2000), which suggests a role for AO in the regulation of auxin levels.

In addition, it is known that AsA and MDHA stimulate the elongation of onion roots and, simultaneously, high vacuolization of meristematic cells (Hidalgo et al. 1989; Cordoba and Gonzalez-Reyes 1994; González-Reyes et al. 1994, 1998). During root elongation, there is an increase in the cell volume by an intake of water by the protoplast and relaxation of the crossed bonds that link several components of the cell wall (McCann and Roberts 1994). In this process, AsA might facilitate elongation by inhibiting apoplastic peroxidases and activating hydroxylation of cell wall proteins as we commented on detail in Sect. 2 (Takahama 1992, 1993).

Knock out (KO) *vtc* Arabidopsis mutants deficient in AsA are very useful to explore the effects of low AsA content in the root and shoot development. Among the five *vtc* mutant lines (*vtc1-5*), only *vtc1* and *vtc2* showed changes in root growth, particularly in the length of primary roots and the presence of lateral roots (LR) (Conklin et al. 2000; Olmos et al. 2006; Dowdle et al. 2007; Tyburski et al. 2012). *Vtc2* mutant, which has 30% less AsA than *vtc1*, showed more and larger primary roots and a greater number of LRs than WT and *vtc1*. Besides, this mutant lost its gravitropism (Olmos et al. 2006) evidencing the importance of controlled oxidation in the tropic responses mediated by ROS and auxins (Joo et al. 2001). However, related to shoot development, both *vtc1* and *vtc2* showed shorter shoots than WT plants (Olmos et al. 2006). The main reasons for these differences seem to reside not only in the low levels of AsA, but also in its cellular location. *Vtc* mutants presented low levels of AsA in the apoplast (Veljovic-Jovanovic et al. 2001a) with the *vtc2* being the one with the lowest values. These experiments support the evidence that the redox state of the apoplast is essential in the modulation of plant growth responses (Olmos et al. 2006).

Another interesting aspect is the relationship between AsA and nutrients as regulators of root growth. It has been reported that MDHAR activity stimulates nutrient intake in Arabidopsis and is required for LR growth (González-Reyes et al. 1994; Foreman et al. 2003; Millar et al. 2003). Different studies using limiting sources of nitrogen and phosphorous have been carried out to analyze the effect on the shoot and root development and the participation of these nutrients in the signaling involved in the root architecture (Zhang and Forde 2000; Signora et al. 2001; Olmos et al. 2006). Low nitrate levels stimulate the formation of LRs in *A. thaliana*, whereas higher levels are inhibitory, and this effect is mitigated by increasing sugar, suggesting the antagonistic interaction between nitrate and sugar in the regulation of LR. However, levels of nitrate in *vtc1* and *vtc2* mutants did not alter the root architecture, suggesting that the enhanced LR growth in *vtc2* may depend entirely on an observed increase in ROS by the activation of the AtrbohC NADPH oxidase (Olmos et al. 2006). Nitrate could exercise its effect through ROS signaling and hence, the oxidative stress in *vtc2* mutant could attenuate the effect of nitrate. Interestingly, the short-root phenotype in *vtc1* has no relation with AsA deficiency

or the accumulation of H_2O_2 (Barth et al. 2010) and this mutant showed a disturbed protein N-glycosylation, which is associated with auxin and ethylene homeostasis and/or nitric oxide signaling (Barth et al. 2010).

Deficiencies in phosphorous (P) provoke a decreasing primary root elongation and development of abundant LRs (López-Bucio et al. 2002; Sánchez-Calderón et al. 2005, 2006; Nacry et al. 2005). Ascorbate can partially reverse the effects of P deficiency so increasing primary root elongation, although under high P levels, AsA inhibits the growth of primary roots. The differences in AsA content and redox state in the apical parts of primary roots of plants grown under various regimes of phosphate availability explain the observed differences. It has been suggested that the effects of AsA on root growth are possibly mediated by its effect on cell division activity in the apical root meristem. However, in these conditions, the behavior of *vtc1* mutant was found to be similar in the presence and absence of P (Tyburski et al. 2012), suggesting that other factors apart from AsA influence the root growth. A poorly adjusted auxin transport and signaling could lead to an auxin accumulation in the root apical meristem and provoke the arrest of root growth under low P availability (Sánchez-Calderón et al. 2005; Nacry et al. 2005).

The cross talk between hormones and redox status is emerging as a key regulatory point of control of plant growth and developmental processes. Emergence of LR is a highly coordinated process in which auxin plays a central role and, in fact, plants containing high levels of auxins produce more LRs than plants impaired in auxin signaling (Fukaki et al. 2007). The importance of auxins in AsA metabolism was also demonstrated by the observed increase in AO protein and transcripts in root tissues incubated with auxin (Pignocchi and Foyer 2003). In this context, it has been suggested that ROS, auxins and antioxidants like AsA and GSH form a redox signaling network are involved in plant development and response to adverse environments (De Tullio et al. 2010). ROS have been proposed as signaling actors during auxin-regulated LR formation although the underlined mechanisms are poorly understood. In *aux* mutants presenting a disruption in the auxin-mediated cell wall accommodation and remodeling in the sites of LR formation, ROS specifically are deposited in the apoplast during LR emergence (Orman-Ligeza et al. 2016). Also in tomato roots, auxin treatments increased the oxidized form DHA and induced root growth arrest but also provoked the production of LRs as an oxidative-mediated response (Tyburski et al. 2008). In Arabidopsis, auxin receptor (TIR1/AFB) mutants *tir1 afb2* presented an increased tolerance to salinity and displayed a reduced accumulation of H_2O_2 as well as enhanced antioxidant activity and showed higher levels of AsA than WT plants, indicating the involvement of auxin/redox interaction in the adaptation (Iglesias et al. 2010).

Therefore, these results indicate that the growth of primary and lateral roots is stimulated by antagonistic mechanisms. Whereas the growth of lateral roots seems to be stimulated by a high oxidative stress which is triggered by auxins and a low AsA availability, the growth of primary roots does not seem to depend on these factors.

6 Flowering

Timing of flowering is crucial for plant reproduction. The initiation of the process under unfavorable conditions can result in an aborted fruit development and hence, the plant will die without leaving offspring. Therefore, plants are able to activate or repress flowering in response to different environmental clues. One of the first indications of the relationship between flowering and environment was reported by J. Tournois (1914), who showed the strong influence of day/night length on flower induction. A century later, several papers have confirmed the influence of light (photoperiod, light quality, light intensity) in the transition from the vegetative to the reproductive state (Imaizumi et al. 2006; Johansson and Staiger 2014; Song and Shim 2015). Temperature is also an important factor and several plants need short exposure to low temperature (vernalization) to sense the end of the cold season (Lokhande et al. 2003; Thines et al. 2014; Airoldi et al. 2015; Capovilla et al. 2015). Other environmental factors such as drought (Kenney et al. 2014), levels of nutrients (Levy and Dean 1998), or CO2 concentration (Jagadish et al. 2016) are equally important. In addition, plants contain a set of internal factors such as hormones (ABA, GA) and antioxidants (AsA, GSH), which participate in the regulation of flowering (Ogawa et al. 2001; Barth et al. 2006; Chai et al. 2012; Weng et al. 2016). Therefore, both internal and external clues are sensed and integrated in the plant in order to establish properly the moment to flourish. In this context, several reports indicate that AsA can act as a repressor or activator of flowering depending on culture conditions such as photoperiod or growth media. Suppressed expression of the apoplastic AO gene delays flowering (Yamamoto et al. 2005) while exogenous addition of AsA or the precursor L-galactono-γ-lactone (GalL) in WT plants delayed growth and flowering in long-day-grown Arabidopsis plants (Attolico and De Tullio 2006) but induced growth and early flowering in short-day-grown plants (Barth et al. 2006). In the opposite case, Arabidopsis AsA-deficient *vtc* mutants showed an earlier flowering compared to WT plants and the addition of exogenous AsA delayed flowering (Conklin and Barth 2004; Kotchoni et al. 2009) irrespective of photoperiod. In this case, although AsA acts as a repressor of flowering, the pathway involved must differ from that suggested by Barth et al. (2006). Interestingly, these results contrast with data obtained by other authors, whose report a delayed instead of early flowering in *vtc1* mutants (Veljovic-Jovanovic et al. 2001b; Pastori 2003). However, in this case, the growth conditions differed, justifying the different behavior. Another set of evidence points to a lack of AsA oxidizing enzymes (APX or AO) as important elements in the regulation of flowering and mutants in genes that showed a delayed flowering with respect to WT (Pnueli et al. 2003). Consequently, all these experiments show that AsA (as repressor or activator) is related to time of flowering and that this is strongly governed by culture conditions. Besides, synthesis and recycling AsA pathways seem equally important in flowering. Nevertheless, the reason for the reported contrasting flowering phenotypes remains unresolved.

The transition of a plant from vegetative to reproductive stage increase the levels of antioxidants and ROS suggesting that plants must undergo an oxidative stress during the flowering process (Hirai et al. 1995; Lokhande et al. 2003; Shen et al. 2009). This oxidative stress has been hypothesized as being the cause of the early flowering in plants from lower latitudes (Lokhande et al. 2003). However, in *vtc* mutants, levels of H_2O_2 are similar to or slightly higher than in WT plants. In addition, the AsA redox state in the low AsA pool of these mutants remained unchanged, suggesting that oxidative stress and AsA redox state are not directly related with the early-flowering phenotype observed in *vtc* mutants. Curiously, other AsA mutants, which showed a delayed-flowering phenotype, such as the double mutant deficient in cytosolic and thylakoid AsA peroxidase (APX) exhibited early flowering under oxidative stress (Pnueli et al. 2003). Moreover, mutants lacking AO displayed both, higher levels of AsA and AsA redox status than WT plants in control conditions (Yamamoto et al. 2005). Therefore, flowering time can be explained by differences in AsA redox state and ROS levels, but only in some mutants. The relationship between the oxidative metabolism, AsA, and flowering has gone further with the studies of (Senthil Kumar et al. 2016) in orchids. The application of AsA to orchids significantly elevated the NO content and enzyme activities associated (nitrate and nitrite reductase). On the other hand, the addition of an NO donor on Arabidopsis *vtc1* mutant delayed flowering and decreased the expression level of some flowering-associated genes suggesting that NO signaling is vital for flowering repression (Senthil Kumar et al. 2016). In contrast, treatment with L-galacturonate of an NO-deficient *noa1* mutant did not alter its flowering time, suggesting that AsA is necessary for NO-biosynthesis involved flowering-repression pathway mediated by NO (Van Ree et al. 2011; Senthil Kumar et al. 2016). Therefore, although the role of AsA in the oxidative metabolism during flowering remains open, the direct implication in nitrosative metabolism is an interesting point of future research into the role of AsA in flowering pathways.

The relationship of AsA with flowering is reinforced when we analyze genetic data. Gene expression studies show that AsA affects the expression of flowering-related genes such as LEAFY, which encode a key transcription factor in flowering-inductive pathways (Attolico and De Tullio 2006). The addition of L-galactono-1,4-lactone to plants under long-day delayed expression of the LEAFY gene. In contrast, the application of GAs, an inductor of flowering (see below), engendered both, an early LEAFY expression and flowering. Later, a more integral study was performed by (Kotchoni et al. 2009). The gene expression analysis of *vtc* mutants showed an upregulation of genes related to flowering, circadian clock, and photoperiod pathway. In contrast, these genes were downregulated in the presence of AsA (Kotchoni et al. 2009), independently of photoperiod. Most important evidence points to AsAs possibly participating downstream in flowering pathways through expression of LEAFY genes, or upstream through circadian clock and photoperiod pathways, although its specific role remains inconclusive.

Flowering is a process intrinsically governed by hormones. It has been demonstrated that the application of GA or salicylic acid (SA) induces early flowering, whereas the application of ABA represses it. As commented in previous sections,

AsA participates as a key cofactor in the biosynthesis of hormones (Arrigoni and De Tullio 2000, 2002), so one would expect the influence of this antioxidant in flowering. Indeed, *vtc* mutants show high levels of ABA (Pastori 2003) and SA (Conklin and Barth 2004) and low contents of GA (Kiddle 2004; Foyer 2007) which is congruent with their delayed-flowering phenotype. The limiting activity of different GA-oxidases, which participates in the biosynthesis of GA (Kiddle 2004; Barth et al. 2006; Foyer 2007) could be responsible for low GA levels. Ascorbate acts as a cofactor of 2-oxoglutarate-dependent dioxygenases (2-ODDs) preventing their inactivation by over-oxidation (De Tullio et al. 2004; Clifton et al. 2006). On the other hand, an increase in NCED dioxygenase activity (Pastori 2003), enzyme involved in ABA biosynthesis pathways, may contribute to the late-flowering phenotype in short days. Surprisingly, these results seem to be contradictory because ABA needs AsA for their biosynthesis. However, Pastori speculates this behavior as a compensation mechanism for the decreased cofactor availability. Abscisic acid may (indirectly) contribute to the downregulation of LEAFY transcription factor, and hence to the delayed flowering.

All in all, a lot of evidence indicates the participation of AsA in flowering; the early or delayed phenotype observed in overexpression and AsA-deficient mutants respectively, the up/downregulation of genes related to flowering in the absence/presence of AsA and the changes in ABA/GA contents with AsA. Thus, the differences observed in the oxidative metabolism in the different mutants point to AsA being a relevant enzymatic cofactor, rather than antioxidant, in the flowering process.

7 Conclusions

The studies reported in this chapter indicate that AsA plays important roles in growth, differentiation, and metabolism. Ascorbate participates in plant growth-promoting cell division in meristems and cell elongation in differentiated tissues. Ascorbate participates in hormone biosynthesis and the AsA redox state is governed by recycling and oxidizing enzymes which respond to hormone stimulus. These elements are integrated in a complex metabolic network which leads to plant growth and development. The role of AsA is supplemented by other antioxidants and redox proteins such as GSH or thioredoxins which participate as signaling molecules and contribute to the redox state of the cell. In this regard, the manipulation of AsA/GSH redox state opens up vast possibilities for their use in the improvement of tissue culture and plant regeneration methods.

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Ascorbate as a Key Player in Plant Abiotic Stress Response and Tolerance

Sonja Veljović-Jovanović, Marija Vidović, and Filis Morina

Abstract During their lifespan, plants are frequently exposed to adverse environmental conditions such as high solar irradiance, drought, heat, chilling, salinity, metal excess, and nutrient deficiency. The effects of these factors on plants are often interrelated and usually result in a decreased capacity of carbon fixation in photosynthesis, disturbed redox homeostasis, and growth arrest. Under severe conditions, increased excitation pressure in the chloroplasts exceeds the antioxidative capacity of plant cells leading to oxidative damage of cellular constituents. Although the plant ascorbate (Asc) level varies depending on external factors, developmental stage, diurnal rhythm, and light, its redox status is related to redox homeostasis in the cell. In chloroplasts, peroxisomes, and cytosol, Asc has a key role in hydrogen peroxide (H_2O_2) scavenging via Asc peroxidase and is efficiently recycled via the ascorbate-glutathione (Asc–GSH) cycle and directly by monodehydroascorbate reductase activity. In apoplast and vacuoles, Asc is the main reductant of phenolic radicals generated under oxidative stress. Besides its antioxidative role, Asc has an important role in a complex and well-orchestrated plant response network to environmental stress, performing multiple tasks in redox signalling, regulation of enzymatic activities, modulation of gene expression, biosynthesis of phytohormones, and growth regulation. The content of Asc and its redox state is tightly related to cellular compartments. Therefore, it is important to emphasize Asc cellular distribution, which has a great impact on reactive oxygen species regulation and signalling. Numerous studies on transgenic plants with altered endogenous Asc levels and redox status were done with the aim to influence plant growth and improve tolerance to various abiotic stressors. In this chapter, we discuss the current understanding of

S. Veljović-Jovanović (✉) · M. Vidović
Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia
e-mail: sonjavel@imsi.rs

F. Morina
Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia

Biology Centre of the Czech Academy of Sciences, Institute of Plant Molecular Biology, České Budejovice, Czechia

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the involvement of Asc metabolism in abiotic stress response. Moreover, the improved resilience to stressors in transgenic plants with altered enzymes involved in Asc biosynthesis and recycling will be discussed.

Keywords Abiotic stress · Anthocyanins · Ascorbate · Cross-tolerance · Signalling · Subcellular localization of ascorbate

1 Introduction

Global climate changes have been recognized as the main threat to living organisms due to sudden and extreme fluctuations in temperature and high insolation accompanied by drought, floods, and altered availability of resources. To cope with the variable and frequently hostile environment, plants employ a vast array of adaptive responses that enable maintaining homeodynamic equilibrium within their phenotypic plasticity (Sultan 2000; Nicotra et al. 2010). When the variation of environment factors brings an organism near to, or over the limits of its ecological niche, these factors become stressors and determinants of plant performance, species distribution, and crop productivity. Understanding stress response and tolerance mechanisms is important for predicting and managing the effects of climate changes on native species and crops.

Disturbance of the redox balance resulting in an increased accumulation of ROS is an intrinsic feature of many, if not all environmental stresses. If the intensity of stress exceeds antioxidative and repair capacity of a cell, accumulated ROS cause oxidative modifications of macromolecules (i.e. oxidative damage) and promote apoptosis and senescence. Accumulation of ROS is a hallmark of cellular molecular response to a wide range of detrimental environmental conditions (light excess, high ultraviolet (UV) doses, drought, heat, chilling, and excess metals). On the other hand, low levels of ROS have an important role in signalling pathways that stimulate adaptive hormonal and metabolic responses. Whether ROS induce extensive cellular damage or trigger acclimation depends on their production site and yield, and is basically determined by antioxidants.

Ascorbate and GSH are central components regulating the level of ROS under abiotic stress in plants (Foyer and Noctor 2011). The content of total Asc in green leaves is higher than GSH, and the chlorophyll content, and more than 10% of total soluble sugars are in the form of Asc, which all illustrate its importance for plants. Due to its high antioxidative capacity, relatively low energetic cost of biosynthesis (Gest et al. 2012) and the existence of an efficient system for redox regeneration, Asc represents the first line of plant defence to oxidative stress.

Ascorbate is present in all plant cellular compartments, and its specific functions are compartmentalized within the cell. It performs multiple tasks such as the regulation of cell division, transcription, epigenetic modifications, cell wall extension,

seed germination, etc. (Noctor and Foyer 1998; Smirnoff 2008; Zhang 2013). Ascorbate is also a key player in cross-tolerance phenomena mediating environmental stimuli and hormonal responses, so that one type of stress enables a plant to respond efficiently to other stresses sharing the same tolerance mechanisms.

Ascorbate participates in two cycles in H_2O_2 scavenging, the classic water–water cycle in chloroplast (Asada 1999) and the Asc–GSH cycle (Foyer and Halliwell 1976; Foyer and Noctor 2011). In addition, the role of 2-Cys peroxiredoxins (PRXs) as major components in the water–water cycle was proposed as an alternative to thylakoid-bound ascorbate peroxidase (APX, EC 1.11.1.11) (Dietz et al. 2006). Ascorbate is the main reductant of phenolic radicals generated during H_2O_2 scavenging in a class III peroxidase/phenolics/Asc (PPA) system in apoplast and vacuoles (Takahama and Oniki 1997).

Numerous studies on transgenic and mutant plants with altered endogenous Asc levels obtained by genetic manipulation of genes involved in biosynthesis, catabolism, and recycling of Asc confirm the importance of Asc in stress tolerance (see references in Table 1.1). Arabidopsis vitamin C-deficient (*vtc*) mutants have increased sensitivity to abiotic stresses, such as high light (HL), UV-B radiation, extreme temperatures, high levels of ozone (O_3), and salt (Table 1.1; Conklin et al. 2000, 2013; Conklin and Barth 2004; Dowdle et al. 2007, Smirnoff 2011; Gallie 2013). References related to the effects of various stresses on ascorbate change induced by particular stress expressed as a percentage of increase/decrease in comparison to untreated control are listed in Table 1 and discussed in Sect. 2.

Despite the ever-growing interest in plant responses to oxidative stress and a great number of published research and review articles every year, certain ambiguities still exist about the mechanisms by which accumulated ROS mediate signals from the environment to specific targets in the plant cell, in spite of the highly efficient antioxidative system (Foyer and Noctor 2016). In this chapter, we discuss recent discoveries and present current understanding of the important role of Asc in a complex and well-orchestrated plant response network to abiotic stress.

2 H_2O_2 Scavenging and Ascorbate Recycling Under Abiotic Stress

Numerous metabolic reactions in plants are capable of producing H_2O_2, although photosynthesis and photorespiration have the highest capacity, especially favoured under HL, drought, or chilling stress (Noctor et al. 2002; Foyer and Noctor 2009). Under abiotic stresses, the level of H_2O_2 in plant tissues is generally elevated. However, the published values assessed in bulk plant tissues, even under optimal conditions, vary by more than three orders of magnitude (Willekens et al. 1997; Karpinski et al. 1999, Neill et al. 2002). The reason for such fluctuations in literature is more of a methodological nature than actual leaf H_2O_2 content (Cheeseman 2006; Shulaev and Oliver 2006; Slesak et al. 2007). For example, Veljović-Jovanović

Table 1 Effects of abiotic stresses on Asc content and Asc redox state (RsA) in wild type (WT) and transgenic/mutant plants and sensitivity to stress

Species	Stressor	WT and transgenic/ mutant plants	Initial Asc difference [%][#]	Stress-induced Asc change [%]*	Stress-induced RsA change [%]*	Growth [%]*	Tolerance	References
Arabidopsis thaliana	High light (HL), 1000 μmol m^{-2} s^{-1}	WT		↑45	n.s.		Decreased	Noshi et al. (2016)
		dhar3[1]	n.s.	↑33	n.s.			
	Excess light (EL), 1500 μmol m^{-2} s^{-1}	WT		↑31	n.s.			
		dhar3[1]	n.s.	↑19	↓15			
Nicotiana tabacum	HL, 2000 μmol m^{-2} s^{-1}	WT		↑25	n.s.			Chen and Gallie (2008)
		DHAR-KD[2]	n.s.	↑19	n.s.			
		DHAR-OX[2]	↑21	↑12	n.s.			
Arabidopsis thaliana	HL, 400 μmol m^{-2} s^{-1}	WT		↑64	n.r.			Gatzek et al. (2002)
		Anti-GalLDH4[3]	n.s.	↑60	n.r.			
		Anti-GalLDH73[3]	n.s.	↑63	n.r.			
		Anti-GalLDH18[3]	↑11	↑48	n.r.			
		Anti-GalLDH42[3]	↓20	↑44	n.r.			
		Anti-GalLDH32[3]	n.s.	↑54	n.r.			
Arabidopsis thaliana	HL, 1800 μmol m^{-2} s^{-1}	WT		↑42	n.s.	↓65	Decreased	Müller-Moulé et al. (2004)
		*vtc2***	↓95	↑92	↓9	↓64		
		vtc2-npq4[4]	↓90	↑84	↑15	↓64		
		vtc2-npq1[4]	↓92	↑87	↑21	↓71		

Arabidopsis thaliana	HL, 550–650 μmol m^{-2} s^{-1}	WT		↑52	n.r.			Page et al. (2012)
		*vtc1***	↓83	↑58	n.r.			
		*vtc2-1***	↓79	↑52	n.r.			
		*vtc2-2***	↓86	↑20	n.r.			
		*vtc3-1***	↓72	↑11	n.r.			
Arabidopsis thaliana	HL, 320 μmol m^{2} s^{-1}	WT		↑56	n.r.			Dowdle et al. (2007)
		*vtc2-1***	↓87	↑78	n.r.			
Arabidopsis thaliana	HL, 1000 μmol m^{-2} s^{-1}	WT		↑45	n.s.		Decreased	Giacomelli et al. (2007)
		sapx[5]	n.s.	↑48	n.s.			
		tapx[5]	n.s.	↑39	↑9			
		*vtc2-2***	↓82	↑38	↑12			
		tapx-sapx[5]	n.s.	↑47	n.s.			
		sapx-vtc2-2[5]	↓84	↑65	n.s.			
		tapx-vtc2-2[5]	↓82	↑50	↑18			
		sapx-tapx-vtc2-2[5]	↓89	↑67	n.s.			
	HL, 2000 μmol m^{-2}s^{-1}	WT		↑56	↓12			
		sapx[5]		↑46	↓34			
		tapx[5]		↑56	↓18			
		vtc2-2[5]		n.r.	n.r.			
		tapx-sapx[5]		↑56	↓20			
		sapx-vtc2-2[5]		n.r.	n.r.			
		tapx-vtc2-2[5]		n.r.	n.r.			
		sapx-tapx-vtc2-2[5]		n.r.	n.r.			

(continued)

Table 1 (continued)

Species	Stressor	WT and transgenic/ mutant plants	Initial Asc difference [%][#]	Stress-induced Asc change [%]*	Stress-induced RsA change [%]*	Growth [%]*	Tolerance	References
Arabidopsis thaliana	HL, 1000 μmol m^{-2} s^{-1}	WT		↑46	n.s.			Giacomelli et al. (2006)
		*vtc2***	↓85	↑56	↑10			
Arabidopsis thaliana	HL, 1000 μmol m^{-2} s^{-1}	WT		↑46	n.s.		Decreased	Müller-Moulé et al. (2003)
		npq1[4]	n.s.	↑38	n.s.			
		*vtc2***	↓86	↑20	↑15			
		vtc2-npq1[4]	↓93	n.s.	n.s.			
Arabidopsis thaliana	HL, 1800 μmol m^{-2} s^{-1}	WT		↑42	n.r.		Decreased	Talla et al. (2011)
		*vtc1***	↓72	↑50	n.r.			
Arabidopsis thaliana	HL, 1000 μmol m^{-2} s^{-1} and high temperature, 40 °C	WT		↑45	↓37		Increased	Wang et al. (2010)
		DHAR-OX[2]	↑67	↑11	n.s.			
	Paraquat, 10 μM	WT		↑41	↓11			
		DHAR-OX[2]	↑67	↑17	n.s.			
Nicotiana tabacum	Low temperature, 4 °C	WT		↓46	n.r.		Increased	Wang et al. (2011)
		T1(+)-6[6]	↑50	↓44	n.r.			
		T1(+)-3[6]	↑52	↓42	n.r.			
	High temperature, 40 °C	WT		↓48	n.r.			
		T1(+)-6[6]	↑51	↓49	n.r.			
		T1(+)-3[6]	↑51	↓40	n.r.			
Lycopersicon esculentum	Low temperature, 4 °C	WT		↑56	n.r.		Decreased	Duan et al. (2012)
		T_1-3[7]	↑31	↑59	n.r.			
		T_1-5[7]	↑33	↑67	n.r.			
		T_1-9[7]	↑17	↑61	n.r.			

Arabidopsis thaliana	Heat stress, 30 °C	WT		↑65	n.r.		Decreased	Conklin et al. (2013)
		*vtc 3-1***	↓73	↑59	n.r.			
		*vtc 3-2***	↓45	↑10	n.r.			
		vtc 3-knockout**	↓39	↑17	n.r.			
Arabidopsis thaliana	Sodium chloride, 100 mM	WT		↑45	n.r.	↓33	Increased	Lisko et al. (2013)
		MIOX4 L3[8]	↑19	↑42	n.r.	↓44		
		GLOase L3[8]	↑22	↑39	n.r.	↓43		
	High temperature, 29 °C	WT		↑19	n.r.			
		MIOX4 L3[8]	↑19	n.s.	n.r.			
		GLOase L3[8]	↑22	↓28	n.r.			
Arabidopsis thaliana	UVB, 0.09 W m^{-2}	WT		↑ 38	n.s.		Decreased	Yao et al. (2015)
		*vtc1***	↓75	↑32	n.s.			
		*vtc2***	↓68	↓9	n.s.			
Arabidopsis thaliana	Short-term UV-B exposure, 0.04 W m^{-2}	WT		↑26	↓51		Decreased	Gao and Zhang (2008)
		*vtc1***	↓80	↑80	n.s.			
Lycopersicon esculentum	Paraquat, 100 μM	WT		n.s.	↓45		Increased	Li et al. (2010)
		s4[9]	n.s.	n.s.	↓22			
		s17[9]	n.s.	n.s.	↓19			
		a1[9]	n.s.	↓29	↓54			
		a14[9]	n.s.	↓23	↓56			
Nicotiana tabacum	Paraquat, 50 μM	WT		↓24	n.s.		Increased	Yabuta et al. (2002)
		Tp TAP-12[10]	n.s.	↓10	n.s.			

(continued)

Table 1 (continued)

Species	Stressor	WT and transgenic/ mutant plants	Initial Asc difference [%][#]	Stress-induced Asc change [%]*	Stress-induced RsA change [%]*	Growth [%]*	Tolerance	References
Solanum tuberosum	Paraquat, 10 μM	UT[11]		↑51	n.s.		Increased	Hemavathi et al. (2010)
		T[11]	↑48	↑41	↑20			
	Sodium chloride, 100 mM	UT[11]		↑45	↑12			
		T[11]	↑48	↑33	↑23			
	Zinc chloride, 20 mM	UT[11]		↑48	↑13			
		T[11]	↑48	↑16	n.s.			
Solanum tuberosum	Sodium chloride, 200 mM	UT[11]		↑22	n.s.	↓74	Increased	Upadhyaya et al. (2011a, b)
		T[11]	↑45	↑38	n.s.	↓61		
Nicotiana tabacum	Sodium chloride, 200 mM	Control		↑34	↓20		Increased	Kavitha et al. (2010)
		MDAR-9[12]	n.s.	↑35	n.s.			
		MDAR-19[12]	n.s.	↑38	n.s.			
Nicotiana tabacum	Sodium chloride, 200 mM	WT		↓20	↓11		Increased	Yamamoto et al. (2005)
		s1[13]	n.s.	↓17	↓36			
		s6[13]	n.s.	↓18	↓34			
		a7[13]	n.s.	↓17	n.s.			
		a56[13]	↑10	↓15	n.s.			
Arabidopsis thaliana	Sodium chloride, 200 mM	WT		↓66	↓56		Decreased	Huang et al. (2005)
		*vtc1***	↓71	↓80	↓87			

Nicotiana tabacum	Aluminium, 400 µM	WT		↑19	↓9		Increased to DHAR overexpressing plants but not for MDAR	Yin et al. (2010)
		DHAR2[2]	↑13	↑17	n.s.			
		DHAR5[2]	↑8	↑22	n.s.			
		DHAR7[2]	↑17	↑13	n.s.			
		MDAR1[12]	n.s.	↑16	↓12			
		MDAR3[12]	n.s.	↑18	↓9			
		MDAR4[12]	n.s.	↑21	↓11			
Oryza sativa	Aluminium, 40 ppm	NT[14]		↑50	n.r.		Increased	Rosa et al. (2010)
		APX1/2s10[14]	↑36	↑39	n.r.			
Arabidopsis thaliana	5 µM Cd^{2+}	WT		↑59	n.s.	↓22	Increased	Jozefczak et al. (2015)
		cad2-1[15]	n.s.	↑54	↑8	↓66		
		*vtc1-1***	↓33	↑20	n.s.	↑9		
		cad2-1 vtc1-1[15]	↓67	↑61	↑12	↓14		
Arabidopsis thaliana	Drought	*vtc1* irrigated	n.r.	↓26	↓35		Decreased	López-Carbonell et al. (2006)
		vtc1 water stressed	n.r.	↓30	n.s.			
Arabidopsis thaliana	Drought	WT		↓46	↓12		Decreased	Munné-Bosch and Alegre (2002)
		*vtc1***	↓96	↑87	↓9			
Arabidopsis thaliana	Drought	WT		↑29	↓44		Decreased	Niu et al. (2013)
		*vtc1***	↓73	↓47	↓70			
Arabidopsis thaliana	Drought	WT		↑24	↓81			Brossa et al. (2011)
		*vtc1***	↓74	↑72	↓71			
Arabidopsis thaliana	Drought	WT		↑22	↑18			Brossa et al. (2013)
		*vtc2***	↓86	↑44	↑9			

(continued)

Table 1 (continued)

Species	Stressor	WT and transgenic/ mutant plants	Initial Asc difference [%][#]	Stress-induced Asc change [%]*	Stress-induced RsA change [%]*	Growth [%]*	Tolerance	References
Nicotiana tabacum	Ozone, acute exposure, 200 ppb	WT		↑11	↓18		Increased	Chen and Gallie (2005)
		DHAR-KD[2]	↓18	↑19	↓22			
		DHAR-OX[2]	↑43	n.s.	↓14			
	Ozone, continuous exposure, 100 ppb	WT		↓36	↓32			
		DHAR-KD[2]	↓22.0	↓36	↓31			
		DHAR-OX[2]	↑18	↓26	↓29			

RsA redox state calculated as reduced ascorbate/total ascorbate, *n.s.* no significant changes, *n.r.* not reported, **, for details see Table 2
[#]Percentage of Asc content in transgenic/mutant plants compared to WT (100%) before stress treatment.
*Percentage of increase (↑) or decrease (↓) in conditions of stress in comparison to untreated control
Description of the transgenic plant:
[1]*dhar3* knockout mutant
[2]*DHAR-KD/OX– DHAR* suppressed/overexpressed transgenic tobacco plant
[3]Antisense suppression of gene encoding L-galactono-lactone-1,4-dehydrogenase (GalLDH, EC 1.3.2.3)
[4]*npq4-npq1*—mutant lacking qE component of NPQ/zeaxanthin and qE
[5]*tapx*, *sapx*—single or double null mutants in thylakoid APX (tAPX) or stromal APX (sAPX) or combined with *vtc2* mutant
[6]T1(+)-6, T1(+)-3, transgenic plants overexpressing GDP-mannose pyrophosphorylase
[7]T1-3, T1-5, and T1-9—transgenic plants carrying the antisense thylakoid APX gene
[8]GLOase, MIOX4—transgenic plant lines overexpressing *myo*-inositol oxygenase or L-gulono-1,4-lactone oxidase
[9]Transgenic plants overexpressing chloroplastic MDAR in sense (s4, s17) and antisense (a1, a14) orientation
[10]Tp TAP-12—transgenic plant overexpressing tAPX
[11]*UT* untransformed control, *T* transgenic line overexpressing galacturonic acid reductase (GalUR)
[12]Transgenic plants overexpressing MDAR
[13]Transgenic plants expressing the apoplastic ascorbate oxidase gene in sense and antisense orientations
[14]*NT* non-transformed, APX1/2s10—transgenic double APX silenced rice plants
[15]*cad2-1*—glutathione-deficient mutant

and co-workers (2002) have shown that endogenous Asc and phenolic compounds from leaf extract interfered with two commonly used H_2O_2 measuring assays and strongly affected the obtained H_2O_2 concentration in the leaf extract. Moreover, the accumulation of H_2O_2 in chloroplasts under abiotic stress would result in the inactivation of Cu/Zn superoxide dismutase (Cu/ZnSOD), PRXs, Rubisco, and thiol-sensitive enzymes of Calvin cycle (Weisiger and Fridovich 1973; Heldt et al. 1978; Kaiser 1979; Badger et al. 1980; Cheeseman 2007).

It is widely accepted that a highly reduced state of Asc, when not affected by the extraction procedure of plant tissue, indicates a healthy plant. Any divergence from the 0.9 to 0.95 in the ratio of the reduced to the total Asc indicates accumulation of ROS beyond the antioxidant capacity of a cell (Foyer and Noctor 2011). The total leaf Asc depends on the developmental and nutritional status, diurnal rhythm, light quantity and quality, and on oxidative pressure imposed on plants under various environmental or experimental conditions (Conklin and Barth 2004; Bartoli et al. 2006, 2009; Diaz-Vivancos et al. 2010; Majer et al. 2016). Ascorbate peroxidase plays an essential role in scavenging H_2O_2 in chloroplasts, due to its high affinity for H_2O_2, utilizing Asc as an electron donor (Sharma and Dubey 2004). Besides stromal APX (sAPX) and thylakoid-bound APX (tAPX), the chloroplastic level of H_2O_2 is controlled by PRXs and glutathione peroxidases (GPXs) (Dietz 2016). In peroxisomes, H_2O_2 is scavenged by catalase (CAT, EC 1.11.1.6), while in the apoplast, cell wall and vacuoles class III peroxidases (POD, EC 1.11.1.7) are the main players in H_2O_2 detoxification.

In chloroplasts, Asc is reduced in two pathways: a classic water–water cycle (Asada 1999) and Asc–GSH cycle (Foyer and Noctor 2011; Smirnoff 2011). The Asc–GSH cycle refers to reduction of H_2O_2 to water, by Asc oxidation via APX, and its further regeneration from monodehydroascorbate radical ($MDA^{\bullet}$) and dehydroascorbate (DHA) by three enzymes (monodehydroascorbate reductase, MDAR, EC 1.6.5.4;dehydroascorbate reductase, DHAR, EC 1.8.5.1; and glutathione reductase, GR, EC 1.6.4.2) and GSH with final consumption of NADPH as an electron donor. Constituents of the Asc–GSH cycle were found in chloroplasts, cytosol, peroxisomes, and mitochondria (Jiménez et al. 1997; Noctor and Foyer 1998; Shigeoka et al. 2002; Mittler et al. 2004; Asada 2006). However, the extent to which Asc and GSH interact in concert to reduce H_2O_2 *in vivo* remains to be established (Foyer and Noctor 2011; Lallement et al. 2016).

Stress-induced enhancement of the activities of Asc–GSH enzymes is correlated with HL, UV-B radiation, salinity, drought, metal excess, and chilling tolerance (Kubo et al. 1999; Oidaira et al. 2000; Gechev et al. 2003; Leterrier et al. 2005; Eltayeb et al. 2007; Stevens et al. 2008; Mishra et al. 2012; Bielen et al. 2013). Increased activities of enzymes involved in the regeneration of Asc, MDAR, and DHAR are commonly obtained for different types of abiotic stressors in many species (Table 1, Schutzendübel et al. 2001, Sharma and Dubey 2005; Azevedo Neto et al. 2006; Moradi and Ismail 2007; Maheshwari and Dubey 2009). Transgenic plants with overexpressed DHAR showed a higher tolerance to salt stress, treatment with Pq and O_3 (Table 1, Chen and Gallie 2005, 2006; Lee et al. 2007), while plants with lower DHAR activity had a lower rate of CO_2 assimilation, lower chlorophyll

content, and slower growth (Chen and Gallie 2006, 2008). Overexpression of GR in tobacco and poplar resulted in higher leaf Asc contents and improved tolerance to oxidative stress (Sharma et al. 2012). Wild type plants, as well as mutants correlated with ascorbate metabolism (i.e. *vtc1, dhar3-1, npq1, vtc2npq1, vtc2npq4, tapx, sapx, tapx-sapx, sapx-vtc2-2, tapx-vtc2-2, sapx-tapx-vtc2-2*) exhibit a stress-induced increase in Asc content and decreased tolerance when they are exposed to variable HL regimes, UV-B radiation, and drought (Munné-Bosch and Alegre 2002; Müller-Moulé et al. 2003, 2004; Giacomelli et al. 2007; Gao and Zhang 2008; Talla et al. 2011; Yao et al. 2015).

However, transgenic plants overexpressing D-galacturonic acid reductase (GalUR) and/or suppressing DHAR, MDAR, double silenced APX, *cad2-1, vtc1-1, and cad2-1-vtc1-1* were more tolerant to metals, O_3, or salt, and had elevated stress-induced ascorbate content, see Table 1 (Chen and Gallie 2005; Kavitha et al. 2010; Rosa et al. 2010; Yin et al. 2010; Upadhyaya et al. 2011a, b; Lisko et al. 2013; Jozefczak et al. 2015). On the other hand, sense and antisense ascorbate oxidase (AO) gene transgenic tobacco plants expressed lower Asc level and elevated tolerance when treated with 200 mM NaCl compared to WT (Yamamoto et al. 2005).

Additionally, plants with altered expression of genes involved in Asc biosynthetic pathway have modified response to various stressors (Table 2). Plants with diminished Asc biosynthesis such as Arabidopsis *vtc* mutants, ranging from 10 to 90% Asc levels compared to WT are rather sensitive to various abiotic stressors (Müller-Moulé et al. 2004; Huang et al. 2005; Alhagdow et al. 2007; Gao and Zhang 2008). In case of overexpressing GDP-D-mannose pyrophosphorylase to low or high temperature, stress-induced Asc content of transgenic and WT plants was depleted, while tolerance increased (Wang et al. 2010). Similar behaviour has been noticed in transgenic lines treated with paraquat (Pq) (Yabuta et al. 2002; Li et al. 2010). In contrast, overexpressing GalUR led to elevated Asc content, as well as increased stress tolerance (Hemavathi et al. 2010).

2.1 Monodehydroascorbate Radical: Generation and Decay

The MDA$^\bullet$ or ascorbyl radical can be measured directly by electron paramagnetic resonance spectroscopy (EPR). It is seen as a doublet at 337 mT spaced by 0.14 mT and can be used as a sensitive endogenous probe of oxidative stress (Buettner and Jurkiewicz 1996, Heber et al. 1996). However, the MDA$^\bullet$ signal from a leaf can be seen only under HL intensity and severe oxidative stress such as herbicide treatment (Westphal et al. 1992; Veljović-Jovanović 1998), high UV-B radiation (Hideg et al. 1997), or sulphite treatment (Veljović-Jovanović et al. 1998).

The concept of delocalized scavenging of H_2O_2 has demonstrated in which HL-induced H_2O_2 leaked from chloroplasts into the cytosol and vacuoles (Bienert and Chaumont 2014), where it was consumed in the POD-Phenolics-Asc (PPA) H_2O_2-scavenging system (Ferreres et al. 2011; Zipor and Oren-Shamir 2013). It has

Table 2 Content of Asc in different Arabidopsis mutants compared to wild type, WT (Col-0)

Mutant	% [Asc] of WT		Point of mutation and defected gene	Note	References
vtc1	*vtc1-1*	≈30	GDP-D-mannose pyrophosphorylase, *VTC1* (At2g39770.1)	65% decreased activity compared to WT	Conklin et al. (1996, 1999)
	vtc1-2	≈30			
vtc2	*vtc2-1*	≈10	GDP-L-galactose phosphorylase, *VTC2* (At4g26850.1)	2% of catalytic efficiency compared to WT	Conklin et al. (2000), Jander et al. (2002), Linster et al. (2007), Dowdle et al. (2007), Laing et al. (2007)
	vtc2-2	10–20			
	vtc2-3	≈50			
	vtc2-4	n.r.			
vtc3	*vtc3-1*	≈50	Polypeptide with dual function of protein kinase/protein phosphatase, *VTC3* (At2g40860)		Conklin et al. (2000, 2013)
	vtc3-2	n.r.			
vtc4	*vtc4-1* *vtc4-2* *vtc4-4*	≈40–50 ≈75 ≈60	L-galactose-1-phosphate phosphatase, *VTC4* (At3g02870.1)		Conklin et al. (2000, 2006), Dowdle et al. (2007), Torabinejad et al. (2009)
vtc5	*vtc5-1*	80–90	GDP-L-galactose phosphorylase, *VTC5* (At5g55120)		Dowdle et al. (2007)
	vtc5-2	80–90			

n.r. not reported, *vtc1* point mutation of *VTC1* gene, *vtc2* missense mutation on *VTC2* gene, *vtc3* missense mutation for *vtc3-1* and nonsense mutation for *vtc3-2* mutant of *VTC3* gene, *vtc4* T-DNA insertion mutation on *VTC4* gene, *vtc5* T-DNA insertion mutation on *VTC5s* gene

been proposed that the reduction of Pq-induced H_2O_2 generation was accompanied by the formation of $MDA^{\bullet}$ in vacuoles in the PPA system (Takahama and Egashira 1991). In accordance with the Asc role in the PPA system, it has been shown that Asc accumulated in vacuoles under HL (Ferreres et al. 2011; Zipor and Oren-Shamir 2013). However, by immunocytochemical detection, it has been shown that vacuole accumulated about five times less Asc than cytosol and chloroplasts (Zechmann et al. 2011; Vidović et al. 2016a).

Neubauer and Schreiber (1989) showed that APX catalysed reduction of externally added H_2O_2 can be as efficient as a Pq-catalysed O_2 reduction in intact isolated chloroplasts. Distinct dynamics of $MDA^{\bullet}$ generation in leaves of *Vicia faba* with the inhibited CAT (aminotriazole) or elevated superoxide anion radical ($O_2^{\bullet -}$) production at Photosystem I (PSI) under Pq and HL indicate different mechanisms for H_2O_2 generation and scavenging in chloroplasts (Veljović-Jovanović 1998). The

reduction of $MDA^{\bullet}$ in the water–water cycle is 1000 fold higher than the production rate of $O_2^{\bullet -}$ in the Mehler reaction, which together with its location close to the PSI complex, imply a high efficiency in the reduction of oxidized forms of Asc (Miyake and Asada 1994; Miyake et al. 1998). Under oxidative stress induced by Pq Asc regeneration is hindered due to inactivation of chloroplastic APXs (Miyake and Asada 1996; Mano et al. 2001). Both chloroplastic APX isoforms, particularly tAPX, are sensitive to inactivation by H_2O_2 (>2 nM) under Asc depletion (Asc content <20 μM) (Miyake and Asada 1992; Ishikawa and Shigeoka 2008), that may imply its role in the regulation of signalling H_2O_2 under abiotic stress. In both cycles, MDAR has a central role in linking Asc and GSH.

2.2 Extracellular ROS Scavenging by Ascorbate in Abiotic Stress

Apoplastic Asc is the first antioxidative barrier to invading pathogens, O_3, metals, and other environmental stressors that disturb redox homeostasis in extracellular space including the cell wall (Kangasjärvi and Kangasjärvi 2014). Ascorbate is considered as the main reductant in an extracellular compartment, and its redox state is regulated mainly by AO (EC 1.10.3.3) (Kato and Esaka 1996; De Tullio et al. 2013). Moreover, the PPA system for H_2O_2 scavenging is an important antioxidative sink in compartments which accumulate phenolic compounds, such as apoplast and vacuole (Takahama and Oniki 1992; Takahama et al. 1992; Takahama and Oniki 1997).

The lowest pool of Asc was found in apoplastic washing fluid (AWF) when extracted mechanically by vacuum infiltration and centrifugation (Lohaus et al. 2001; Nouchi et al. 2012; O'Leary et al. 2014). Though the technique is robust and invasive to some extent, it has been commonly used for studying the apoplastic composition and the effects of various stressors (Polle et al. 1990; Takahama et al. 1992; Luwe et al. 1993; Veljović-Jovanović et al. 2001; Pignocchi et al. 2003; Sanmartin et al. 2003; De Pinto and De Gara 2004; Yamamoto et al. 2005; Kärkönen and Fry 2006; Delaunois et al. 2013). Pignocchi and co-authors (2006) showed that changes in the redox state in the apoplast mediated plant growth and antioxidative defence responses, by regulation of signal transduction pathways and expression of genes involved in cell wall metabolism. The redox state of Asc is also important in a number of stress responses, such as the control of guard cell signalling and stomatal movement during drought (Chen et al. 2014). A significant negative correlation between the Asc apoplastic level along hypocotyls and the growth rate was observed (Córdoba-Pedregosa et al. 2003; Pedreira et al. 2004; Rodriguez-Serrano et al. 2006) suggesting the role of Asc in the regulation of trade-off between growth and defence.

In contrast to the redox state of intracellular Asc pool that is kept largely reduced even under conditions of oxidative stress, a high accumulation of DHA

in the apoplast is commonly obtained, and its content and redox state may vary during a day or season and developmental stage of the plant. Luwe (1996) showed that in AWF of beech leaves less than 20% of Asc was reduced and that both, content and redox state, increased to 60–75% in summer. While damage of cells firstly was considered to be the reason for Asc oxidation during the procedure of extraction, it is now widely accepted that apoplast is the compartment of Asc degradation and that abundance of DHA is a natural process (Green and Fry 2005; Parsons and Fry 2012). Moreover, since Asc is a cofactor of endoplasmic reticulum-located prolyl hydroxylase that produces the hydroxyproline-rich glycoproteins required for cell division and expansion, the requirements of cell wall cross-linking are compatible only with a completely oxidized apoplastic Asc pool (Kärkönen and Fry 2006). In addition, apoplastic Asc, DHA, diketogulonate, and other products are able to modulate the H_2O_2 concentration, thus providing potential control mechanisms for growth and lignification under stress. Enzymes involved in recycling of Asc and GSH have not been reported in the apoplast (Castillo and Greppin 1988). Though GSH was not found in leaf apoplast except in γ-glutamyl transferase (*ggt*) mutants of Arabidopsis (Zechmann 2014), it has been proposed that GSH degradation and recovery operate in the apoplast in a so-called γ-glutamyl cycle with a role in redox sensing during UV-B stress (Masi et al. 2016).

Oxidation of Asc in the apoplast may occur during scavenging of hydroxyl radical (•OH) generated on the cell wall in the hydroxylic POD cycle (Chen and Schopfer 1999). Efficient scavenging of •OH in isolated cell walls was observed by addition of exogenous Asc (Veljović-Jovanović et al. 2005). Formation of MDA• in the cell wall isolates after the addition of Asc was accompanied by the decrease in EPR signals due to quinhydrone (QH) and •OH in the cell wall of pea root (Kukavica et al. 2008). The results imply that the redox state of the apoplastic Asc pool could be one of the main regulators of •OH formation and the cell wall loosening/stiffening (Veljović-Jovanović et al. 2005; Morina et al. 2010). We have previously shown accumulation of •OH and carbon-centred (•CH_3) radicals in the root cell wall of *Verbascum thapsus* plants treated with excess Zn^{2+} and Cu^{2+} (Morina et al. 2010; Morina et al. 2012) by EPR analysis, accompanied with decreased Asc, and increased DHA content.

The obtained products, MDA• and H_2O_2, are further regulated by enzymatic reactions, regenerating reduced Asc in the Asc–GSH cycle. There is also evidence that NADPH is not available in the apoplast; therefore, recycling of Asc and GSH must take place using the cytoplasmic machinery. In the apoplast, Asc is also converted into MDA•, mainly by the action of PPA system (Takahama and Oniki 1992), As mentioned above, apoplastic MDA• generated in the vicinity of plasma membrane might accept electrons from membrane-bound cytochrome *b* and then be converted into Asc and DHA by the action of membrane-bound MDAR (Drazkiewicz et al. 2003). The DHA is further transported to the cytoplasm where it is reduced to Asc in Asc–GSH cycle (Horemans et al. 2000).

3 Non-enzymatic Antioxidative and Pro-oxidative Activities of Ascorbate

Ascorbate is a very effective reducing agent and antioxidant in biological systems. It can donate one or two electrons and MDA$^{\bullet}$ (formed after loss of one electron from the C2–C3 enediol group) is relatively unreactive and unusually long-lived for a free radical species, due to resonance stabilization of unpaired electrons (the unpaired electron is part of delocalized π-system above 3 C atoms) (Fig. 1). Free MDA$^{\bullet}$ radicals spontaneously convert to Asc and DHA ($k = 10^5$ M^{-1} s^{-1} at pH 7; Hossain and Asada 1985). If DHA does not quickly reduce to Asc, it will be irreversibly hydrolyzed, via 2,3-diketogulonate to L-threonate, oxalate, or tartrate (Deutsch 2000; Debolt et al. 2007).

Besides its role in H_2O_2 scavenging in enzymatic reactions, Asc can directly react with singlet oxygen ($^1O_2$), $O_2^{\bullet-}$, $^{\bullet}OH$, and hydroperoxyl radical ($HO_2^{\bullet}$), which are generated to a higher extent under unfavourable conditions such as HL, drought, metal excess, and biotic stress (Aver'yanov 1985; Kuchitsu et al. 1995, Schopfer et al. 2001; Rodriguez-Serrano et al. 2006). Various cellular components including plasma membranes may be capable of generating $O_2^{\bullet-}$ (Mojović et al. 2004) with a plasma membrane-bound NAD(P)H oxidase as a crucial enzyme responsible for the generation of $O_2^{\bullet-}$ in the apoplast (Doke 1985; Murphy and Auh 1996; Van Gestelen et al. 1997). Furthermore, $^1O_2$ is a major ROS produced under photooxidative stress conditions. It is produced in chloroplasts at the Photosystem II (PSII) (Hideg et al. 1994; Triantaphylidès et al. 2008). Most $^1O_2$ is quenched by carotenoids and tocopherols or reacts with galactolipids in thylakoid membranes, yielding galactolipid hydroperoxides (Zoeller et al. 2012; Farmer and Mueller 2013). Ascorbate and MDA$^{\bullet}$ are indirectly involved in the detoxification of lipid peroxides and radicals, through regeneration of tocopheroxyl radicals, thus enabling membrane protection and decreasing lipid peroxidation (Thomas et al. 1992; Szarka et al. 2012).

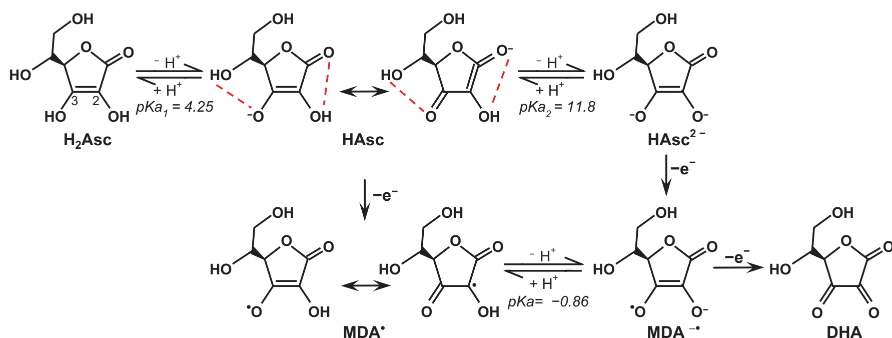
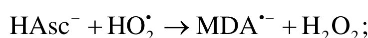


Fig. 1 Chemical structure of ascorbate (Asc) with its oxidation and dissociation products. At pH 7, Asc is in the form of monoanion ($HAsc^-$) which is stabilized by resonant forms. It easily oxidizes to monodehydroascorbate radical (MDA$^{\bullet}$), also stabilized by resonant forms, and then to dehydroascorbate (DHA). Red dashed lines present intramolecular hydrogen bonds in $HAsc^-$

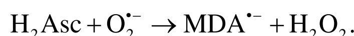
Also, Asc readily reacts with $^1O_2$ yielding H_2O_2 as shown by Kramarenko et al. (2006) in a fast reaction *in vitro* ($k = 3.2 \times 10^8\ M^{-1}\ s^{-1}$).

Bielski and co-workers (1981) proposed a reaction mechanism of oxidation of H_2Asc and its monoanion, $HAsc^-$ by $HO_2^{\bullet}$ and $O_2^{\bullet-}$ at a wide range of pH (0.3–11) as follows (Bielski et al. 1981; Cabelli and Bielski 1983):

$$H_2Asc + HO_2^{\bullet} \rightarrow MDA^{\bullet-} + H^+ + H_2O_2;$$



$$HAsc^- + HO_2^{\bullet} \rightarrow MDA^{\bullet-} + H_2O_2;$$



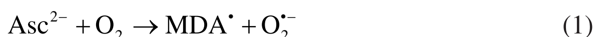
$$H_2Asc + O_2^{\bullet-} \rightarrow MDA^{\bullet-} + H_2O_2.$$

Light-induced formation of $O_2^{\bullet-}$ in the cytochrome *b6f* complex was detected in spinach leaves (Sang et al. 2011) by EPR spectroscopy using a 5-ethoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide (EMPO) spin-trapping agent. Addition of antioxidants, Asc, β-carotene, and GSH, could efficiently scavenge $O_2^{\bullet-}$ *in vitro* indicating their direct involvement in photoprotection. This reactivity of Asc in chloroplasts may be of particular importance under conditions where the activity of Cu/ZnSOD is suppressed. For example, although chloroplastic Cu/ZnSOD activity was sensitive to Zn treatment (Morina 2011), the detoxification mechanism of ROS in the leaves of *V. thapsus* efficiently relied on the strongly induced biosynthesis of Asc (Morina et al. 2010). Furthermore, an earlier study on the effects of O_3 on *Poa pratensis*, *Lolium perenne,* and leaf pieces of *Raphanus sativus* (Runeckles and Vaartnou 1997) showed that generation of $O_2^{\bullet-}$ was suppressed in the leaf samples previously vacuum infiltrated with Asc. Although the differentiation between $O_2^{\bullet-}$ generated in the apoplast and chloroplasts was not evident, an important role of Asc in $O_2^{\bullet-}$ scavenging in the apoplast in response to O_3 was proposed.

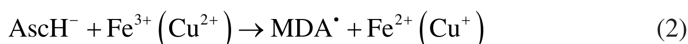
Ascorbate was the main scavenger of $O_2^{\bullet-}$ in Arabidopsis under optimal conditions as indicated by EPR measurements (Nagata et al. 2003). Furthermore, following γ-radiation treatment accumulation of ROS was associated with the increase in Asc and anthocyanin content. In addition, UV-B radiation promoted accumulation of $^{\bullet}OH$ and $^{\bullet}CH_3$ radicals in isolated thylakoids (Hideg and Vass 1996), and their reactivity with Asc was demonstrated by increased EPR signals of $MDA^{\bullet}$ radicals in leaves of *V. faba* (Hideg et al. 1997). Taken together, the direct reactions of Asc with ROS are very fast. This way the damage of biomolecules is prevented prior to activation of antioxidative enzymes.

On the other hand, Asc can act as a pro-oxidant, particularly at higher pH as demonstrated *in vitro* (Buettner 1993; Buettner and Jurkiewicz 1996). The biological relevance of this reaction has been debated in medicinal biology (Halliwell and Gutteridge 2007; Du et al. 2015). However, although concentrations of trace metal ions, e.g., Cu^{2+}, range from 2 to 50 μg g^{-1} dry weight in plant cells (Burkhead et al. 2009), up to date the pro-oxidative properties of Asc have not been fully explored *in planta* (Majer et al. 2016). Under neutral pH, Asc oxidizes spontaneously and very slowly (*1*; $k \approx 300\ M^{-1}\ s^{-1}$), via Asc dianion, Asc^{2-}, which consists 0.005% of total Asc at pH 7 (Du et al. 2012). However, in the presence of traces of catalytic,

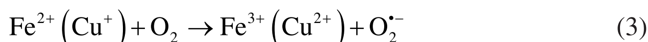
redox-active, metals (e.g. Cu^{2+}, Fe^{3+}), Asc oxidation is accelerated: it reduces transition metals (*2*), and activates them enabling their interaction with oxygen, and production of $O_2^{\bullet-}$ (*3*) that subsequently converts to H_2O_2 and O_2 (*4*, Buettner and Jurkiewicz 1996; Du et al. 2012). Reduced Fe^{2+} or Cu^+ can react with H_2O_2 (or some organic peroxide) and form a notorious $^{\bullet}OH$ in a so-called Fenton reaction (*5*; Buettner 1993).



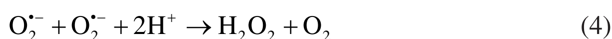
$$Asc^{2-} + O_2 \rightarrow MDA^{\bullet} + O_2^{\bullet-} \quad (1)$$



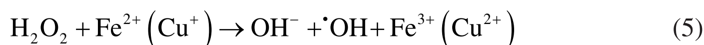
$$AscH^- + Fe^{3+}\left(Cu^{2+}\right) \rightarrow MDA^{\bullet} + Fe^{2+}\left(Cu^+\right) \quad (2)$$



$$Fe^{2+}\left(Cu^+\right) + O_2 \rightarrow Fe^{3+}\left(Cu^{2+}\right) + O_2^{\bullet-} \quad (3)$$



$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$



$$H_2O_2 + Fe^{2+}\left(Cu^+\right) \rightarrow OH^- + {}^{\bullet}OH + Fe^{3+}\left(Cu^{2+}\right) \quad (5)$$

A study by Samuni et al. (1983) demonstrated a 'site-specific' Fenton mechanism initiated by binding transition metal ions to biomolecules. The bound metal was reduced by reductants such as Asc and subsequently reoxidized by H_2O_2 yielding $^{\bullet}OH$. The production of $^{\bullet}OH$ radicals in the proximity of biomolecules induced direct damage that preceded scavenging of the antioxidative system.

The ability of Asc to reduce Fe^{3+} makes it suitable to act as a reducing cofactor for many enzymes, such as hydroxylases that belong to the Fe^{2+}-2-oxoglutarate-dependent families of dioxygenases.

4 Signalling Role of Ascorbate Under Abiotic Stress

Although ROS can cause oxidative cellular damage, at low levels they activate distinct signalling pathways either directly, or by oxidation of different metabolites or signalling proteins (Mock and Dietz 2016). Redox-related post-translational modification of proteins may affect their stability, catalytic activity, and interaction with other molecules (Dietz 2014). Hydrogen peroxide is an important signalling molecule in retrograde signalling, which modulates the expression of nuclear genes (Pogson et al. 2008; Bienert and Chaumont 2014). Fine redox tuning of redox-active target signalling proteins occurs through regulation of the level and spatial distribution of primary ($O_2^{\bullet-}$; H_2O_2; $^1O_2$; nitric oxide radical, $NO^{\bullet}$) and secondary pro-oxidants (peroxynitrite, $ONOO^-$; *S*-nitrosoglutathione, GSNO; and DHA), which are finally involved in the redox regulation of protein and non-protein thiols (Dietz 2016). Indeed, Asc together with APX in chloroplasts controls the content of H_2O_2. However, its oxidized form, DHA, drains electrons from the thiol-disulphide redox regulatory network, via DHAR. New reports suggest that cytosolic DHAR is

significant for coupling the cellular H_2O_2 metabolism to GSH oxidation (Rahantaniaina et al. 2017). The oxidized form of GSH: GSSG and oxidized 2-CysPRXs transmit signals further, towards transcriptional factors in the nucleus (Mock and Dietz 2016). Oxidized 2-CysPRXs act as redox sensors, and they can be reduced by thioredoxins, and glutaredoxins, which receive electrons from NADPH and/or ferredoxin. In this way, they act as thiol switches in various cellular pathways. Recently, it was shown that 2-CysPRXs are involved in the regulation of numerous chloroplastic processes, such as photosynthetic light reactions, carbon metabolism, nitrogen and sulphur metabolism, antioxidant defence (SOD, GR), and secondary compound synthesis (Liebthal et al. 2017).

Double *tapx-sapx* mutants acclimated to HL stress showed enhanced levels of 2-CysPRXs compared with WT plants (Kangasjärvi et al. 2008). Under HL stress, 2-CysPRXs are more dominant than tAPX in the water-water cycle, which was confirmed in comparative studies with the respective mutants (Awad et al. 2015). Supplementation with exogenous Asc declines the transcript levels of chloroplastic *PRX* genes; however, the Asc redox state is the crucial signal for regulation of *PRX* expression (Horling et al. 2003). In this context, Asc could act as a suppressor, while DHA and/or $MDA^{\bullet}$, could act as stimulators of chloroplastic *PRX* expression.

Due to the propensity of chloroplastic APX to inactivation in the absence of reduced Asc can be regarded as a signalling function (Dietz 2016; Exposito-Rodriguez et al. 2017).

Cell wall loosening is very important under osmotic, drought and salt stress for maintaining the possibility of cells and organs to expand (Tenhaken 2014). Moreover, DHA is involved in cell wall loosening and reorganization which contributes to cell expansion (Kukavica et al. 2008). DHA reacts with lysine and arginine of side chain residues of cell wall proteins, thus preventing their bonding with pectin and other polysaccharides. In the apoplast, DHA is degraded to L-tartrate, oxalate, and L-threonate via several intermediates, which could have important roles in plant growth and development (Green and Fry 2005; Parsons and Fry 2012). In addition, oxalate can extract calcium from calcium-pectin complexes, leading to reduced pectin cross-linking and cell wall loosening (Hocking et al. 2016). On the other hand, Fry's team has proposed a mechanism for the generation of $^{\bullet}OH$ in an Asc-dependent way in the presence of Cu^{2+}, or from H_2O_2 formed during Asc and DHA degradation in the cell wall (Fry 1998; Green and Fry 2005; Kärkönen and Fry 2006). The $^{\bullet}OH$ further causes backbone cleavage of pectin and xyloglucan in the cell wall (Dumville and Fry 2003; Spasojević and Bogdanović-Pristov 2010). Finally, AO is involved in the degradation of auxin, a growth regulator important in controlling responses to osmotic stress imposed by salinity, drought, and low-temperature conditions (Pignocchi et al. 2003). Among other phytohormones involved in abiotic stress response in plants, salicylic acid (SA) and abscisic acid (ABA) act as negative, while jasmonic acid (JA) acts as a positive regulator of AO expression (Sanmartin et al. 2007). Moreover, a higher level of DHA in AO overexpressing tobacco plants compared to WT plants was responsible for enhanced H_2O_2 and ABA levels and control of guard cell signalling and stomatal movement, probably via reversible redox regulation of specific protein thiols (Fotopoulos et al. 2008).

Ascorbate is the reducing cofactor for Cu^{+}-dependent monooxygenases and for Fe^{2+}/2-oxoglutarate-dependent dioxygenases (2-OGDs, e.g. prolyl-lysyl-hydroxylases) in plants (Smirnoff 2000; Foyer and Noctor 2011; Zhang 2013; Bánhegyi et al. 2014). 2-OGDs catalyse oxygenation/hydroxylation reactions in various biological processes, including proline hydroxylation, DNA demethylation, phytohormone biosynthesis, and the biosynthesis of various secondary metabolites, such as anthocyanins and flavon-3-ols, considered as markers of HL and UV exposure (Kawai et al. 2014). A wide range of functionally diverse 2-OGDs is responsible for biosynthesis of numerous metabolites that contribute to defence responses against various biotic stresses. These dioxygenases require Fe^{2+} in the active site to catalyse oxidation (and oxygenation) of a substrate with concomitant decarboxylation of 2-oxoglutarate. Ascorbate is required for maintaining iron in the reduced state (Fe^{2+}) thereby maintaining full activity of this class of enzymes (Arrigoni and De Tullio 2002; Ozer and Bruick 2007). In this way, Asc is involved in the production of hydroxyproline-rich glycoproteins, such as expansins required for vacuole formation, cell wall extension, and cell division (Córdoba and González-Reyes 1994; Brosche and Kangasjarvi 2012). Expansin expression is usually up-regulated by abiotic stress conditions, particularly drought and salinity stresses (Tenhaken 2014).

In addition, Asc is the coenzyme for aminocyclopropane carboxylate (ACC) oxidase, gibberellin 2-oxidases (GA2oxs), and 9-*cis*-epoxycarotenoid dioxygenase (NCED) required for the biosynthesis of ethylene, gibberellic acid (GA), and ABA, respectively (Arrigoni and De Tullio 2002; Pastori et al. 2003). On the other hand, in Arabidopsis, Asc is a cofactor for salicylic acid 3-hydroxylase, involved in salicylic acid (SA) catabolism during senescence (Kawai et al. 2014). Therefore, Asc might be involved in the regulation of plant growth and defence against biotic and abiotic stressors through interaction with phytohormones, see Sect. 6 (Foyer and Noctor 2011).

Finally, Asc is involved in catalysis of epigenetically relevant reactions in the nucleus serving as a cofactor for a group of 2-OGD responsible for oxidative dealkylation (e.g. in *Escherichia coli*-AlkB dioxygenase) (Fedeles et al. 2015). Thus, Asc promotes methylcytosine dioxygenases-catalysed cascade oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, to 5-formylcytosine, and to 5-carboxylcytosine modulating gene expression (Camarena and Wang 2016). Epigenomic reprogramming including histone post-translational modifications (methylation, phosphorylation) and DNA methylation are critical for plant responses to abiotic stresses (e.g. cold, salinity, drought, osmolality, or mineral nutrition; reviewed by Fortes and Gallusci 2017). From 5 to 14 different genes encoding these histone and DNA demethylases were found in different plant species (Mielecki et al. 2012; Kawai et al. 2014). In mammalian cells, Asc in the nucleus regulates the activity of various 2-OGDs catalysing the demethylation of histones and nucleic acids, and the hydroxylation of certain histones, while several 2-OGDs can also participate in DNA repair (Bánhegyi et al. 2014). Since altered AlkB proteins were found in several tumours and diseases, numerous studies related to their role in medicinal biology have been recently conducted. However, so far they were not fully explored in plants, particularly under stress conditions (Kuiper and Vissers

2014; Fedeles et al. 2015; Camarena and Wang 2016). In spite of the scarce information, recent reports showing an abundance of Asc in the nucleus supports the importance of its particular role in cellular signalling (see the next section).

4.1 Subcellular Distribution of Ascorbate Under Abiotic Stress

Various abiotic and biotic stressors trigger enhanced production of ROS in different compartments. High light, cold, drought, and low CO_2 stimulate Mehler reaction (generation of H_2O_2, $O_2^{\bullet-}$) and $^1O_2$ production in chloroplasts, while HL and low CO_2 provoke H_2O_2 generation in peroxisomes (Foyer and Noctor 2016). Biotic stressors, high O_3 levels, metal excess, and HL intensities stimulate the production of $NO^{\bullet}$ and $O_2^{\bullet-}$ in the plasma membrane and apoplast (Dietz 2014).

Ascorbate is found in almost all plant tissues and organs, with the exception of dry seeds (De Gara et al. 1997). However, the total content of Asc in a particular plant tissue depends on the species, but also on the developmental stage, as well as on environmental conditions, such as light quality and quantity, drought, temperature, metal excess, O_3, and nutrient availability (Veljović-Jovanović et al. 2001; Kukavica and Veljović-Jovanović 2004; Bartoli et al. 2009; Morina et al. 2010; Gest et al. 2012; Page et al. 2012; Heyneke et al. 2013).

Ascorbate is a multifaceted molecule and its specific functions are compartmentalized within the eukaryotic cell. However, for relevant interpretation of the Asc metabolism under stress conditions and a better understanding of defence mechanisms, the tissue- and compartment-specific changes should be analysed and discussed (Majer et al. 2016). In contrast to many studies which deal with the total cellular Asc level and redox state, only several reports present subcellular levels of Asc under unfavourable environmental conditions (Zechmann et al. 2011; Heyneke et al. 2013; Koffler et al. 2014a, b). Immunolocalization studies suggest that changes in Asc and GSH compartmentation are crucial in various stress responses (Zechmann 2014; Heyneke et al. 2013). In mammalian cells, disturbances in Asc subcellular distribution can be manifested in compartmentation diseases (Bánhegyi et al. 2014).

The majority of Asc biosynthesis pathways occur in the cytosol, while the final step of Asc biosynthesis takes place in the inner membrane of mitochondria (Smirnoff 2011; Schertl et al. 2012; Szarka et al. 2013). Since diffusion of Asc (charged) and DHA (hydrophilic) through lipid bilayers is unlikely, both forms are transported via specific transporters: uptake of DHA and glucose in mitochondria occurs by facilitated diffusion and is mediated by the same transporter, while Asc transfer from cytosol to chloroplasts stroma in Arabidopsis occurs via a membrane potential- and Cl^--dependent transporter AtPHT4;4, localized to the chloroplast envelope membrane, while transporter AtPHT4;1 is found in the thylakoid membranes, and its transport function and physiological role still have to elucidate (Miyaji et al. 2015).

Ascorbate is present in all subcellular compartments in concentrations ranging from 20 to 300 mM depending on the plant organ and species (Badejo and Esaka

2010). In studies using isolated intact organelles, Asc was detected in chloroplasts (Gillham and Dodge 1986; Foyer and Noctor 2003), peroxisomes (Jiménez et al. 1997), mitochondria (Jiménez et al. 1997; Bartoli et al. 2000), vacuoles (Takahama 2004), and apoplastic fluid (Polle et al. 1990; Takahama and Oniki 1992; Vanacker et al. 1998; Veljović-Jovanović et al. 2001; De Pinto and De Gara 2004) of various plant species.

A second approach, immunocytochemical detection (that does not discriminate between Asc and DHA) has been employed to analyse the subcellular Asc content. The highest concentration of Asc-specific labelling (immunogold particles) was found in peroxisomes, cytosol, and nuclei followed by chloroplasts, mitochondria, and vacuoles of mesophyll cells of Arabidopsis plants grown under optimal conditions (Table 3). These results corresponded to the following Asc concentrations: 22.8 mM in peroxisomes, 21.7 mM in the cytosol, 16.3 mM in nuclei, 10.8 mM in chloroplasts, 10.4 mM in mitochondria, and 2.3 mM in vacuoles (Zechmann et al. 2011). Ascorbate-deficient mutants, *vtc1-2* and *vtc2-1,* showed a similar Asc subcellular distribution, with a slightly higher proportion in plastids and vacuoles, and a lower one in peroxisomes compared to WT. However, in the cells of *N. tabacum*, the highest content of Asc was found in the nucleus and cytosol, followed by chloroplasts, mitochondria, and peroxisomes (Table 3). In both species, the Asc content in chloroplasts and mitochondria was similar, and it was localized in the mitochondrial matrix, chloroplast stroma, and outer side of thylakoid membranes. Ascorbate was not detected in the cell wall in either of the two species (Zechmann et al. 2011), although Asc was detected in extracted apoplastic fluid, but the redox state was significantly lower than in intracellular compartments (Sect. 2.2).

Using the same immunolocalization approach, Zechmann's group (Koffler et al. 2014a, b) showed the importance of monitoring compartment-specific redistribution of Asc and GSH in response to Cd excess and drought. Upon Cd treatment of Arabidopsis *Col-0* with 50 μM Cd, the first observed response was very rapid (after 12h) reduction of Asc and GSH contents in all compartments. On the fourth day,

Table 3 Ratio (in %) of the distribution of gold particles bound to ascorbate, Asc (labelling density, LD, of gold particles per μm^2) between mesophyll cell compartments of *Arabidopsis thaliana, Nicotiana tabacum* and green (photosynthetic) and white (non-photosynthetic) leaf parts of *Pelargonium zonale* and estimated sub-cellular Asc contents ($\mu mol\ g^{-1}FW$)

Cellular compartment	Nucleus	Plastid	Mitochondrion	Peroxisome	Cytosol	Vacuole
LD, %						
A. thaliana (Col-0)	19.4	12.8	12.2	27.1	25.8	2.7
A. thaliana vtc1-2	20.4	16.5	15.6	18.9	25.0	3.6
A. thaliana vtc2-1	20.6	19.5	10.7	19.4	25.8	4.0
N. tabacum	31.9	10.7	11.9	10.0	31.4	4.1
P. zonale						
Green sectors	32.1	18.5	17.6	21.3	7.4	3.1
White sectors	34.5	14.1	20.0	n.o.	28.0	3.4

Data were taken from: Zechmann et al. (2011), Vidović et al. (2016a). n.o., peroxisomes were not observed in non-photosynthetic cells of variegated species

elevation of the Asc content in chloroplasts, peroxisomes, and nuclei was observed, while in mitochondria this increase was delayed (seventh day). At the end of the experiment (14 days), the depletion of Asc was observed in all cellular compartments except in chloroplasts, supporting its crucial antioxidant role in this compartment, and the importance of maintaining the process of photosynthesis (Koffler et al. 2014a).

Under drought, the first significant change in Asc content in WT Arabidopsis was observed in the vacuole, where both H_2O_2 and Asc accumulated, indicating the role of Asc in the recovery of phenolics used for scavenging of H_2O_2 by POD (Koffler et al. 2014b). The first decrease of Asc content was observed in cytosol and nuclei (eighth and ninth day), while after the tenth day, the Asc content had significantly declined in all compartments concomitantly with the observed H_2O_2 accumulation in chloroplasts, cytosol, and peroxisomes. On the other hand, the Asc content increased in isolated chloroplasts of three Mediterranean water-stressed plants (rosemary, sage, and lemon balm), especially in lemon balm (Munné-Bosch and Alegre, 2003).

Apoplastic Asc, as the most important antioxidant in the apoplast and at the plasma membrane, is responsible for O_3 detoxification (D'Haese et al. 2005). Ozone fumigation studies (Luwe et al. 1993; Sanmartin et al. 2003) showed a decline of apoplastic Asc concomitantly with the Asc redox state after O_3 treatment. Similar responses were observed during pathogen attack and ethylene and SA treatment, suggesting an important role of the apoplastic Asc redox state in plant defence (Sandermann et al. 1998; Pignocchi and Foyer 2003).

In order to correlate Asc subcellular distribution with photosynthesis (the main sites of ROS generation in green leaves), variegated *Pelargonium zonale* has been used as a model species (Vidović et al. 2016a), with both photosynthetically active and non-photosynthetically active tissues within the same leaf. The main characteristic of photosynthetically non-active mesophyll cells was that they contained smaller plastids lacking thylakoid membranes or starch granules, and had no peroxisomes, while the relative vacuole volumes were greater than in photosynthetic cells (Vidović et al. 2015a). Although the total content of Asc was higher in autotrophic cells compared with heterotrophic ones, the latter contained a twice higher Asc content in the cytosol, while in other compartments (nuclei, plastids, peroxisomes, and vacuoles), a higher content of Asc was found within the autotrophic cells (Table 3).

An immunolocalization study of Asc distribution in another heterotrophic tissue, the root of *Cucurbita maxima*, showed the highest Asc content in the cytosol, nuclei (nuclear membrane and nucleoli), and plasma membrane (together with AO; Liso et al. 2004).

Altogether, these findings emphasize the need for measuring Asc (and GSH) content at the subcellular level rather than in bulk tissue in order to understand their role in different cellular compartments as a part of abiotic stress tolerance. The significant ratio of Asc-specific labelling found in nuclei re-opens the question about the roles of this molecule in the regulation of gene expression (Zechmann et al. 2011; Vidović et al. 2016a). Although the role of GSH in the nucleus related to regu-

lation of cell proliferation and development via interactions with transcription factors showing redox-sensitive activation was described (Diaz-Vivancos et al. 2015), relatively little is known about the role of nuclear Asc in photosynthetic cells. It was shown that under HL, the content of nuclear Asc increased (see Sect. 5; Heyneke et al. 2013). A possible involvement of Asc in cell proliferation, by influencing the RNA synthesis via an interaction with nuclear proteins, was proposed (Liso et al. 2004). We believe that high Asc abundance in the nuclei is in correlation with the recently revealed role of Asc in epigenetic regulation (see the previous section). Subcellular mapping of Asc concentrations, determination of the Asc redox state and exploration of Asc/DHA transporters in organelles are future challenges, which will likely lead to a more detailed understanding of Asc compartmentation under physiological and stress conditions.

5 Photoprotective Role of Ascorbate to High Light

In their natural environment, plants are inevitably exposed to fluctuating photosynthetically active radiation (PAR) intensities (Mullineaux and Karpinski 2002; Lichtenthaler 2007). When the PAR intensity overcomes metabolic requirements and the capacity of dissipation mechanisms, the rate of ROS generation increases, inhibiting photosynthesis and CO_2 assimilation (Li et al. 2010). This condition is called photooxidative stress (Asada 2006; Foyer and Shigeoka 2011). It is difficult to define which PAR intensity is a stressor for a particular plant species since that depends on the developmental stage, previous light adaptation, and on other environmental conditions, including drought, high salinity, nutrient deprivation, and temperature stress (Ort 2001; Li et al. 2010). Even optimal PAR intensity might be a stressor in combination with limited CO_2 availability or decreased rate of the Calvin-Benson cycle (Mittler et al. 2004; Baker 2008). Under such conditions, down-regulation of PSII is a major mechanism of photoprotection, dissipating efficiently the excess photon energy by low pH and zeaxanthin accumulation (Russell et al. 1995; Demmig-Adams and Adams 1996; Karpinski et al. 1999). Molecular oxygen has an important role in energy dissipation as it serves as an electron acceptor in two photosynthetic processes, photorespiration (Noctor et al. 2002) and in Mehler reaction coupled to the water–water cycle (Asada 1999; Ort and Baker 2002).

Ascorbate is involved in the dissipation of excess of energy in chloroplasts and thus has an important role in the protection of photosynthesis against HL stress: (1) in non-photochemical quenching (NPQ), as a cofactor for the VDE-catalysed de-epoxidation of violaxanthin and antheraxanthin to zeaxanthin (Neubauer and Yamamoto 1992; Demmig-Adams and Adams 1996); (2) in the water–water cycle, as a electron donor for APX in H_2O_2 scavenging (Asada 1999); (3) in the prevention of lipid peroxidation, through regeneration of oxidized tocopherol (Foyer and Shigeoka 2011; Szarka et al. 2012), and (4) in the protection of PSII reaction centres from photo-oxidation, serving as an as an alternative electron donor to PSII *in vivo*

under conditions where the oxygen-evolving complex is inactivated (Mano et al. 1997; Tóth et al. 2009, 2011; Nagy et al. 2012; Munné-Bosch et al. 2013).

Cytosolic APX (Karpinski et al. 1997; Mullineaux and Karpinski 2002; Fryer et al. 2003), Asc (Bartoli et al. 2006; Zechmann et al. 2011; Heyneke et al. 2013), other components of the Asc–GSH cycle (Gechev et al. 2003; Heyneke et al. 2013; Szechyńska-Hebda and Karpiński 2013), anthocyanins and flavonols (Neill and Gould 2003; Agati et al. 2009; Pollastri and Tattini 2011), α-tocopherol (Munné-Bosch et al. 2013), and xanthophylls (Rockholm and Yamamoto 1996; Lichtenthaler 2007) are all HL-induced components of antioxidative defence.

High light provokes rapid enhancement (already after 1 h) of the leaf Asc content, depending on the irradiance increase (Bartoli et al. 2006; Page et al. 2012; Noshi et al. 2016; Vidović et al. 2016b), supporting the significance of Asc in photoprotection. Briefly, HL induced a 2–3 fold increase of the total content Asc in the leaves of various plant species, both, after short-term (<1 day) (Bartoli et al. 2006; Zechmann et al. 2011; Gest et al. 2012; Laing et al. 2017), and after long-term exposure (1–15 days) (Golan et al. 2006; Page et al. 2012; Heyneke et al. 2013; Vidović et al. 2015a, b).

A deeper immunolocalization study of subcellular changes of the Asc content after short- and long-term HL stress in the mesophyll cells of Arabidopsis showed that increasing irradiance from 700 and 1500 μmol $m^{-2}s^{-1}$ progressively elevated the Asc content in chloroplasts and vacuoles (Heyneke et al. 2013). At very high PAR intensities, Asc was detected within the lumen of thylakoids. After a 2-week exposure to 700 μmol $m^{-2}s^{-1}$, Asc increased in all compartments. The most drastic increase in Asc relative distribution was in vacuoles (4 times) and it doubled in chloroplasts. In both cases, the Asc relative ratio in peroxisomes decreased under HL. The Asc increase in chloroplasts is in accordance with the above-mentioned photoprotective roles. Ascorbate increase in vacuoles is related to an H_2O_2-scavenging system that comprises PODs and phenolics (see Sect. 2.1), which are usually increased under HL (Ferreres et al. 2011; Zipor and Oren-Shamir 2013). In order to elucidate the mechanisms of H_2O_2 regulation in green and white tissues with respect to the photosynthetically dependent generation of ROS, we exposed variegated leaves of *P. zonale* to strong sunlight (>1000 μmol $m^{-2}s^{-1}$ PAR; Vidović et al. 2016b). Previously, we revealed that antioxidative defence in photosynthetic cells relied on Asc, tAPX, and CAT, while in non-photosynthetic cells it relied on GSH, Asc–GSH cycle enzymes, and MnSOD and Cu/ZnSOD (Vidović et al. 2016a). Sudden exposure to pro-oxidative conditions caused distinctive responses in these two cell types. Strong PAR increased the Asc level 2.5 times already after 1 h only in green sectors, while in white sectors, soluble APX (mainly consisted of cytosolic isoform) was increased. This can be correlated with a 2.6-fold higher level of Asc in the cytoplasm of white sectors compared with cells from green ones. Under more pro-oxidative conditions, induced by Pq treatment, the Asc level was increased in non-photosynthetic tissue, together with MDAR and DHAR activities. These findings suggest that in non-photosynthetically active cells, a high redox state is maintained by efficient Asc recycling (unchanged Asc content, increased APX, DHAR, and GR activities) rather than Asc biosynthesis, which is favoured in photosyntheti-

cally active cells. These different, tissue-specific strategies in maintaining the Asc redox status may be attributed to the non-photosynthetic tissue dependence for Asc precursors (D-mannose, L-galactose; Smirnoff 2011) deriving from the photosynthetic tissue. The molecular mechanism of regulation of the Asc level and related enzymes coupled to the Asc–GSH cycle in the heterotrophic tissue of variegated leaves should be further examined in the future.

5.1 Ascorbate in Cross-Tolerance to High Light and UV-B Radiation

Besides high PAR, plants are naturally exposed to UV radiation, which makes up about 7% from the total solar radiation, consisting of UV-B (290–315 nm) and UV-A (315–400 nm) radiation (Frohnmeyer and Staiger 2003; Kerr and Fioletov 2008). Initial investigation of the influence of UV-B radiation on plant growth and development revealed its detrimental effects, such as forming cyclobutane pyrimidine dimers in a DNA strand, acceleration of ROS generation, oxidative stress, and damaging of cellular membrane and proteins, including D1 and D2 subunits of PSII, and Rubisco (Jansen et al. 1998; Jenkins 2009; Lidon et al. 2012; Hu et al. 2013). However, these detrimental effects of UV-B were attributed to high fluence rates and artificial UV-B: UV-A: PAR ratio in the growing chambers, or to some other unfavourable factor in the field (high temperature, high PAR, drought) (Aphalo et al. 2012; Hideg et al. 2013). During the last decade, it was revealed that ecologically relevant UV-B radiation is an important environmental cue and regulator of plant growth and development (Jenkins 2009; Heijde and Ulm 2012). The UV Response Locus 8 (UVR8)-dependent signalling pathway is responsible for the expression of protective genes involved in the biosynthesis and metabolism of flavonoids and antioxidative defence, related to the GSH metabolism (e.g. GR, GSH-peroxidase, GSH-*S*-transferase, PRXs, and glutaredoxin; Brown et al. 2005; Favory et al. 2009; Wu et al. 2012a).

High levels of UV-B radiation decreased Asc redox state in the leaves of pea (Kalbin et al. 1997) and barley (Hideg et al. 2006) as a consequence of increased ROS accumulation. However, ecologically relevant UV-B radiation activates an antioxidative defence via UVR-8 signalling. The UVR-8-dependent signalling pathway includes constitutively photomorphogenic 1 (COP1), elongated hypocotyl 5 (HY5), and HY5 homologue (HYH) components already involved in HL signalling (Vidović et al. 2017). Therefore, under natural conditions, plant responses to solar radiation are a combination of UV-A, UV-B, and high PAR responses, and they might overlap, implying cross-tolerance (Bolink et al. 2001; Behn et al. 2010; Götz et al. 2010; Klem et al. 2012; Majer and Hideg 2012).

PPA and Asc/APX systems were responsible for efficient H_2O_2 scavenging and high Asc redox state in the photosynthetically active tissue of *P. coleoides* leaves after 10-day exposure to HL, Fig. 2 (Vidović et al. 2015b). UV-B radiation in combination with high PAR induced no changes in the Asc redox state, but diminished

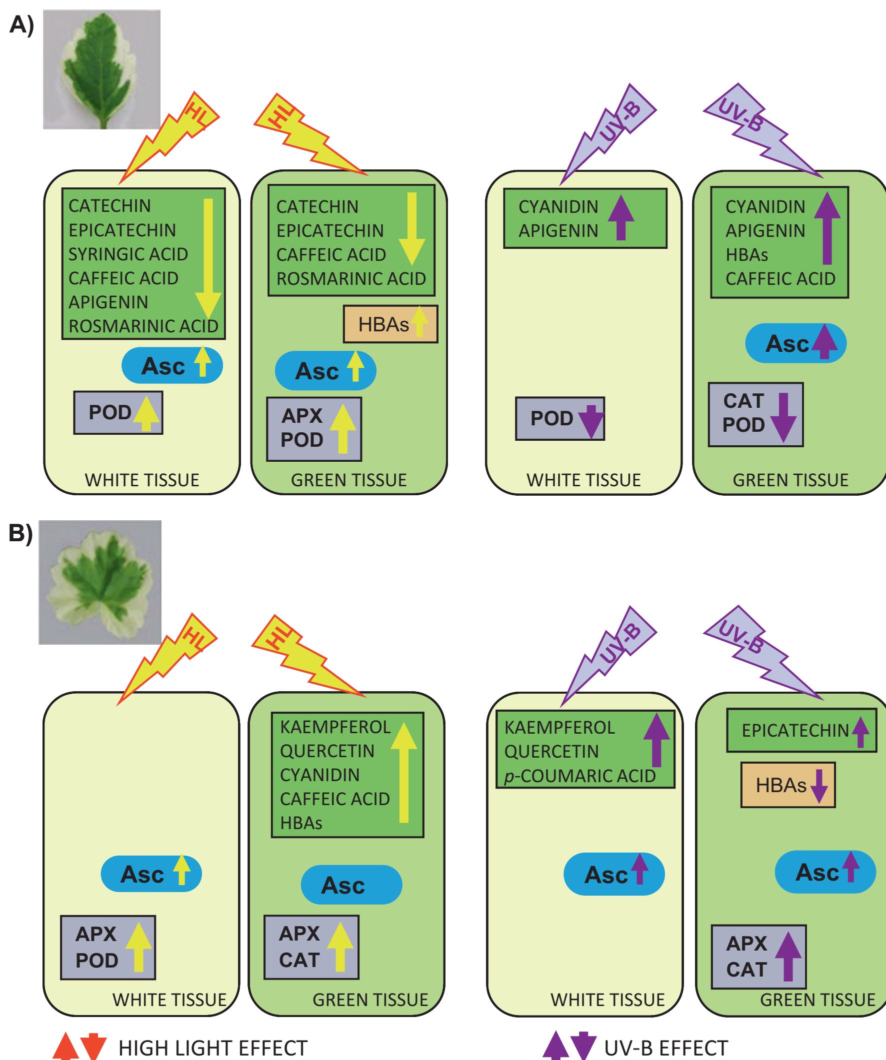


Fig. 2 Schematic overview of high light (HL) and UV-B radiation effect on the H_2O_2 regulating system in variegated leaves. (**a**) Variegated *P. coleoides* and (**b**) *P. zonale*. Arrow directions indicate increased or reduced metabolite content/enzyme activity. Plants were exposed to: UV-B_{BE}: 7.0 kJ m^{-2} day^{-1} combined with 48.8 mol m^{-2} day^{-1} of PAR (HL). *HBAs* hydroxybenzoic acids, *APX* ascorbate peroxidase, *CAT* catalase, *POD* class III peroxidase. For detailed results, see Vidović et al. (2015a, b)

POD and CAT activity, indicating that UV-B radiation (independently of background PAR) did not provoke oxidative stress, Fig. 2. This is in accordance with the overall observation that UV-B-mediated stress in plants in the field is relatively rare (Ballaré et al. 2011; Hideg et al. 2013).

High light in combination with UV-B radiation provoked greater enhancement of APX and CAT activities and Asc accumulation than without UV-B radiation, indicating that the two factors synergistically stimulate antioxidative defence only in green sectors of variegated *P. zonale* leaves, Fig. 2 (Vidović et al. 2015a). Similarly, high PAR-induced antioxidative defence in bean and pea plants previously treated with UV-B radiation was stronger than under high PAR alone, implying a protective role of UV-B under high PAR intensity (Bolink et al. 2001). Moreover, in tobacco leaves, UV-B-acclimation responses overlapped with HL responses, while a higher Asc redox state under UV-B radiation was observed in previously HL acclimated tobacco plants (Majer and Hideg 2012).

5.2 Ascorbate and Anthocyanins Biosynthesis

Biosynthesis of flavones, flavonols, and anthocyanins comprises hydroxylation of C-ring catalysed by Asc-dependent Fe^{2+}/2-OGDs: flavanone 3-β-hydroxylase (F3H), flavonol synthase (FLS), and anthocyanin synthase (ANS) (Springob et al. 2003; Turnbull et al. 2004; Martens et al. 2010). Giacomelli and co-workers (2006, 2007) reported that Asc-deficient Arabidopsis mutant *vtc2-1* accumulates much less anthocyanins under HL. In addition, Page et al. (2012) showed that *vtc1*, *vtc2,* and *vtc3*, mutants under high PAR (Table 4), produced lower amounts of cyanide glycoside, while the content of kaempferol glycosides was not changed. An additional analysis with *cat2* mutants with only 20% residual CAT activity compared with WT, showed diminished anthocyanin accumulation and lower expression of genes involved in anthocyanin biosynthesis, glycosylation, and sequestration, as well as two known regulatory MYB transcription factors production of anthocyanin pigment 1/2 (PAP1/PAP2). These results indicated that peroxisomal H_2O_2 might be involved in the regulation of anthocyanin biosynthesis (Vanderauwera et al. 2005). On the other hand, mutants deficient in cytosolic APX (APX1) and tAPX showed increased levels of cyanidin glycosides after exposure to HL (Miller et al. 2007). It should be noticed that all these mutants deficient in APX isoforms or CAT do not exhibit the significant difference in the Asc content, Table 4. Based on these studies, an accumulationof H_2O_2 might be important for HL-dependent induction of anthocyanins.

In addition, Maruta and co-workers (2014) showed that Pq treatment induced marked accumulation of anthocyanins and up-regulation of all main genes involved in the phenylpropanoid anthocyanin pathway in WT Arabidopsis plants, even under 100 μmol m^{-2} s^{-1}. Studies using mutants with decreased tAPX additionally supported the findings that chloroplastic H_2O_2 generated under photooxidative stress enhanced the expression of the genes involved in the regulation and biosynthesis of

Table 4 Relation between Asc content and high PAR induced anthocyanin (Anth) and flavonol (Fl) accumulation determined in Arabidopsis mutants compared to the WT plants

A. thaliana mutant	[Asc], mutant/WT ratio	[Anth], mutant/WT ratio	[Fl], mutant/WT ratio	High PAR, μmol m^{-2} s^{-1}	References
vtc1	0.17	0.08	1.11	550–650	Page et al. (2012)
vtc2-1	0.20 0.23	0.30 0.11	n.a. 1.11	1000 550–650	Giacomelli et al. (2007); Page et al. (2012)
vtc2-2	0.07	0.03	0.91	550–650	Page et al. (2012)
vtc3-1	0.21	0.46	0.83	550–650	Page et al. (2012)
apx1	≈1.00	1.22	n.a.	750 + 4 °C[a]	Asai et al. (2004)
tapx	0.90 n.a. ≈1.00[b]	n.a. (Anth↑)[c] 1.50 2.75	n.a. n.a. n.a.	1000 900 1000	Giacomelli et al. (2007); Miller et al. (2007); Maruta et al. (2010, 2014)
sapx	1.02 ≈1.00	n.a. (Anth↑)[c] n.a.	n.a. n.a.	1000 1000	Giacomelli et al. (2007); Maruta et al. (2010)
sapx/tapx	≈1.00	0.80	n.a.	1000	Giacomelli et al. (2007)
apx1/tapx	n.a.	2.46	n.a.	900	Miller et al. (2007)
cat2-2	1–2 n.a.	n.a. abolished[d]	n.a. n.a.	200 + ↑CO_2 1600	Queval et al. (2007); Mhamdi et al. (2010); Vandenabeele et al. (2004); Vanderauwera et al. (2005)
2cp/ *2cpa2cpb*	1.2–1.4 ≈1.00	n.a. 0.33–0.50	n.a. n.a.	75 900	Baier et al. (2000); Awad et al. (2015)
trol	≈1.00	decreased[d]	n.a.	800	Vojta et al. (2015)

n.a. not available, *n.d., apx1* knock-out (KO) mutant deficient in cytosolic APX1 (At1g07890), *tapx* KO mutant deficient in tAPX (At1g77490), *sapx* KO mutant deficient in stromal APX (At4g08390), *cat2-2* KO mutants with 20% of total residual catalase (At4g35090) activity level in WT, *2cp* antisense mutants with decreased 2-cysteine peroxiredoxin (PRX), *2cpa-2cpb* KO mutant deficient in 2-Cys PRX A (At3g11630) and 2-Cys PRX B (At5g06290), *trol* KO mutant deficient in thylakoid rhodanese-like protein (TROL, At4g01050)

[a]Differences in [Anth] between *apx1* and WT was observed only when HL was followed by 4 °C, not by 25 °C

[b]IS-tAPX-19 plants were sprayed with estrogen to induce RNAi for *tAPX* silencing (≈30% reduced tAPX activity)

[c]Only accumulation of Anth similar as in WT was reported

[d]Only relative accumulation of Anth in relation to WT was reported

anthocyanins (Maruta et al. 2014; Table 4). In addition, *2cpa-2cpb* double-mutant plants also generated elevated levels of H_2O_2 under HL stress, but lacked anthocyanin accumulation (Awad et al. 2015). Recently, groups of Smirnoff and Mullineaux (Exposito-Rodriguez et al. 2017) used HyPer2, a genetically encoded fluorescent H_2O_2 sensor, to show that direct H_2O_2 transfer from chloroplasts to nuclei, avoiding the cytosol, enables photosynthetic control over gene expression.

More evidence supporting that the signal for the activation of anthocyanins accumulation derived from photosynthesizing chloroplasts was presented in the study of *Lemna giba* under conditions provoking higher [PQH_2]/[PQ] ratio (Akhtar et al. 2010). In this study, it was demonstrated that chloroplast-derived ROS were not

associated with promoted photosynthetic electron transport chain (PETC) reduction and were not required for flavonoid biosynthesis, Table 4. In favour of these findings, Arabidopsis *trol* mutant deficient in thylakoid rhodanase-like (TROL) protein responsible for interaction with ferredoxin:NADP$^+$ oxidoreductase (FNR) and partitioning between energy-conserving and energy-dissipating pathways, has abolished production of anthocyanins although no difference in Asc content in comparison with WT plants was observed (Vojta et al. 2015; Table 4).

As an additional support for the assumption that PETC and electron partitioning play a crucial role in activating anthocyanins biosynthetic pathways, and that Asc can be involved in this mechanism indirectly, through altering electron flow at PSI via MDAR and DHAR activity, is that HL-induced accumulation of anthocyanins presents an additional valve for energy dissipation. In general, anthocyanins and flavonoids in plants are glycosylated, with two or three sugar moieties per aglycone, and their synthesis in the cells requires triosephosphates, ATP, NADPH, and malonic acid (Hernández and Van Breusegem 2010). Exposing Arabidopsis plants to conditions stimulating an increased CO_2 assimilation rate (including high PAR, glucose supplementation, and nitrogen and phosphate deprivation) resulted in increased expression of 16 enzymes involved in flavonoid biosynthesis, while the darkness and limited CO_2 suppressed anthocyanin production (Vanderauwera et al. 2005).

We propose that the signal for anthocyanins biosynthesis is related to the overreduced linear PETC induced by H_2O_2. The PETC overreduction can be provoked by depletion of Asc since electron flow through the MDAR-dependent alternative sink (Miyake et al. 1998) is decreased (Fig. 3).

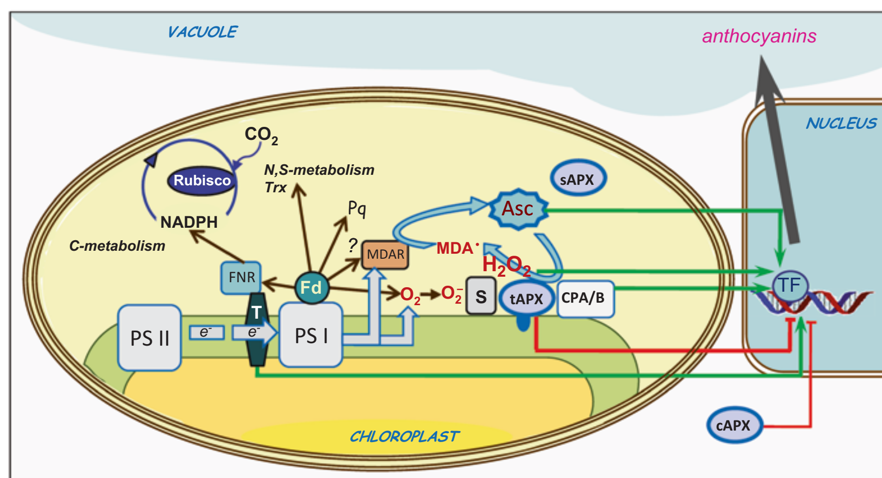


Fig. 3 Schematic overview of the proposed pathways of redox retrograde signalling responsible for anthocyanin induction under HL. The scheme was created according to data presented in Table 3. *PSI/II* photosystem I/II, *Fd* ferredoxin; *FNR* Fd-NADP$^+$ oxidoreductase, *Trx* thioredoxin, *Pq* paraquat, *CPA/B* 2-Cys peroxiredoxins A and B, *sAPX* stromal APX, *cAPX* cytosolic APX, *T* Trol protein, *TF* transcriptional factor

6 Ascorbate and Phytohormones in Cross-Tolerance

In their natural environment, plants are exposed to simultaneous effects of abiotic and biotic stressors, and they have evolved various stress-responsive synergistic and coordinated mechanisms to maintain cellular homeostasis. The term cross-tolerance refers to triggering plant resistance to different stressors by previous exposure to another stressor, and this trait can be very common in plants (Knight and Knight 2001; Pastori and Foyer 2002; Mittler 2006). This indicates an overlap and cross-talk between stress-responsive signalling pathways. Understanding these mechanisms has a great importance for plant breeding and selection of tolerant genotypes. Numerous studies have implicated the signalling role of H_2O_2 and hormones in response to abiotic and biotic stress through modulation of stress-tolerant genes, thus supporting the view of sharing hormonal and ROS signalling pathways under abiotic and biotic stress (Bartoli et al. 2013; Perez and Brown 2014; Nguyen et al. 2016). Although cross-tolerance has been explored in different model plants, there is still a lack of data about plant responses to multiple stressors under field conditions, especially considering the duration and intensity of stressors, species-specific responses and the importance of the plant development stage (Mittler 2006; Hossain et al. 2017). Having in mind that exposure to abiotic and biotic stressors leads to disturbance of metabolic processes which is inevitably accompanied by increased generation of ROS and oxidative stress, the interactions between cellular redox signalling and plant hormones should be considered (Bartoli et al. 2013).

Auxin regulates plant development through the action of transport inhibitor resistant 1/auxin signalling (TIR1/AFB) receptors, auxin/indole-3-acetic acid (Aux/IAA) transcriptional co-regulators and auxin response factors (ARFs) transcription factors (Weijers and Wagner 2016). It was shown that auxin signalling is repressed under biotic and abiotic stresses such as oxidative stress and salt stress (Navarro et al. 2006; Iglesias et al. 2010). The study using a double Arabidopsis mutant *tir1-afb2* showed reduced accumulation of H_2O_2 and $O_2^{\bullet-}$ and increased Asc content as well as the activity of antioxidative enzymes under oxidative and salt stress compared to the WT (Iglesias et al. 2010). The authors suggested that suppression of auxin signalling may enhance stress tolerance. Furthermore, *apx6* mutant seeds showed higher ROS accumulation and oxidative damage compared to the WT. This was accompanied by an increased content of ABA and auxin and their metabolites during drying. The authors suggested a new protective role of APX6 during seed desiccation and germination involving regulation of ROS levels and hormone signalling (Chen et al. 2014). Extensive meta-transcriptomic analysis of gene expression in willow growing in uncontaminated and soil contaminated with petroleum hydrocarbons (Gonzalez et al. 2015) suggested a cross-tolerance mechanism in which resistance to abiotic stress contributed to biotic resistance against spider mite. Potential candidates in the cross-tolerance interactions included secondary metabolites as well as a regulatory network of stress-responsive hormones (ABA, SA, ethylene, and auxin). On the other hand, Shi and co-workers (2014) demonstrated positive regulation of multiple abiotic stress-related genes and antioxidative

enzyme activities contributing to enhanced drought stress resistance in plants pretreated with exogenous IAA and in *iaaM-OX* transgenic lines with higher endogenous IAA levels.

The *abscisic acid* signalling pathway is a central regulator of abiotic stress response in general, including metal excess, HL intensity, and salt stress, triggering changes in gene expression and physiological responses (Galvez-Valdivieso et al. 2009; Zörb et al. 2013; Morina et al. 2016; Saxena et al. 2016). As a primary stress hormone, the increase of ABA coupled with H_2O_2 accumulation was reported following temperature priming (reviewed by Hossain et al. 2017), salt and drought cross-tolerance (Suzuki et al. 2016), and drought and heat cross-tolerance (Zandalinas et al. 2016). It was shown that a number of transcripts which accumulated in response to salt and heat stress combination in Arabidopsis were also upregulated by light stress. Compared to other hormone-induced transcripts, those associated with ABA under heat and salt combined stress were the most highly represented (Suzuki et al. 2016). Furthermore, using Arabidopsis *abi1-1* mutants (with lower ABA content) Zandalinas and co-workers (2016) showed that the levels of cytosolic APX1 and multiprotein bridging factor 1c (MBF1c) proteins, which are required for acclimation to a combination of water deficit and heat stress, were significantly reduced compared to WT, thus suggesting the key role of ABA in regulation of proteins important for cross-tolerance to drought and heat stress.

In order to elucidate whether plants differing in metal tolerance differ in sensitivity to drought stress, we used previously characterized *V. thapsus* plants from two populations, metal tolerant (Vt) and sensitive (Vs) (Morina et al. 2016). A higher content of reduced and total Asc was detected in well-watered Vt than in Vs, while water stress (5% H_2O in soil compared to 30% for well-watered plants) caused a decrease in total leaf area to a higher extent in Vs than in Vt, accompanied by higher inhibition of stomata opening (Prokić et al. 2013). The total Asc content decreased in the leaves of drought-stressed Vt and increased in the leaves of Vs reaching similar values. Moreover, the activity of APX was unchanged by drought in Vt but not in Vs, while 1.5 h after re-watering, APX increased in the leaves of plants from both populations, although to a higher extent in Vt (unpublished results). These results imply that a constitutively higher Asc content and capacity for APX induction in metal tolerant plants can contribute to drought cross-tolerance. In addition, the decrease in Asc content after re-watering may indicate an increased oxidative load although further work is needed to understand the events occurring during rehydration.

Salicylic acid is a phenolic phytohormone, with a key role in both regulation of plant growth and development, and in the responses to environmental stresses (Rivas-San Vicente and Plasencia 2011; Bartoli et al. 2013). Besides its well-recognized role in basal resistance, effector triggered and induction of systemic acquired resistance in response to pathogen attack, it regulates responses to drought, metal tolerance, cold, and heat and osmotic stress (reviewed by Rivas-San Vicente and Plasencia 2011; Miura and Tada 2014). Exogenous application of SA may enhance antioxidative capacity and tolerance to stress (Horváth et al. 2015; Giménez et al. 2017); however, at higher concentrations, depending on the species, it increases

ROS accumulation and plant sensitivity to abiotic stress (Hayat et al. 2010; Miura and Tada 2014). Salicylic acid signalling mediates activation of defence genes via its receptor, non-expressor of pathogenesis-related genes1 (*NRP1*), and its two homologues, *NPR3* and *NPR4* (Moreau et al. 2012; Wu et al. 2012b). A low apoplastic Asc concentration in the *vtc1* mutant correlates with higher ROS production under stress, followed by enhanced SA biosynthesis and expression of SA-regulated defence signalling pathways (Brosche and Kangasjarvi 2012). The same study showed that another stress hormone, JA, interacts with Asc and GSH in the regulation of the defence-gene expression. Interestingly, NPR1 translocation from cytoplasm to nucleus is redox regulated, while a number of SA-binding proteins (SABPs) identified, including CAT and cytosolic APX, are involved in redox regulation, thus further linking SA signalling with redox regulation (Manohar et al. 2015 and references therein). A recent study indicated the role of DHAR in activation of signalling pathways mediated by SA through alterations in intracellular GSH status under oxidative stress (Rahantaniaina et al. 2017). Exogenous SA increased drought resistance in wheat through increased accumulation of Asc and GSH as well as transcripts of GSH-transferase 1/2 (GST1/2), GR, and MDAR (Kang et al. 2013).

Jasmonates are multifunctional cyclopentanone phytohormones deriving from fatty acids metabolism. They are involved in plant development and reproduction, fruiting and senescence, as well as defence responses (Wasternack and Hause 2013). Jasmonic acid activates defence responses to pathogen attack and herbivory, but also to abiotic stresses such as drought, salinity, and UV radiation, thus conferring its role in cross-tolerance to both biotic and abiotic stresses. It was shown that ABSCISIC ACID INSENSITIVE-4 (ABI4) and Asc regulation of growth and defence-gene expression were modulated thought JA signalling using Arabidopsis mutants *abi4, vtc1*, and *vtc2*. Interestingly, there was a significant overlap in transcription signatures of all three mutant lines (Kerchev et al. 2011). The authors proposed that low Asc levels triggered JA and ABA-dependent signalling pathways. Stimulating effects of exogenous methyl-JA on Asc biosynthesis were shown in Arabidopsis and tobacco BY-2 suspension cells (Wolucka et al. 2005). Enhanced transcription of methyl-JA-responsive genes involved in Asc biosynthesis was observed, leading to its increased *de novo* synthesis, and in turn provided redox homeostasis in plant cells. Increased Asc content and/or enzymes in the Asc–GSH cycle in relation to elevated endogenous JA under water stress were reported in several studies (Jubany-Mari et al. 2010; Shan and Liang 2010; Brossa et al. 2011). Integrated action between Asc, JA, and ABA in modulation of water stress responses has been proposed in a study showing higher sensitivity of JA deficient (*aos*), *aba2,* and *vtc1* mutants compared to WT (Brossa et al. 2011).

The role of Asc in SA and JA signalling pathways has been noted in several studies. Mukherjee and co-workers (2010) showed that *vtc1-1, vtc2-1*, and *vtc3-1* mutants were more tolerant to a bacterial pathogen than WT, and this was attributed to up-regulated H_2O_2 levels and higher abundance of SA and mRNA of pathogenesis-related (PR) genes. The authors suggested that elevated H_2O_2 levels due to Asc deficiency may act as a priming mechanism that stimulates SA accumulation and increases disease resistance. Similar observations of enhanced basal resistance to

pathogens and constitutive PR genes and other SA-inducible proteins were observed in *vtc* mutants (Kiddle et al. 2003; Pavet et al. 2005; Brosche and Kangasjarvi 2012), which in turn had inhibited growth, suggesting a negative correlation between cell growth and activation of innate immune responses under Asc-deficient conditions.

Ethylene modulates a variety of processes in plants, including germination and growth, senescence, fruit ripening, and stomatal opening, as well as growth arrest and programmed cell death in biotic and abiotic stress responses (Skirycz et al. 2011; Mase et al. 2013; Poór et al. 2013; Wang et al. 2013; Zhang et al. 2016). The ethylene signalling pathway starts with endoplasmic reticulum-localized receptor kinases. The following ones were identified in Arabidopsis: ethylene response1 (ETR1), ETR2, ethylene insensitive4 (EIN4), ethylene response sensor1 (ERS1), and ERS2 (Zhang et al. 2016 and references therein). Transcription factor genes, ethylene-responsive factors, such as ERF1, which activates a specific set of stress response genes, are direct targets of EIN3. Involvement of ethylene in cross-tolerance is supported by reports showing that ERF gene expression is common for a number of abiotic stresses (salt, drought, cold, light) as recently reviewed by Müller and Munné-Bosch (2015). ERF is a large gene family including 122 genes in Arabidopsis and the roles of different ERFs (e.g. ERF105 and ERF74) have recently been explored (Bolt et al. 2017; Yao et al. 2017).

The direct effects of ethylene on Asc content and redox state, in relation to senescence, were investigated using detached spinach leaves treated with ethephon and stored in the dark (Gergoff et al. 2010). The authors showed a rapid decrease in Asc and increase in DHA content without effects on MDAR and DHAR activities before senescence parameters changed. Ethylene-insensitive Arabidopsis mutants (*ein3-1* and *ein4*) had a higher Asc content than WT before darkness treatment and showed a slower decrease in Asc content than WT during dark-induced senescence, thus indicating the important role of ethylene in modulating Asc levels in leaves. Similarly, it was shown that AtERF98 regulates Asc levels through direct control of genes involved in Asc biosynthesis. The knockout mutant *aterf98-1* had increased sensitivity to salt stress due to inhibited Asc synthesis (Zhang et al. 2012).

Interestingly, it was observed that ERF1 expression is rapidly induced by JA, implying synergistic signalling pathways of both JA and ethylene in defence responses (Lorenzo et al. 2003; Cheng et al. 2013; Huang et al. 2015). Another ethylene response factor, JERF3, regulates the oxidative stress response and expression of antioxidative enzymes, including SOD, APX1, APX2, and GPX, contributing to increased tolerance to salt, drought, and cold stress in tobacco (Wu et al. 2008). Similarly, a positive regulation of antioxidants (MDAR3, CAT3) under abiotic and biotic stress by ERF6 in Arabidopsis was shown by Sewelam and co-workers (2013). On the other hand, Mehlhorn (1990) demonstrated that ethylene pre-treatment of *Vigna radiata* and *Pisum sativum* increased tolerance to O_3, H_2O_2, and Pq through enhanced activity of APX.

Ascorbate can influence ethylene and ABA levels and signalling, as shown by Pastori and co-workers (2003). Exogenous Asc up-regulated the ethylene-responsive transcription factor in *vtc1*. Furthermore, the role of Asc in hormone-mediated signalling pathway linking O_3 and pathogen response was also demonstrated (Conklin

and Barth 2004) through up-regulated defence genes. It seems likely that ERF and ROS act synergistically in the regulation of stress-responsive genes; however, more research is needed to fully understand these mechanisms in cross-tolerance.

In addition to hormones mentioned in this chapter, the synergistic/antagonistic effects of other hormones such as cytokinins, gibberellins, and brassinosteroids and redox regulation in stress response should be studied in more detail.

The cross-talk between hormone-mediated responses to the environment and ROS signalling is largely determined by the spatial and temporal accumulation of ROS (Xia et al. 2015; Saxena et al. 2016). This perspective has received great attention; however, the data regarding subcellular ROS and hormone function are still emerging and more research is needed to fully understand this complex integrated signalling network.

In conclusion, further work is needed to fully understand the role of hormones in the regulation of cellular redox responses to abiotic and biotic stressors in different plant species and *vice versa*, especially in relation to cross-tolerance mechanisms.

7 Role of Ascorbate in Response to Drought

One of the most important constraints to plant growth and crop productivity nowadays is water deficit and global warming (Chaves et al. 2003). Climatic models predict that global warming will further escalate drought as a result of increasing evapotranspiration and decreased rainfall in many regions all over the world (Salinger 2005; Metz et al. 2007). Hallmarks of drought stress are stomatal closure, regulated by hydraulic and chemical (ABA) signals (Tombesi et al. 2015), and changes in apoplastic and xylem pH which mediate ABA distribution (Gloser et al. 2016). Drought stress promotes ABA biosynthesis through up-regulation of *NCED3*. Significant increases in ABA levels are detected in immediate plant responses to water deficit (McAdam and Brodribb 2016). In addition to biosynthesis, drought promotes deconjugation of ABA-glucose esters stored in the vacuoles enabling its circulation within the plant (Seiler et al. 2011).

Besides stomatal closure, plant's response to drought stress includes reduced growth, accumulation of osmotic compounds, lignification, changes in antioxidative metabolism, and induction of senescence (Chaves et al. 2003). Three main sites of ROS production during drought stress have been recognized: H_2O_2 generation in peroxisomes, due to increased activity of glycolate oxidase (Noctor et al. 2002), and chloroplasts, due to acceleration of the Mehler reaction, and accumulation of $^1O_2$ at PSII (De Carvalho 2008; Noctor et al. 2014). In order to maintain cellular redox balance under drought-induced oxidative pressure, plants employ an efficient antioxidative network. However, the response of antioxidative enzymes and low molecular weight antioxidants to drought is very variable and inconsistent reports can be found, related to the severity of stress, plant development stage, species, and stress duration.

Perception of drought in plants is inevitably linked to hormone and ROS-regulated signalling pathways. Under water deficit conditions, H_2O_2 accumulates in the stomata functioning as an intermediary in ABA-induced stomatal closure (Pei et al. 2000; Zhang et al. 2001). Under physiologically optimal conditions, Chen and Gallie (2004) observed that the levels of Asc redox state and H_2O_2 in both guard cells and the whole leaf have diurnal dynamics and are negatively correlated. In the afternoon, the H_2O_2 level is lower while the redox state of Asc is higher. Furthermore, in plants overexpressing *DHAR* stomata were open and less responsive to H_2O_2 and ABA signalling, while suppressed *DHAR* expression contributed to drought tolerance. Exogenously applied ABA increased drought tolerance in wheat, and this was partly explained by elevated contents of GSH and Asc in leaves and roots as well as up-regulation of genes regulating enzymes of the Asc–GSH cycle (Wei et al. 2015). A study of early response to water deficit in soybean (Xing et al. 2016) showed that already 3 h after the onset of drought, the levels of ABA, H_2O_2, and $O_2^{\bullet-}$ significantly increased, simultaneously with up-regulation of Asc and GSH levels as well as induction of enzymes in the Asc–GSH cycle. Addition of both ROS scavengers and the ABA biosynthesis inhibitor diminished the induction of antioxidative metabolism. On the other hand, a study by Bartoli and co-workers (2005) on two wheat cultivars with contrasting levels of Asc showed no change in Asc levels under drought, regardless of the increased abundance of the L-galactono-1,4-lactone-dehydrogenase (GalLDH, EC 1.3.2.3), enzyme involved in the last step of Asc biosynthesis, localized in mitochondria. However, although both cultivars reacted to drought in a similar way regarding the decrease of stomatal conductance and net CO_2 assimilation, the cultivar with higher Asc content was able to recover to the control state upon rehydration, unlike the sensitive one.

As roots are the first organs to experience and sense water deficiency, recent reviews and studies have focused on the redox changes and mechanisms of root to shoot signalling through ABA, including changes in the antioxidative metabolism and activation of various transcription factors (Janiak et al. 2015). In a study by Pyngrope and co-workers (2013), comparison of responses to drought of two rice cultivars (drought sensitive and drought tolerant) showed that in the sensitive cultivar drought induced a decrease in Asc and increase of DHA to a higher extent than in the tolerant one, suggesting that the tolerant cultivar was more efficient in maintaining the redox state under drought-imposed oxidative stress. This was confirmed by a lower ROS content in the seedlings of the tolerant cultivar following polyethylene glycol treatment.

Although these results imply that Asc metabolism may be differentially affected by drought (increased, decreased, unchanged) as discussed recently by Noctor and co-workers (2016), it should be emphasized that the majority of studies report Asc and GSH levels in the whole tissue, thus not distinguishing changes on the subcellular level. Only a few studies report on temporal changes in subcellular Asc distribution under drought (see Sect. 4.1). The involvement of Asc and GSH during advanced drought treatment is supported in the study by Koffler and co-workers (2014b), where WT plants were better adapted to drought conditions than the mutants deficient in Asc (*vtc2-1*) and GSH (*pad2-1*) as they had less visible symptoms, an absence of necrosis and the highest NPQ levels.

Furthermore, the importance of Asc in the process of rehydration should be investigated in more detail since under natural conditions periods of drought are replaced by re-watering. In this respect, Brossa and co-workers (2013) reported differential effects of water stress on the redox status in mitochondria and cytosol in Arabidopsis and the *vtc2* mutant. Differential effects of drought on Asc and DHA contents were observed in young and old leaves, in both WT and *vtc2* (Table 1). An increased total Asc content and increased DHA content was more pronounced in younger leaves under water stress, while following rehydration the DHA content decreased. The dynamics in the total leaf Asc redox state was related to changes in the redox potential, in the cytosol and mitochondria determined by a redox-sensitive, compartment-specific molecular probe, roGFP, while no effects in GSH levels were observed (Brossa et al. 2013). The authors suggested the existence of an alternative route for Asc regeneration and accumulation besides the Asc–GSH cycle. Another recent study showed that the *vtc1* mutant (Table 1) was more sensitive to drought stress than WT regardless of increased GSH levels after 48 h of drought (Niu et al. 2013).

The importance of cytosolic isoforms of APX and Cu/ZnSOD in plant response to drought stress has been emphasized in a number of studies (Mittler and Zilinskas 1994; Rossel et al. 2006; Miller et al. 2007; Sofo et al. 2015). Transgenic tobacco lines with two copies of cytosolic APX (line 51) and one copy of cytosolic APX and cytosolic Cu/ZnSOD (line 51) were shown to be more tolerant to mild drought stress compared to the WT (Faize et al. 2015). Transgenic lines were less susceptible to drought-induced oxidative stress, evident by a lower amount of H_2O_2, thiobarbituric acid reactive substances (TBARs) and a lower percentage of electrolyte leakage, at the same time having a higher water use efficiency and better growth performance. Following initial decrease of activity, APX increased in response to drought with time in the roots of soybean seedlings (Kausar et al. 2012), similarly as in an experiment with four *Prunus* hybrids where about 3 weeks after onset of water stress activities of APX, MDAR, and DHAR increased in parallel to Asc and DHA accumulation and continued to increase compared to well-watered plants with severity of the drought treatment. The increase in GR activity was delayed compared to other antioxidative enzymes; however, it was also parallel to the accumulation of GSH. Interestingly, the level of H_2O_2 continually increased with drought treatment (70 days), while, after re-watering, the activity of the Asc–GSH cycle was down-regulated to control levels, possibly due to decreased accumulation of ROS and requirement for their detoxification (Sofo et al. 2005).

A recent study on drought stress effects in soybean showed a decrease in Asc but not in DHA in the leaves and stems. These results were correlated with the activity of GalLDH; however, no difference in the level of GalLDH transcripts was observed between well-watered and drought-stressed plants (Seminario et al. 2017). The authors suggested possible post-translational modifications of the GalLDH enzyme, opening new questions about regulation of the Asc metabolism in abiotic stress. A number of studies investigated the role of miRNA in the regulation of gene expression at the transcription and post-transcription level in drought-tolerant plants (reviewed by Zhang 2015; Xie et al. 2017). Besides targeting transcription factors,

miRNA may target genes involved in abiotic stress response, in particular, the antioxidative response. For example, it was shown that all three *CSD* genes in Arabidopsis are targeted by heat-inducible miR398 (Guan et al. 2013). Another study by Lin and co-workers (2012) demonstrated that expression of miR828 in response to wounding controls H_2O_2 and lignin accumulation through repression of *IbMYB* and *IbTLD*. In transgenic tobacco overexpressing *IbTLD* expression levels of *NtAPX*, *NtCAT,* and *NtCZS* were up-regulated, indicating that *IbTLD* may regulate genes coding for antioxidative enzymes in response to wounding. Possible regulation of other components of the Asc metabolism by miRNA in response to drought should be investigated further.

8 Role of Ascorbate Under Metal Excess

Over the last decades, environmental pollution by metals and metalloids has raised great concern due to their persistence in the ecosystem, their ability to bioaccumulate, and the tendency for bio-enrichment through the food chain. Excess metals in soils are mainly the result of industrial, mining and urban activities, and agriculture. Their bioavailability depends on a number of factors, including the pH, amount of organic matter, soil cation exchange capacity, etc. While some metal ions are essential for plant growth and development, such as Zn, Cu, Fe, and Mn, others are non-essential (Pb, Cd, Al) and they compete for uptake with other elements causing metabolic disturbances.

The toxicity mechanisms of excess metals include increased generation of ROS leading to redox perturbations and modifications in antioxidative defence; displacement of essential cations (e.g. Mg in chlorophylls); and interactions with proteins especially with histidyl, carboxyl, and thiol groups leading to their dysfunction (reviewed by Sharma and Dietz 2009; Hossain et al. 2012; Küpper and Andresen 2016).

Overall, the result of metal toxicity is inhibition of photosynthesis and energy production, lipid peroxidation and DNA damage, and finally reduced plant growth and productivity. It should be mentioned that the effects of metal toxicity are species-specific, and strongly depend on the concentration and duration of metal exposure, as well as on the plant developmental stage (Anjum et al. 2014; Koffler et al. 2014a). Redox-active metals can directly induce $O_2^{\bullet-}$ and subsequently H_2O_2 and $^{\bullet}OH$ production via Haber-Weiss and Fenton reactions (reviewed by Demidchik 2015). Inside the cell, metal ions are mostly bound by ligands: amino acids, organic acids, phytochelatins, and metallothioneins (Hossain et al. 2012). However, in the root apoplast, Asc is in direct contact with metal ions and may act as a pro-oxidant (see Sect. 3). On the other hand, redox-inactive metals such as Cd, Ni, and Zn induce ROS through indirect mechanisms, such as inhibition of antioxidative enzymes (Morina et al. 2010; Anjum et al. 2014) and stimulation of the activity of NADPH oxidase (Horemans et al. 2007). An additional mechanism for Zn-induced oxidative stress was proposed through stabilization of phenoxyl radicals in the cell

wall leading to accumulation of charge transfer complex, QH, which exhibits prooxidant behaviour (Morina et al. 2010).

Initial events related to metal excess sensing occur in the apoplast. Zinc treatment induced a shift towards a more oxidized state in the apoplast (decreased Asc/DHA ratio) in the roots but not in leaves of *V. thapsus* (Morina et al. 2010). Strong accumulation of DHA in roots was attributed to the accumulation of a QH located in the cell wall, and to inhibition of activities of all enzymes in the Asc–GSH cycle. Similarly, Zn decreased the activity of AO activity in *Zea mays* roots, while Cu had no effect (Vuletić et al. 2014), and Cd inhibited AO activity in barley roots (Tamás et al. 2006). Furthermore, application of exogenous Asc ameliorated the toxic effects of NaCl (Shalata and Neumann 2001).

Information on metal-induced changes in Asc content in the apoplast is scarce. For example, Fecht–Christoffers and co-workers (2003) implicated that apoplastic Asc and soluble PODs were involved in Mn tolerance in a study with two *Vigna unguiculata* cultivars. In this study, Mn induced a decrease in total Asc content and redox state in the apoplast of the sensitive cultivar, but had no effect on the tolerant one. Apoplastic DHA can be transported in the cell (Horemans et al. 2000), altering the cell cycle, similarly as observed for BY-2 tobacco cell lines (Potters et al. 2004). The same authors suggested that a challenging environment, such as metal-imposed oxidative stress, and the effects of redox signals (increased DHA content) act in a similar way, so that the cell cycle is halted allowing the cell to adjust to the new environment.

Ascorbate biosynthesis and recycling through the Asc–GSH cycle have been implicated in metal tolerance in non-hyperaccumulating species. Some of these aspects have been extensively discussed in recent reviews (Hossain et al. 2012; Bielen et al. 2013; Anjum et al. 2014). Moreover, the role of the Asc metabolism for maintaining the redox state and metal tolerance was observed in Zn/Cd hyperaccumulator *Arabidopsis halleri*. A higher level of expression of *APX1, APX3,* and *MDAR4* was observed in *A. halleri* compared to Arabidopsis, which was also confirmed by higher APX activity and higher efficiency in H_2O_2 detoxification (Chiang et al. 2006). Similarly, Opdenakker and co-workers (2012) showed that expression of *APX1* was induced in early response in Arabidopsis roots to Cu, contrary to Cd treatment which had no effect on this gene. The same authors proposed a possible signalling pathway mediated by transcription factor ZAT12 which induces *APX1* expression in response to ROS, thus emphasizing the role of cytosolic APX1 in Cu-induced oxidative stress. Indeed, in our study, Zn increased the activities of APX and MDAR in the shoots of *V. thapsus* (Morina et al. 2010). However, in addition to efficient Asc recycling, its biosynthesis, shown by the increased activity of GalLDH, was stimulated as well. In addition, the levels of total and reduced GSH decreased by Zn in both tissues indicating interrupted Asc recovery beyond MDAR activity (unpublished). Lucini and Bernardo (2015) showed that long-term exposure to Zn increased the abundance of GalLDH in lettuce. Depletion of Asc and increase of DHA content under Cd treatment were reported in rice leaves (Chao et al. 2010). In this time-course study, it was shown that oxidation of Asc was associated with the increase of the $MDA^{\bullet}$ content, indicating its antioxidative role. In addition, when plants were pre-treated with exogenous Asc or

L-galactono-1,4-lactone (GalL), its precursor, toxicity effects of Cd were alleviated as well as the decrease in Asc content. Interestingly, exogenous Asc and GalL increased the expression of *OsAPX2*, *OsAPX3*, *OsAPX4*, *OsAPX5*, *OsAPX6*, *OsAPX7*, and *OsGR1* genes. Addition of exogenous Asc increased the content of cell wall-bound proteins and proline in the roots of Cu-treated wheat thus contributing to ameliorating effects against Cu toxicity.

The role of Asc in metal tolerance has been confirmed by other transgenic and mutant studies. Overexpression of Arabidopsis cytosolic *DHAR* in tobacco roots led to increased tolerance to Al observed through lower H_2O_2 accumulation and lipid peroxidation, and increased content of Asc and APX activity compared to the WT (Yin et al. 2010). Interestingly, overexpression of cytosolic *MDAR* did not contribute to Al tolerance although the Asc level was higher than in the WT in the absence of Al. The results indicate that efficient recovery of Asc through DHA reduction in the cytosol may provide antioxidative defence both in the apoplast and symplast. However, these may be limited by available GSH in the cell.

The fate and metabolism of Asc in the apoplast under Zn excess is presented in Fig. 4 in a simplified form in order to emphasize its multiple involvements in several aspects of metal toxicity and regulation of antioxidative stress response. Further work is needed for better understanding of redox reactions in the apoplast and cell wall which act as signals towards intercellular space and distinct organelles.

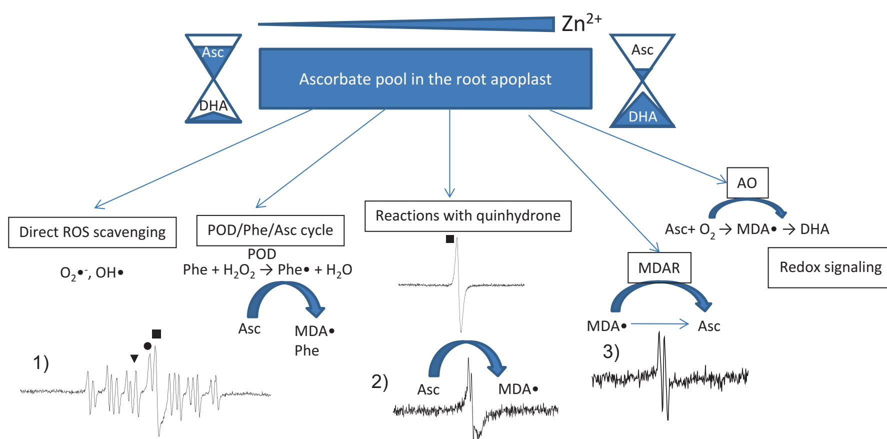


Fig. 4 Zn-induced changes in the redox state of apoplastic Asc in the roots of *V. thapsus* plants. Increasing Zn accumulation in the root cell wall contributes to stabilization of phenoxyl radicals and formation of quinhydrone (QH) which can directly react with Asc. Direct ROS scavenging and activities of POD and AO all contribute to depletion of Asc and accumulation of DHA, while MDAR enables Asc recycling. (1) EPR spectrum of isolated root cell wall of Zn-treated plants showing a characteristic peak of DEPMPO/OH (filled circle), DEPMPO/CH_3 (filled inverted triangle) and QH (filled square); (2) EPR spectrum of QH (filled square) in isolated root cell wall, and combined with $MDA^•$ immediately after addition of Asc; (3) EPR spectrum showing a characteristic peak of $MDA^•$. For more details, see Morina et al. (2010). *AO* ascorbate oxidase, *MDAR* monodehydroascorbate reductase, *POD* class III peroxidase

9 Ascorbate as a Major Player in Growth-Defence Trade-Offs

Reprogramming growth and development is a key to plant survival under non-favourable conditions. A growth arrest is one of the critical responses to various environmental stresses (e.g. Acevedo et al. 1971; Cramer and Bowman 1991; Munns 2002, and references therein). These trade-offs have profound implications for agriculture and natural ecosystems (Huot et al. 2014). While this concept may be explained by resource restrictions and reallocation of photosynthates from growth towards defence under abiotic stresses, molecular mechanisms of the underlying growth and defence trade-offs remain to be elucidated. Plants possess a complex and well-orchestrated response network to adverse stimuli with the essential role of Asc metabolism in stress tolerance mechanisms (Fig. 5). In addition to its role as antioxidant and mediator in signalling pathways, Asc is also integrated into growth regulation and development (Córdoba and González-Reyes 1994; Noctor and Foyer 1998; Smirnoff and Wheeler 2000). Increase in Asc leads to the promotion of

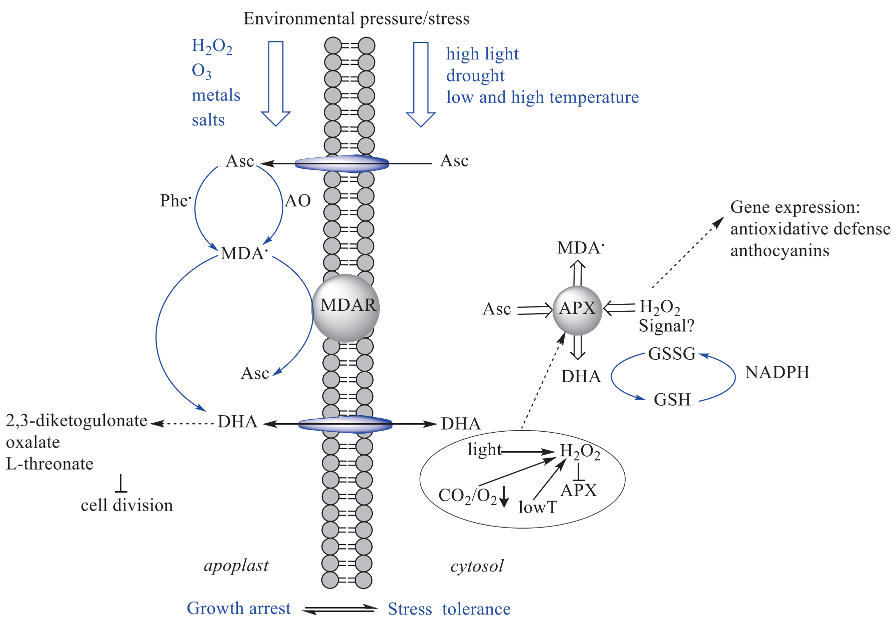


Fig. 5 Schematic overview of the key role of Asc redox state in the stress tolerance mechanisms and the trade-off strategy between growth and defence. Particular stressors are differentiated according to the first cellular target of H_2O_2 generation—apoplast (pathogen attack, wounding, oxidative burst, metal excess, ozone) or chloroplast (high light, drought, high and low temperatures). Asc and its oxidation products (MDA· and DHA) are important regulators of different acclimative responses in the apoplast and symplast. Intracellular transport of H_2O_2 and its signalling role are described in the text. Redox signals modulate expression of stress tolerance genes and accumulation of secondary metabolites

mitosis and cell division, as shown for cell and tissue cultures (Arrigoni et al. 1989; De Pinto et al. 1999). On the other hand, a repression of GalLDH expression in tobacco BY-2 cell lines resulted in an arrest of cell division and growth by decreasing the Asc pool for 30% (Tabata et al. 2001).

Numerous studies with Asc-deficient mutants show that a 10–90% depletion of the total Asc pool has a profound influence on plant growth and stress tolerance (Tables 1 and 2). While a minimal amount of Asc may be necessary for its role as cofactor, an overwhelming body of evidence suggests that metabolism and gene expression are highly sensitive either to changes in the Asc pool size or redox state (Müller-Moulé et al. 2004; Huang et al. 2005; Chen and Gallie 2006; Alhagdow et al. 2007; Gao and Zhang 2008; Foyer and Noctor 2016). In addition to being hypersensitive to O_3 *vtc1* plants exhibit growth retardation together with delayed flowering and premature senescence (Veljović-Jovanović et al. 2001). Even lower amounts of Asc (10–15% of the WT) in transgenic tomato as a result of the suppressed gene encoding the GDP-D-mannose-3,5-epimerase (GME) led to severe growth inhibition (Zhang 2013). The mutants lacking Asc are not viable. The growth of *vtc2-vtc5* double-mutant seedlings is recovered when supplemented with Asc or L-galactose (Dowdle et al. 2007). Higher sensitivity of Asc-deficient mutants *vtc1* to abiotic stresses was, however, accompanied by growth retardation and an induction of the PR-1 and PR-5 proteins upon infection (Barth et al. 2004; Conklin and Barth 2004; Pavet et al. 2005), implying that low Asc content, $O_{3,}$ or pathogens share the same defence system mediated by SA (Pastori et al. 2003). The increased resistance of the *vtc1* and *vtc2* mutants to pathogens like *Pseudomonas syringae* and *Peronospora parasitica* has also been reported (Gallie 2013).

Contrary to Asc-deficient plants, the Asc content can be improved by genetic modification of certain enzymes involved in its biosynthesis. It has been shown that transgenic plants overexpressing such genes exhibit enhanced tolerance to abiotic stress (Akram et al. 2017). One of the genetic approaches to increase the Asc pool is activation of alternative biosynthetic pathways, which bring about up to sevenfold more Asc (Laing et al. 2007; Bulley et al. 2009). Zhang and co-workers (2011) also showed that overexpression of GME in tomato led to an increased Asc content and higher stress tolerance. Jain and co-workers (2000) showed that transgenic plants (tobacco and lettuce) with overexpressed rat cDNA encoding L-gulono-1,4-lactone oxidase contained 4–7 fold more Asc than untransformed controls. Overexpression of *myo*-inositol oxygenase or L-gulono-1,4-lactone oxidase in Arabidopsis elevated Asc content but also increased biomass and tolerance to abiotic stresses (Lisko et al. 2013). On the other hand, overexpression of L-galactose dehydrogenase did not show any effect on the foliar Asc concentration, while antisense suppression reduced it (Gatzek et al. 2002). In conclusion, attempts to change the Asc level are a promising tool in genetic engineering with the aim to improve both stress tolerance and crop yield under unfavourable conditions (Gallie 2013). However, understanding the underlying mechanism of the trade-off strategy between growth and defence and the complex role of Asc in the regulation of these physiological processes is yet to be resolved.

10 Conclusions and Future Perspectives

As compartmentalisation is a major factor in redox signal specificity under stress, increasing emphasis should be placed on the subcellular distribution and redox state of Asc and GSH and their function in the regulation of redox-sensitive proteins and processes. Recent evidence on Asc content and redox state in the apoplast, vacuole, and nucleus should stimulate future research of its role in abiotic stresses signalling and tolerance mechanisms.

The multiple roles of Asc in the control of plant growth and development under abiotic stress and high Asc level in the nucleus address future research to genetic and epigenetic factors that modulate the growth and fitness of crop plants, particularly within an agricultural context. Thus, genetic manipulation of plants under stress conditions with the goal of obtaining a high accumulation of Asc is an essential area to be considered.

Because of the complexity of Asc functions, and its interplay between ROS, antioxidants, and hormone signalling pathways, any attempts to engineer its content may improve one aspect, but may also have a negative impact on other physiological parameters, such as growth. Thus, further genetic manipulation will require highly targeted genetic approaches and a comprehensive study of the impacts on all other aspects of plant growth, development, and responses to biotic and abiotic stresses under field conditions.

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Ascorbate Peroxidases: Crucial Roles of Antioxidant Enzymes in Plant Stress Responses

Takanori Maruta and Takahiro Ishikawa

Abstract Ascorbate peroxidases (APXs) are, in general, photosynthetic eukaryote-specific enzymes, which catalyze the reduction of H_2O_2 using ascorbate as an electron donor. Considering the very low affinity of ascorbate with H_2O_2, the acquisition of APX was certainly an important event, allowing plants to use ascorbate for H_2O_2 metabolism. This also provides a plausible explanation for why plants accumulate a massive amount of ascorbate because this substrate is also required for stabilizing fragile APX enzymes (particularly chloroplastic isoforms). In higher plants, APXs are distributed in the cytosol, mitochondria, chloroplasts (both stroma and thylakoid membrane), and peroxisomes to modulate organellar and cellular levels of H_2O_2. Despite its potential toxicity, H_2O_2 is a relatively stable form of a reactive oxygen species, and consequently it can act as a key signaling molecule for plant stress responses. From this point of view, APXs also have a dual role, being antioxidant enzymes and H_2O_2 signaling regulators, and their balance is crucial for fine-tuning stress responses. In this chapter, we describe the physiological roles of APX isoforms in plants by overviewing the findings of biochemical, physiological, and genetic studies.

Keywords Ascorbate peroxidase · Oxidative stress · Oxidative signaling · Redox regulation · Stress response

1 Introduction

Suboptimal growth conditions caused by environmental changes, such as light, drought, and temperature, lead to yield losses in crops. Under these environmental stresses, enhanced production of reactive oxygen species (ROS) originates from

T. Maruta · T. Ishikawa (✉)
Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane, Japan
e-mail: ishikawa@life.shimane-u.ac.jp

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photosynthesis, respiration, and photorespiration, or through several oxidases (Mittler et al. 2004; Shigeoka and Maruta 2014). ROS are potentially cytotoxic molecules that can oxidize any cellular component, causing oxidative damage. Among ROS, H_2O_2 is a relatively stable form and poorly oxidizes cellular components, such as nucleic acids, proteins, sugars, and lipids, with some exceptions (e.g., cysteine is a good target for this ROS). Thus, H_2O_2 itself is not very toxic for plants, as well as for other organisms (Mittler 2017). In the presence of free iron, however, the Fenton reaction occurs and converts H_2O_2 to a hydroxyl radical ($OH^•$), which is the most reactive form of ROS and oxidizes any component randomly and rapidly (Mittler 2017). The very short half-life of $OH^•$ (approximately 1 ns) indicates that the selective scavenging of this ROS is impossible in cells. Strict control of H_2O_2 levels is therefore essential in the prevention of oxidative damage from $OH^•$.

It is well known that higher plants accumulate a large amount of ascorbate. The high accumulation occurs mainly in photosynthetic tissues, such as leaves. Because these tissues are the main targets of light-dependent oxidative stress (photooxidative stress) (Asada 1999), it is easy to imagine that ascorbate plays a key role in protecting cells from photooxidative damage. Although ascorbate is indeed a powerful antioxidant, this chemical itself does not efficiently interact with H_2O_2. Acquisition of ascorbate peroxidases (APXs), which convert H_2O_2 to water using ascorbate as an electron donor, during evolution has allowed plants to use ascorbate for H_2O_2 metabolism (Gest et al. 2013). In higher plants, APXs are distributed in the cytosol, chloroplasts, mitochondria, and peroxisomes (Mittler et al. 2004; Maruta et al. 2016), together with several layers of the ascorbate recycling system that supplies ascorbate for the APX reaction (Gallie 2013). The antioxidant ability of ascorbate in plant cells has been maximized by the evolution of ascorbate metabolism (Gest et al. 2013).

For the last few decades, physiological function of APXs, as well as that of other antioxidant enzymes, was analyzed based only on the oxidative stress theory, in which ROS are only cytotoxic molecules. However, it is now widely accepted that ROS, especially H_2O_2, have another face, functioning as signaling molecules to control a diverse range of physiological processes, such as stress responses, growth, and development. From this point of view, APXs also play a dual role, being antioxidant enzymes and H_2O_2 signaling regulators, and their balance is crucial for fine-tuning stress responses. In this chapter, we describe the physiological roles of APX isoforms in plants by summarized the findings of biochemical, physiological, and genetic studies.

2 Distribution of APX Isoforms in Plant Cells

APXs are heme peroxidases and members of Class I non-animal peroxidases, which also include cytochrome *c* peroxidases (CCPs) and bacterial catalase peroxidases (CPs) (Welinder 1992; Passardi et al. 2007). APXs are only found in plastid-containing organisms with some exceptions (Teixeira et al. 2004; Passardi et al.

2007; Nedelcu et al. 2008). As supported by genome-sequencing studies (Passardi et al. 2007), no *APX* gene has ever been found in cyanobacteria. In contrast, most eukaryotic algae analyzed possessed more than one *APX* gene (Maruta et al. 2016). Two types of hybrid peroxidases, atypical APX-CCP hybrid A1 and A2, were found in non-photosynthetic kinetoplastids and photosynthetic euglenids, respectively (Zámocký et al. 2014; Ishikawa et al. 2010). Mono-functional plant APXs are considered evolutionary descendants of hybrid A1, and they evolved in parallel with hybrid A2 (Zámocký et al. 2014).

All *APX* genes are nuclear encoded (Mittler et al. 2004). In higher plants, APX isoforms are distributed in the cytosol (cAPX), chloroplasts (chlAPX), mitochondria (mitAPX), and peroxisomes (pAPX), which are key sites for H_2O_2 production and/or scavenging (Fig. 1) (Shigeoka et al. 2002). Two chloroplastic isoforms, stromal sAPX and thylakoid membrane-bound tAPX, are found in chloroplasts of land plants (Maruta et al. 2016). They form the water–water cycle in a powerful ROS regulation system (see below; Asada 1999). The existence of an additional isoform in the chloroplast lumen (e.g., *Arabidopsis* At-APX4/TL29) was proposed in *Arabidopsis* (Kieselbach et al. 2000). The proposed isoform is highly conserved in other plant species, but the protein lacks some amino acid residues that are essential for APX activity. Indeed, its knockout has no effect on APX activity (Granlund et al. 2009), indicating TL29 is not a functional APX. This is apparently supported by a structural analysis (Lundberg et al. 2011).

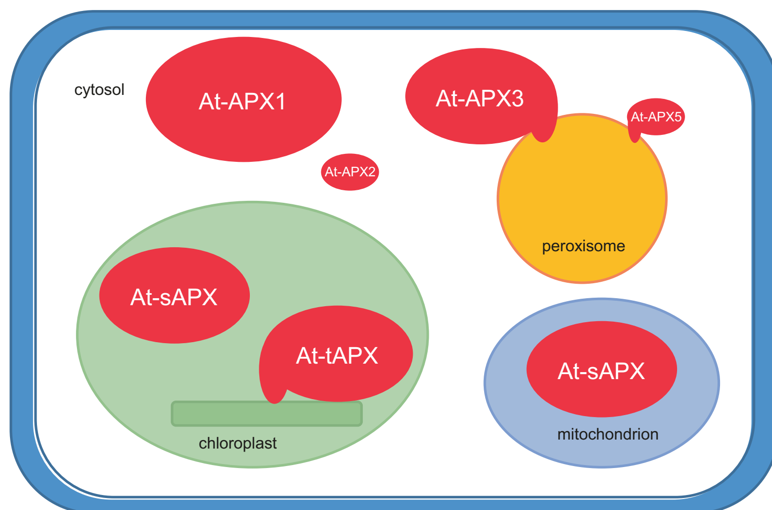

Fig. 1 Distribution of APX isoforms in *Arabidopsis* leaf cells. At-APX1 and 2 are cytosolic isoforms, while At-APX3 and 5 are peroxisomal. At-sAPX is a dual-targeting protein in both the chloroplast stroma and mitochondrial matrix. At-tAPX is solely distributed in chloroplasts and attached to thylakoid membrane. Size of red circles indicate expression levels of the enzymes. Expression level of At-APX2 is very low under normal growth conditions, but highly induced by high light

Previously, *Arabidopsis* was reported to have nine *APX* genes (Mittler et al. 2004), i.e., *At-APX1–7*, *At-sAPX*, and *At-tAPX*. Among them, At-APX6 and At-APX4 lack Arg-172, which is essential for the efficient use of ascorbate (Bursey and Poulos 2000), and At-APX6 is now annotated as an APX-related (APX-R) protein (Lazzarotto et al. 2011). In addition, At-APX7 (At1g33660) is described as a pseudogene in current databases, such as The Arabidopsis Information Resource (TAIR). Therefore, Arabidopsis has six functional *APX* genes. At-APX1 and 2 are cytosolic, whereas At-APX3 and 5 are peroxisomal (Mittler et al. 2004). At-tAPX is distributed throughout the thylakoid membrane although At-sAPX is a dual-targeting protein in both the chloroplast stroma and mitochondrial matrix (Chew et al. 2003; Maruta et al. 2016). In contrast to *Arabidopsis*, rice plants have 8 APX isoforms (Teixeira et al. 2004, 2006). Os-APX5 and Os-APX6 are targeted solely to mitochondria, and Os-APX7 (sAPX) and Os-APX8 (tAPX) to chloroplasts (Xu et al. 2013). Our recent comprehensive mining of *APX* genes in plant species whose genomes are already sequenced indicated that all monocot plants may have APX isoform(s) solely targeted to the mitochondria (Maruta et al. 2016). Although *Physcomitrella patens* APX (Pp-APX1), which is the most orthologous to *Arabidopsis* sAPX, is only targeted to chloroplasts, *Picea glauca* APX (Pg-APX1) is dual-targeted to both chloroplasts and mitochondria (Xu et al. 2013). Based on these findings, it was suggested that the dual-targeting ability of APX developed after the split between *Physcomitrella patens* and *Picea glauca* and was subsequently lost in rice following monocot divergence (Xu et al. 2013).

3 Expression and Regulation

3.1 Transcriptional Regulation

Cytosolic APXs are highly responsive to environmental stimuli, especially high irradiance, whereas other isoforms are not (Yoshimura et al. 2000). Expression of the *Arabidopsis At-APX2* gene has been thoroughly analyzed, and it is the representative stress marker gene. The first important finding was that high light-induced *At-APX2* is largely affected by the redox state of the photosynthetic electron transport (PET) chain (Karpinski et al. 1997). The PET inhibitors, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) block reduction and oxidation, respectively, of the plastoquinone pool. High light-induced *At-APX2* expression is almost completely inhibited by DCMU, whereas its expression is enhanced by DBMIB. This is also the case in the tobacco *cAPX* gene (Yabuta et al. 2004). These findings indicate that the plastoquinone redox state acts as a retrograde signal from chloroplasts to the nucleus for the regulation of *At-APX2* expression under high irradiance. Exogenous application of glutathione also suppresses *At-APX2* expression under high light intensity (Karpinski et al. 1997). Identification of an *Arabidopsis regulator of APX2 1–1* (*rax1–1*)

mutant, an allele of gamma-glutamylcysteine synthetase 1, consolidated the role of glutathione as a mediator of *At-APX2* induction (Ball et al. 2004). These signals are associated with photoelectrophysiological signaling (PEPS) in a light wavelength-specific manner (Szechyńska-Hebda et al. 2010).

Another *Arabidopsis* mutant, *altered expression of APX2* (*alx8*), provides an alternative retrograde signal from chloroplasts (Rossel et al. 2006). The *ALX8* gene encodes SAL1 that dephosphorylates 3′-phosphoadenosine 5′-phosphate (PAP) in chloroplasts. The PAP accumulated in chloroplasts of sal1 mutants can be transferred to the nucleus to inhibit 5′ to 3′ exoribonucleases (XRNs) that modulate thousands of mRNA expressions (Estavillo et al. 2011). This regulation is associated with the abscisic acid (ABA) pathway, and *alx8* as well as other *sal1* mutants accumulate ABA, being highly tolerant to drought stress (Rossel et al. 2006). Considering the fact that ABA is essential for *At-APX2* induction under high light intensity (Galvez-Valdivieso et al. 2009), a plausible explanation is that high irradiance stimulates the SAL1-PAP pathway, which in turn, activates ABA signaling for the gene expression; however, whether the SAL1-PAP pathway is active in wild-type plants exposed to high light intensity requires further validation.

H_2O_2 also acts as a signal for the regulation of *At-APX2* expression. Pre-infiltrating leaves with catalase, but not with superoxide dismutase, strongly inhibits the gene expression under high light intensity (Karpinski et al. 1999). Considering that catalase proteins cannot pass through the plasma membrane, the strong inhibition of *At-APX2* expression must be caused by a decrease in extracellular H_2O_2 levels. This is clearly supported by the finding that NADPH oxidases, which produce ROS in the apoplast, are essential for the full expression of *At-APX2* under high light intensity (Bechtold et al. 2008). A recent pioneering work using HyPer2, a genetically encoded fluorescent H_2O_2 sensor, demonstrated that photosynthesis-produced H_2O_2 is directly transferred from chloroplasts to nuclei and, then, induces tobacco cytosolic *APX* expression under high light stress (Exposito-Rodriguez et al. 2017). Thus, both intracellular and extracellular H_2O_2 can activate *cAPX* expression, possibly through the redox modification of heat-shock transcription factors (Jung et al. 2013).

Taken together, a variety of signals have been found to regulate cytosolic *APX* expression under high light intensity although it is still unclear how these signals are integrated or coordinated to fine-tune the gene expression in plant cells.

3.2 Post-transcriptional Regulation

In some plant species, sAPX and tAPX are encoded by a single gene, which produces both isoforms by alternative splicing in a tissue-specific manner. This regulation occurs in tobacco, spinach, pumpkin, and ice plants (Ishikawa and Shigeoka 2008). In the case of tobacco and spinach, chloroplastic APX pre-mRNA produces four types of mRNA variants, one *tAPX* and three *sAPX* forms (*sAPX-I*, *-II*, and *-III*).

The ratio of the level of *sAPX* mRNAs to *tAPX* is close to 1 in leaves, whereas the ratio in roots is largely elevated because of the increase in *sAPX-III* and decrease in *tAPX* (Yoshimura et al. 2002). The splicing regulatory cis element (SRE) sequence located between exons 12 and 13 of the *chlAPX* gene is required for tissue-specific splicing efficiency. Gel-shift assays revealed that SRE strongly interacts with nuclear protein(s) extracted from leaves, but not with those from roots (Yoshimura et al. 2002). Thus, SRE is anticipated to act as a splicing enhancer that regulates the tissue-specific alternative splicing of chloroplastic *APX* pre-mRNA.

3.3 Post-transcriptional Regulation

In addition to transcriptional regulation, cytosolic APX is also regulated at the post-translational level. This involves the redox modification of Cys-32, which is highly conserved in APXs. Nitric oxide and *S*-nitrosoglutathione react with Cys-32 of cytosolic APX to form *S*-nitrosylation. This modification has a positive effect on cytosolic APX activity in *Arabidopsis* (Yang et al. 2015), but an inhibitory effect in tobacco Bright Yellow-2 cells (de Pinto et al. 2013). *S*-nitrosylation also occurs in Cys-49 of At-APX1 although the modification has no effect on enzyme activity (Yang et al. 2015). Similarly, *S*-sulfhydration by hydrogen sulfide occurs at Cys-32 and activates APX activity (Aroca et al. 2015). This cysteine is a target of thioredoxins (Trxs), which are ubiquitous small disulfide oxidoreductases. Reduction of cytosolic APX by Trxs, as well as by reducing chemicals (such as DTT and glutathione), inactivates the peroxidase activity (Gelhaye et al. 2006).

One of characteristics of chloroplastic APXs is that these enzymes are extremely sensitive to H_2O_2 under low ascorbate levels compared to cytosolic and peroxisomal isoforms (Chen and Asada 1989; Miyake and Asada 1996). The half-inactivation time of chloroplastic APXs is 15 s when the concentration of ascorbate is less than 10 μM, whereas that of the cytosolic enzyme is more than 40 min (Kitajima 2008). The irreversible cross-linking of heme to the distal Trp-41 and radical formation in Cys-31 and Cys-125 are involved in this process. It should be noted that these amino acids are generally conserved in the stable cytosolic isoform (see Maruta et al. 2016). An insertion of amino acids specific to chloroplastic isoforms (chloroplastic domain 2) moves a loop structure, which is in the vicinity of the propionate side chains of heme, away from the propionate side chains. This structural property may facilitate the cross-linking process (Kitajima 2008). Triple mutations in the amino acids described above and deletion of the chloroplastic domain 2 have improved the H_2O_2 sensitivity of tobacco sAPX (Kitajima et al. 2008, 2010). Consequently, a rapid inactivation of chloroplastic APXs is observed in plants exposed to photooxidative stress (Miyake et al. 2006; Yoshimura et al. 2000). However, there have been difficulties with the elucidation of the exact relationship between the inactivation of these enzymes and ascorbate levels in vivo (see Maruta et al. 2016). It is possible that another unknown mechanism(s) may be involved in the inactivation process.

tAPX activity in vivo was also recently shown to be inactivated through direct phosphorylation by a specific kinase in wheat during pathogen infections (Gou et al. 2015). Indeed, phosphoproteomic studies have successfully identified *Arabidopsis* tAPX and sAPX as phosphorylated proteins (for example, Roitinger et al. 2015). A heme-containing APX-related (APX-R) protein (also referred to as At-APX6 in Arabidopsis) is located in chloroplasts and mitochondria, in which it physically interacts with APX, possibly to modulate its activity (Lazzarotto et al. 2011).

4 Role as Antioxidant Enzymes

4.1 Chloroplastic APXs

The APX reaction in chloroplasts is coupled with the photosynthetic electron transport chain to form the water–water cycle (Fig. 2). In this cycle, electrons excised from water in photosystem II (PSII) are transferred to oxygen by PSI, resulting in the formation of O_2^- (Asada 1999). Membrane-attached copper/zinc superoxide dismutase (Cu/Zn-SOD) converts O_2^- into H_2O_2, which is further reduced into water by tAPX. Even if they escaped from this system, ROS would be attacked by the second layer of ROS scavenging, consisting of iron SOD (Fe-SOD) and sAPX in the stroma. The oxidized form of ascorbate generated by the APX reaction is reduced by ferredoxin-, glutathione-, and NAD(P)H-dependent pathways. The water–water cycle acts as both an antioxidant system and a system for dissipating excess electrons from PET, i.e., an electron sink (Asada 1999).

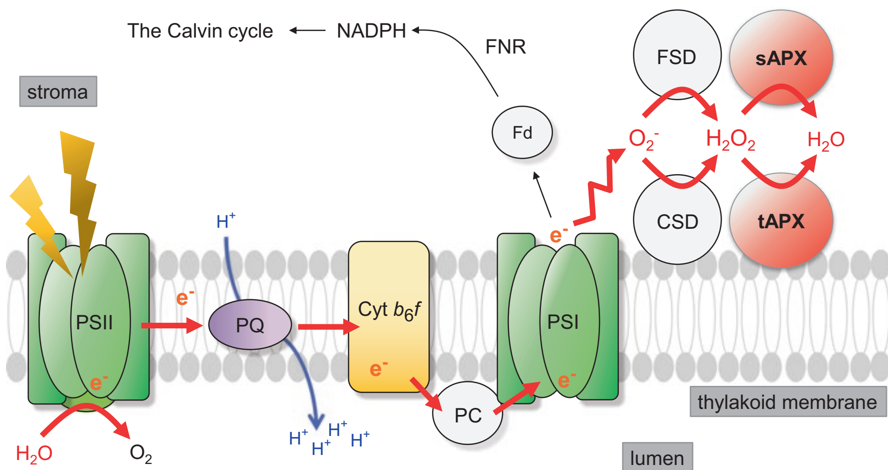

Fig. 2 The water–water cycle. Notes: CSD Cu/Zn-SOD, Cyt cytochrome, Fd ferredoxin, FNR Fd-NADP+ reductase, FSD Fe-SOD, PC plastocyanin, PQ plastoquinone, PS, photosystem

Considering the fragile nature of chloroplastic APXs, it was a plausible hypothesis that APX activity in chloroplasts is a bottleneck for plant stress tolerance. This has been clearly supported by a number of findings showing that overexpression of peroxidases or catalases within chloroplasts results in enhanced stress tolerance in plants (Foyer and Shigeoka 2011). For example, overexpression of *Escherichia coli* catalase (KatE) with a chloroplast-targeting signal protects thiol-modulated enzymes in the Calvin cycle in tobacco plants, thereby mitigating the inhibition of photosynthesis under photooxidative stress (Shikanai et al. 1998; Miyagawa et al. 2000). One of the major causes of photoinhibition is the inhibition of D1 protein translation (Nishiyama et al. 2001). This inhibition is also alleviated in the KatE transgenic tobacco under a combination of salt and high light intensity (Al-Taweel et al. 2007). Overexpression of spinach tAPX has similar effects on tobacco stress tolerance (Yabuta et al. 2002). These lines of evidence strongly indicate that the inactivation of APXs is significant for photooxidative damage from abiotic stress in plant cells, and further suggested that the loss-of-function mutants of chloroplastic APXs should cause severe growth defects or lethality in plants under illumination (Yabuta et al. 2002).

Nevertheless, researchers have unexpectedly failed to find a "stress-sensitive phenotype" among loss-of-function mutants, at least in the laboratory conditions. In *Arabidopsis* mutants lacking sAPX and/or tAPX, the accumulation of H_2O_2 and decrease in PET activity are slightly pronounced after short-term application of high light intensity (more than 1000 μmol photons/m^2/s for up to 6 h) (Kangasjärvi et al. 2008; Maruta et al. 2010). However, no obvious phenotypic difference was found between these mutants and the wild type under short- and even long-term application of high irradiance (Giacomelli et al. 2007; Kangasjärvi et al. 2008; Maruta et al. 2010). A hexaploid wheat mutant S-SV8, which lacks one of three *tAPX* genes, was found to exhibit a growth retardation under mild light stress (Danna et al. 2003) although it is unclear whether *tAPX-6B* is the only gene absent in the mutant and responsible for its phenotype.

Compensation by other antioxidant enzyme(s) is a plausible explanation for the negligible phenotype of chloroplastic APX mutants under laboratory conditions. A complete double mutant lacking 2CPA and 2CPB, which are chloroplastic 2-Cys peroxiredoxins, exhibits growth retardation under a light intensity of 160 μmol photons/m^2/s, and this phenotype is further facilitated by an additional defect in At-tAPX (Awad et al. 2015). *Arabidopsis* chloroplastic glutathione peroxidases (GPX1 and GPX7) also provide an alternative route for the scavenging of H_2O_2 in the water–water cycle (Chang et al. 2009). Other mechanisms are also involved in regulating the production of ROS from photosynthesis. In addition to linear electron transport, cyclic electron transport (CET) around PSI via the proton gradient regulation 5 (PGR5)- and chloroplast NADH dehydrogenase-like (NDH) complex-dependent pathways largely contribute to the formation of a proton gradient across the thylakoid membrane (i.e., low pH in the lumen), which activates the xanthophyll cycle to dissipate excess light energy as heat (Shikanai 2014).

4.2 Cytosolic APXs

The cytosol is not a major site for ROS production. However, from the viewpoint of stress sensitivity of knockout mutants, cytosolic APX is likely to play a key role in cellular redox regulation. An *Arabidopsis apx1* mutant (ecotype Ws) exhibits a growth defect even under normal growth conditions, with an altered stomatal response and decreased photosynthetic activity because of cellular oxidative damage (Pnueli et al. 2003). These phenotypes might occur in an ecotype-dependent manner because our *apx1* mutant (ecotype Col-0) grows at wild-type levels under similar growth conditions (Maruta et al. 2012a). However, *apx1* (Ws) exhibits a severe sensitivity to high light intensity, methyl viologen-induced oxidative stress, and a combination of drought and heat (Davletova et al. 2005; Koussevitzky et al. 2008); *apx1* (Col-0) is also very sensitive to wounding (Maruta et al. 2012a). Thus, the lack of At-APX1 actually weakens plants against a wide range of stresses. Interestingly, oxidation of not only the cytosolic proteins, but also organellar ones is enhanced in the *apx1* mutants during stress (Davletova et al. 2005; Maruta et al. 2012a). The cytosol is in cellular compartments across organelles, such as chloroplasts, mitochondria, peroxisomes, and the nucleus. Thus, cAPX can protect organelles from oxidative stress by preventing H_2O_2 from flowing into one organelle from another. This is known as cross-compartment protection (Davletova et al. 2005).

4.3 Peroxisomal and Mitochondrial APXs

Peroxisomes are considered the most significant site for H_2O_2 production in C_3 leaves during photorespiration (Foyer and Noctor 2003) and therefore accumulate a large amount of catalase to scavenge and regulate H_2O_2 levels. Physiological importance of catalase has been demonstrated by knockout mutants of the *Arabidopsis CAT2* gene, which exhibit a severe bleaching phenotype under photorespiratory conditions (i.e., ambient air with high light intensity) (Mhamdi et al. 2012; Queval et al. 2007; Vandenabeele et al. 2004). In addition to catalase, APXs are also distributed in peroxisomes (Yamaguchi et al. 1995). These peroxisomal isoforms, for example, *Arabidopsis* At-APX3 and At-APX5, have a transmembrane domain with which they attach to the peroxisomal membrane, but their catalytic domain faces the cytosol (Ishikawa et al. 1998; Shen et al. 2010). Affinities for H_2O_2 are substantially different between catalase and APX, whose *K*m values for ROS are approximately 40–600 mM and 10–100 μM, respectively (Mhamdi et al. 2012; Shigeoka and Maruta 2014). Thus, peroxisomal APXs may react with a low concentration of H_2O_2, which escaped from the catalase reaction, to fine-tune the cellular H_2O_2 levels. Overexpression of peroxisomal APX is likely to enhance plant stress tolerance (for example, Wang et al. 1999). In contrast, knockout of At-APX3 had no effect on

plant tolerance to various abiotic stresses (Narendra et al. 2006). This might have been caused by compensation by catalase or another isoform At-APX5. Physiological significance of ascorbate metabolism in peroxisomes was indicated by Eastmond (2007), who showed that peroxisomal At-MDAR4 (monodehydroascorbate reductase) is essential for autotrophic growth although it is unclear if the At-MDAR4 function is coupled with the APX reaction. In contrast to that of peroxisomal APX, the catalytic domain of the MDAR isoform is in the peroxisomal matrix, which results in a question regarding how MDHA produced in the cytosol is reduced in the peroxisome matrix.

Mitochondria would be a significant site for H_2O_2 production, at least in non-photosynthetic tissues, such as roots. Nevertheless, H_2O_2 metabolism in the organelles and its physiological significance are poorly understood in plants. As described above, At-sAPX is a dual-targeting enzyme for both the chloroplast stroma and mitochondrial matrix (Chew et al. 2003). However, the knockout of the gene had little effect on plant tolerance for oxidative stress (Davletova et al. 2005; Maruta et al. 2010). Other thiol-dependent peroxidases, such as peroxiredoxin II F and glutathione peroxidase 6 in *Arabidopsis*, have been found to function in the mitochondria.

5 Role as Redox Signaling Regulators

H_2O_2 is currently recognized to act as a signal for regulating a wide range of physiological processes, including abiotic and biotic stress responses (Apel and Hirt 2004; Foyer and Shigeoka 2011; Mittler et al. 2011). Accumulating transcriptome data from plants subjected to oxidative stress or redox mutants, in which one or more antioxidant enzymes are knocked out/down, have revealed the existence of a production site- and type-specific pathways for ROS signaling (Gadjev et al. 2006; Vaahtera et al. 2014; Shigeoka and Maruta 2014; Willems et al. 2016). Although the mode of action of each pathway remains largely unclear, the integration and crosstalk of multiple pathways in plants have been considered to fine-tune stress responses. This must be based on the strict spatiotemporal control of ROS levels through a diverse set of antioxidant enzymes, including APXs, in various cellular compartments.

Because of the dual face of ROS actions, some redox mutants are paradoxically more resistant to some circumstances compared to the wild type. One of the clearest examples is that the photorespiratory oxidative stress phenotype of the *Arabidopsis cat2* mutant is largely mitigated by additional mutation in the cytosolic *At-APX1* gene (Vanderauwera et al. 2011). Specific activation of DNA damage response occurs in the *cat2 apx1* double mutant (Vanderauwera et al. 2011), probably through an interaction between cytosolic and peroxisomal H_2O_2 signals, leading to the stress-tolerant phenotype. A similar paradoxical phenotype is observed in the *apx1*

single mutants, which are highly tolerant to selenium and lead (Jiang et al. 2016, 2017). These observations clearly show that cAPX plays a key role in balancing the dual faces of ROS actions.

cAPX is also involved in plant immunity. Expression of tobacco cAPX is post-transcriptionally, but not transcriptionally, suppressed upon pathogen infection (Mittler et al. 1998). This has a negative correlation with enhanced ion leakage (cell death) and *pathogenesis-related 1* (*PR1*) gene expression. Hypersensitive response (HR) during pathogen attack is highly accelerated in transgenic tobacco plants with decreased cAPX expression (Mittler et al. 1999). There is also increasing experimental evidence for the involvement of chloroplastic APXs in plant immunity. Phosphorylative inactivation of tAPX through protein kinase wheat kinase start 1.1 (WKS1.1) occurs in wheat upon pathogen attack, resulting in enhanced H_2O_2 levels (Gou et al. 2015). In *Arabidopsis*, knockdown of tAPX enhances the accumulation of salicylic acid and subsequent transcriptional activation of defense-related genes without the application of any stress (Maruta et al. 2012b). These findings clearly indicate that cytosolic and chloroplastic APXs regulate immune responses by regulating H_2O_2 levels.

To identify chloroplastic H_2O_2-responsive genes, a conditional system for tAPX silencing in *Arabidopsis* has been developed using an estrogen-inducible RNAi method (Maruta et al. 2012b). Although no obvious oxidative stress symptom was observed in the tAPX-silenced plants, 365 and 409 genes were at least two-fold ($P < 0.05$) up- and down-regulated, respectively, in response to tAPX silencing. Interestingly, these genes rarely included typical marker genes for oxidative stress, which have been identified by comparing the transcriptomic data of several ROS-related mutants and plants treated with ROS-producing agents. Indeed, these genes only slightly overlapped with genes whose expression was affected by cytosolic and peroxisomal H_2O_2 (i.e., in the *apx1* and *cat2* mutant, respectively) (Maruta et al. 2012b: Queval and Foyer 2012). Classification and comprehensive analysis of these genes have indicated a regulatory role for tAPX in metabolic pathways related to abiotic stress acclimation in plants. For example, tAPX silencing enhances γ-amino aminobutyric acid (GABA) and anthocyanin metabolisms, which may protect chloroplastic APXs mutants from photooxidative stress (Maruta et al. 2013, 2014).

How does chloroplast-produced H_2O_2 act as signal to modulate expression of nuclear genes? A recent finding using HyPer2 revealed the mode of action of chloroplastic H_2O_2 signaling in *Nicotiana benthamiana* epidermal cells under high irradiation. Exposito-Rodriguez et al. (2017) found that chloroplast-produced H_2O_2 is directly transferred to nuclei, avoiding the cytosol. Nuclear H_2O_2 accumulation and subsequent high light-responsive gene expression were critically attenuated by sAPX overexpression or DCMU treatment, but not by cAPX overexpression. This clearly indicates the involvement of chloroplastic APXs in chloroplast-to-nucleus H_2O_2 signaling.

6 Conclusion and Future Perspectives

More than three decades have passed since the APX enzyme was first characterized in Euglena (Shigeoka et al. 1980). During this period, basic information on APX isoforms in higher plants has accumulated in the context of their enzymological properties, distribution, and functions as antioxidant enzymes. However, this is largely restricted to model plants, such as *Arabidopsis* and rice, and there are still more questions than answers. For example, the physiological significance of organellar isoforms in plant stress tolerance remains largely unclear. Furthermore, the validation of APXs as signaling modulators has just started. H_2O_2 has multiple signaling roles in a production site-specific manner. Because their crosstalk is believed to fine-tune plant stress responses, it will be interesting to clarify how APX isoforms functionally interact with each other under stressful conditions to achieve spatio-temporal tuning of H_2O_2 signaling pathways.

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Molecular and Functional Characterization of Monodehydroascorbate and Dehydroascorbate Reductases

Satoshi Sano

Abstract When ascorbate (AsA) plays a role in cells as an antioxidant or a substrate for some enzymes including ascorbate peroxidase (APX), it is primarily oxidized to the monodehydroascorbate (MDHA) radical and then dehydroascorbate (DHA) is formed via the disproportionation of MDHA. The regeneration of AsA from these oxidation products is essential to maintain cellular levels of AsA. Plant cells contain enzymes catalyzing the reductions of MDHA and DHA, known as MDHA reductase (MDHAR) and DHA reductase (DHAR), respectively. Both reductases are components of the ascorbate-glutathione (AsA-GSH) cycle. MDHAR and DHAR use NADH and GSH as electron donors, respectively. MDHAR is an FAD-containing enzyme that forms a charge-transfer complex with NADH, and DHAR contains a conserved catalytic cysteine residue that forms a mixed disulfide with GSH. Several paralogous genes of MDHAR and DHAR can be found in plant genomes. The isoforms of MDHAR and DHAR, encoded by these genes, show diverse subcellular localization and biochemical properties. Recently, several structures of MDHAR and DHAR from plants were determined independently. These results and the biochemical data obtained represent the reaction mechanism of AsA regeneration by these reductases. This chapter focuses on the molecular properties of these AsA-regenerating enzymes and their biochemical functions.

Keywords Monodehydroascorbate reductase · FAD enzyme · NADH · Bacterial iron-sulfur reductase · Dehydroascorbate reductase · Catalytic cysteine residue · Glutathione · Glutathione transferase

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S. Sano (✉)
Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan
e-mail: satsano@kpu.ac.jp

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1 Introduction

Ascorbate (AsA) plays a central role to protect eukaryotes from oxidative stress. When AsA acts as an antioxidant in cells, in most cases, the one-electron oxidation product, the monodehydroascorbate (MDHA) radical, is produced as the primary oxidation product. In plants, the scavenging reaction of hydrogen peroxide, which is one of the reactive oxygen species (ROS), catalyzed by AsA peroxidase is a major source of the MDHA radical (Hossain et al. 1984). Additionally, AsA is oxidized to MDHA in the reactions shown in Fig. 1. The other ROS, superoxide anions and hydroxyl radicals, directly oxidize AsA to MDHA (Bielski 1982). The autoxidation of AsA by molecular oxygen is mediated by superoxide (Scarpa et al. 1983). When glutathione (GSH) acts as an antioxidant, the glutathionyl thiyl radical ($GS^{\bullet-}$) is formed (Winterbourn and Metodiewa 1994). This radical oxidizes AsA to MDHA (Forni et al. 1983). The tocopherol radical is generated from tocopherol with the scavenging of lipid radicals. This radical is regenerated to tocopherol by AsA producing MDHA (Munné-Bosch and Alegre 2002). Other radicals such as carbon-centered, alkoxy, peroxy, and phenoxy radicals also generate MDHA (Bielski 1982). Consequently, MDHA is produced whenever AsA scavenges radicals. In addition, cytochrome b_{561} in plasma membrane and tonoplast oxidizes AsA to MDHA (Asard et al. 2001; Griesen et al. 2004). Furthermore, MDHA is generated at the lumen of the thylakoid membrane in chloroplasts. In the xanthophyll cycle, which plays an important role in protecting photosystems against excess light, AsA is oxidized to MDHA accompanying the de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin with violaxanthin de-epoxidase (Neubauer and Yamamoto 1994). AsA can donate electrons to photosystems I and II, producing MDHA, when

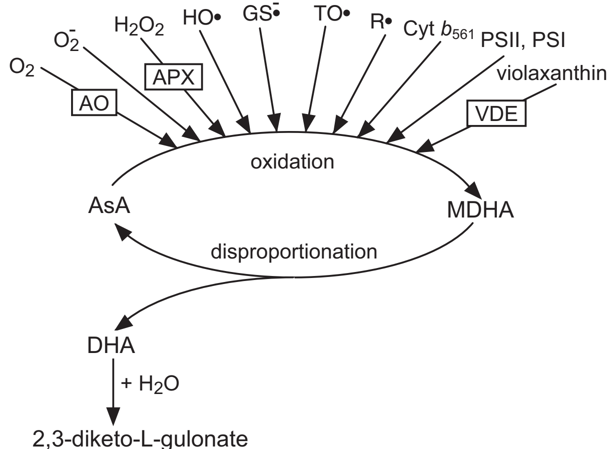


Fig. 1 Production of monodehydroascorbate (MDHA) and dehydroascorbate (DHA). *AO* ascorbate oxidase, *APX* ascorbate peroxidase, *Cyt* cytochrome, $GS^{\bullet-}$ glutathione thiyl radical, *PS* photosystem, $R^{\bullet}$ organic radical, $TO^{\bullet}$ tocopherol radical, *VDE* violaxanthin de-epoxidase. Molar stoichiometry is not shown

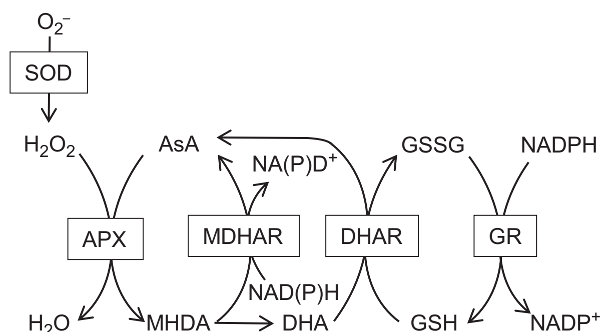


Fig. 2 The ascorbate-glutathione cycle and superoxide dismutase in plants. *APX* ascorbate peroxidase, *AsA* ascorbate, *DHA* dehydroascorbate, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *MDHA* monodehydroascorbate, *MDHAR* monodehydroascorbate reductase, *SOD* superoxide dismutase. Molar stoichiometry is not shown

the primary electron donor system is inactivated (Mano et al. 1997, 2004). Furthermore, AsA oxidase catalyzes the univalent oxidation of AsA by dioxygen to MDHA, especially in cucurbitaceous plants (Yamazaki and Piette 1961).

If MDHA escapes from biochemical reduction to AsA, AsA and dehydroascorbate (DHA), which is the two-electron oxidation product of AsA, are generated via the disproportionation of MDHA. DHA is unstable at a physiological pH and is hydrolyzed irreversibly to 2,3-diketo-L-gulonate spontaneously and enzymatically (Deutsch 2000), and this subsequently breaks down further to various compounds including oxalate and tartrate (Keates et al. 2000).

To maintain the pool of AsA in cells, the regeneration of AsA from MDHA and DHA is clearly indispensable. For the regeneration of AsA from MDHA and DHA, plants possess MDHA reductase (MDHAR) and DHA reductase (DHAR). These enzymes constitute the AsA-GSH cycle (also known as the Foyer-Halliwell-Asada cycle) with other antioxidative enzymes (Fig. 2) and the water-water cycle in chloroplasts as well (Smirnoff and Pallanca 1996; Noctor and Foyer 1998; Asada 1999). This chapter deals with the molecular properties and functions of MDHAR and DHAR.

2 Monodehydroascorbate Reductase (MDHAR)

2.1 Molecular Properties

MDHA reductase (MDHAR, EC 1.6.5.4) is a soluble monomeric flavin enzyme that catalyzes the reduction to AsA from the MDHA radical using NADH as an electron donor (Hossain and Asada 1985). MDHAR was the first reported enzyme to use an organic radical as an enzyme substrate:

$$2\text{MDHA} + \text{NADH} \rightarrow 2\text{AsA} + \text{NAD}^+$$

MDHAR has been purified from cucumber fruit (Hossain and Asada 1985), potato tuber (Borraccino et al. 1986), the soybean root nodule (Dalton et al. 1992), potato tuber mitochondria (De Leonardis et al. 1995), and spinach chloroplast (Sano et al. 2005b) and has undergone enzymatic characterization. The partially purified MDHAR from *Euglena gracilis* was also characterized (Shigeoka et al. 1987). The cDNAs of MDHAR were initially cloned from the cucumber (Sano and Asada 1994) and pea (Murthy and Zilinskas 1994).

MDHAR contains one molecule of FAD per enzyme molecule as a prosthetic molecule. MDHAR in plants is usually composed of approximately 430 amino acid residues, and its molecular mass is approximately 47 kDa (Sano and Asada 1994). The amino acid sequence of MDHAR has the FAD and pyridine nucleotide-binding regions with the consensus motif V/IXG$_{1-2}$XGXXGXXG/A (Kleiger and Eisenberg 2002). However, MDHAR shares only limited homology with flavin oxidoreductase from eukaryotes, such as ferredoxin-NADP reductase functioning in the photosynthetic system, but exhibits a high level of sequence homology with bacterial flavin oxidoreductase (Sano and Asada 1994). A homology search indicates that bacterial NADH-dependent iron-sulfur protein reductases are responsible for the high homology score on comparison with MDHAR using BLAST. For example, the FAD-containing ferredoxin reductases from *Acidovorax* sp. CHX100 (AKJ87747) (Salamanca et al. 2015), *Sphingomonas* sp. KA1 (BAE75877) (Urata et al. 2006), *Streptomyces tubercidius* R-922 (AAT45308) (Molnár et al. 2005), and *Novosphingobium* sp. SB32149 (BAX09275) (Kozono et al. 2017) exhibit sequence identities of 33, 32, 30, and 32% with *E*-values of $6 \times e^{-43}$, $1 \times e^{-41}$, $2 \times e^{-41}$, and $2 \times e^{-41}$ on comparison with MDHAR from cucumber (BAA05408), respectively. Most of the bacterial iron-sulfur protein reductases similar to MDHAR are dependent on NADH and involved in the metabolism of various organic compounds with oxygenases and iron-sulfur protein including ferredoxins, putidaredoxin, and rubredoxin. An electron transfer system that is composed of iron-sulfur protein and iron-sulfur protein reductase donates electrons to oxygenase for its catalytic reaction from NADH (Senda et al. 2009). Intriguingly, the mammalian apoptosis-inducing factor (AIF) shares a close homology with bacterial ferredoxin reductase and MDHAR (Candé et al. 2002). AIF exhibits an NADH-dependent reduction of MDHA, but this activity is inhibited by SOD, indicating that the reaction may be mediated by superoxide radicals generated by the NADH oxidase activity of AIF (Miramar et al. 2001).

MDHARs with a lower molecular mass have also been isolated: the 40-kDa enzyme from soybean root nodules (Dalton et al. 1992), and the 32-kDa enzyme from the glyoxysome of the castor bean (Luster et al. 1988; Bowditch and Donaldson 1990) and the peroxisomal membrane of the pea (Lopez-Huertas et al. 1999). Interestingly, the 32-kDa MDHAR from the pea was recognized by polyclonal antibody raised against a 47-kDa cucumber MDHAR (Sano et al. 1995; Lopez-Huertas et al. 1999). Storage proteins like dioscorin, the nectar protein Nectarin III, and the antimicrobial protein defensin have been reported to exhibit MDHAR activity (Hou et al. 1999; Carter and Thornburg 2004; Huang et al. 2008). Discorin and Nectarin III have molecular masses resembling these of smaller MDHARs, but their association is unclear.

In mammals, some proteins which regenerate AsA from MDHA has also been reported (Martin-Romero et al. 2004; Kobayashi 2016). They are classified into six types: transmembrane NADH:MDHA oxidoreductase (Goldenberg et al. 1983; Coassin et al. 1991), cytochrome b_5 reductase (Iyanagi and Yamazaki 1969; Ito et al. 1981), soluble NADH-dependent MDHAR (Bando and Obazawa 1994; Bando et al. 2004), transmembrane AsA:MDHA reductase (Schipfer et al. 1985; Van Duijn et al. 1998), cytochrome b_{561} (Wakefield et al. 1986; Njus and Kelley 1993), and thioredoxin reductase (May et al. 1998). The plasma membrane MDHAR reductase activities are considered to be part of the plasma membrane electron transport chain (May 1999; Martin-Romero et al. 2004). It is possible that the trans-plasma membrane AsA:MDHA reductase is just a partial reaction of the trans-plasma membrane NADH:MDHA oxidoreductase enzyme complex (May 1999; Martin-Romero et al. 2004). Plant MDHAR can clearly be distinguished from these mammalian enzymes.

In fungi, a 66-kDa enzyme containing no prosthetic groups and a 127-kDa homodimeric enzyme containing FMN have been found in *Neurospora crassa* and *Pleurotus ostreatus*, respectively (Schulze et al. 1972; Yu et al. 1999).

2.2 Isoforms and Their Localization

In plant cells, MDHAR occurs not only in chloroplasts (Hossain and Asada 1984), but also in the cytosol (Dalton et al. 1993), mitochondria (Arrigoni et al. 1981; De Leonardis et al. 1995), peroxisomes (Jiménez et al. 1997; Lisenbee et al. 2005), glyoxysomes (Bowditch and Donaldson 1990; Bunkelmann and Trelease 1996), and the plasma membrane (Bérczi and Møller 1998), like other enzymes constituting the AsA-GSH cycle.

In *Arabidopsis*, five genes encoding MDHAR are present in the genome, from which six MDHAR isoforms, AtMDHAR1 to AtMDHAR6, may be predicted (Chew et al. 2003). Among the genes of AtMDHAR, the gene encoding AtMDHAR1 (At3g52880) shows the closest homology with the cDNAs of MDHAR which have been cloned from various plants including cucumber (Sano and Asada 1994), pea (Murthy and Zilinskas 1994), tomato (Grantz et al. 1995), Chinese cabbage (Yoon et al. 2004), sweet potato (Huang et al. 2010), and blueberry (Harb et al. 2010). The amino acid sequences of these MDARs possess a C-terminal tripeptide that resembles the motif (-SKL) of peroxisomal targeting signal type 1 (PTS1) (Mullen 2002; Lisenbee et al. 2005). In databases, some other MDHAR genes similar to that of AtMDAHAR1 and containing the PST1-like motif can be found. The myc epitope-tagged AtMDHAR1 and pea MDHAR (PsMDHAR) as well as eGFP-fused PsMDHAR have been sorted to the matrix of peroxisomes (Leterrier et al. 2005; Lisenbee et al. 2005). Furthermore, in proteome analyses, AtMDHAR1 and the corresponding MDHARs of rice were found in peroxisomes (Eubel et al. 2008; Kaur and Hu 2011). However, the sorting of the epitope-tagged AtMDHAR1 and PsMDHAR to peroxisomes was incomplete (Lisenbee et al. 2005), suggesting the possibility that MDHAR similar to AtMDHAR1 functions in not only peroxisomes

but also the cytosol. AtMDHAR2 (At5g03630) and AtMDHAR3 (At3g09940), which include no characteristic sequence that predicts specific subcellular localization, accumulate in the cytosol (Lisenbee et al. 2005). T-DNA insertion mutants of AtMDHAR3 (and AtDHAR1) showed that these enzymes are crucial for the promotion of growth and seed production of *Arabidopsis* by the mutualistic endophytic fungus *Piriformospora indica* (Vadassery et al. 2009). This result suggests the redox state of ascorbate in *Arabidopsis* cells affects their interaction. AtMDHAR4 (At3g27820) has a molecular mass of about 54 kDa and an amino acid sequence similar to membrane PTS (mPTS) at the C-terminus, which consists of a hydrophobic transmembrane domain and an adjacent cluster of basic amino acids (Mullen and Trelease 2000). Analyses of the epitope-tagged AtMDAHR4 and GFP-fused AtMDHAR4 indicate that this isoform sorts to the peroxisomal membrane and is inserted in such a way that its N-terminus is exposed to the cytosol, and its mPTS is necessary for targeting the peroxisomal membrane (Lisenbee et al. 2005). Subsequently, AtMDHAR4 was identified as a peroxisomal membrane protein in proteomic studies of *Arabidopsis* (Reumann et al. 2009; Kaur and Hu 2011). *Arabidopsis* with AtMDHAR4 mutation exhibits a seedling-lethal phenotype in the absence of sucrose (Eastmond 2007). In the oilseed biology of *Arabidopsis*, a large amount of hydrogen peroxide is generated within the peroxisome by the β-oxidation of fatty acids (Graham and Eastmond 2002). The phenotype of the AtMDHAR4 mutant plant is due to the inactivation of lipase on the oil body surface participating in the supply of carbon for the establishment of seedlings by hydrogen peroxide that has escaped the peroxisome (Eastmond 2007). The last MDHAR gene of *Arabidopsis* (At1g63940) encodes a 55-kDa protein containing an additional part in the N-terminus as a transit sequence for targeting organelles. This gene has multiple transcription initiation sites that generate two different isoforms, AtMDHAR5 and AtMDHAR6, differing in length by seven amino acid residues (Obara et al. 2002). AtMDHAR5 with a longer N-terminal region is imported into mitochondria and AtMDHAR6 with a shorter N-terminal region is imported into chloroplasts, being confirmed by data on GFP fusion protein and the proteome (Obara et al. 2002; Chew et al. 2003). An *Arabidopsis* mutant lacking AtMDHAR5/6 was insensitive to 2,4,6-trinitrotoluene (TNT), a toxic and persistent environmental pollutant, and was not more susceptible to environmental stress (Johnston et al. 2015).

The bryophyte *Physcomitrella patens* has three genes encoding a 47-kDa MDHAR isoform, all of which resemble the cytosolic isoforms from vascular plants lacking organelle-targeting sequences (Lunde et al. 2006). The enzymatic properties of these MDHARs are markedly similar to those of the corresponding enzymes from vascular plants (Drew et al. 2007).

The phylogenetic tree of MDHAR forms three main clades (Fig. 3). These three clades represent MDHAR isoforms located in the same cellular compartment: (a) a group of cytoloic and peroxisomal matrix isoforms including AtMDHAR1, AtMDHAR2, and AtMDHAR3; (b) a group of peroxisomal membrane-bound isoforms including AtMDHAR4; (c) a group of chloroplastic and mitochondrial isoforms including AtMDHAR5 (and AtMDHAR6). All three MDHARs from *Physcomitrella* are found in the first clade.

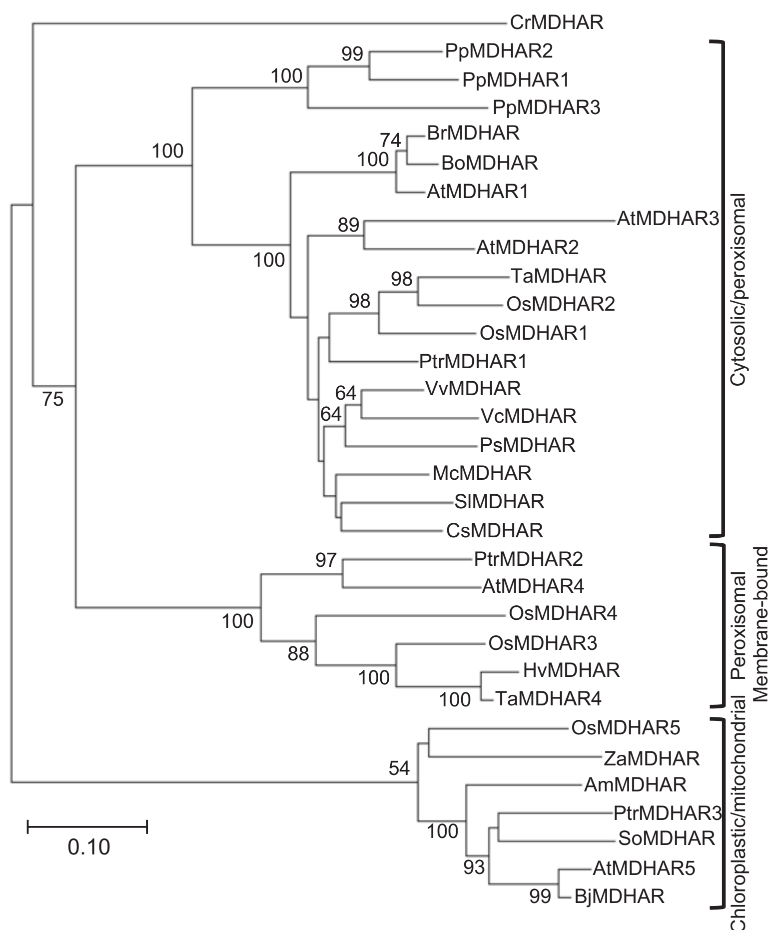


Fig. 3 Phylogenetic analysis of MDHAR from plants. The phylogenetic tree was constructed with MEGA7.0 software. The scale bar on the figure shows the substitution rate against one amino acid residue. The bootstrap values calculated from 1000 replicates (only values ≥50% are indicated) are shown as percentages on branches. This phylogenetic tree was generated using the following amino acid sequences: AtMDHAR1 (NP_190856), AtMDHAR2 (NP_568125), AtMDHAR3 (NP_566361), AtMDHAR4 (NP_189420), and AtMDHAR5 (NP_564818) from *Arabidopsis thaliana*; AmMDHAR (ACP31193) from *Avicennia marina*; BcMDHAR (AAD28178) from *Brassica juncea*; BoMDHAR (BAD14934) from *Brassica oleracea*; BrMDHAR (AAK72107) from *Brassica rapa*; CrMDHAR (EDO98161) from *Chlamydomonas reinhardtii*; CsMDHAR (BAA05408) from *Cucumis saivas*; HvMDHAR (CAC69935) from *Hordeum vulgare*; McMDHAR (CAC82727) from *Mesembryanthemum crystallinum*; OsMDHAR1 (BAD46251), OsMDHAR2 (BAD09086), OsMDHAR3 (BAD08054), OsMDHAR4 (BAD08053), and OsMDHAR5 (BAC98552) from *Oryza sativa*; PpMDHAR1 (ABA47446), PpMDHAR2 (ABA47447), and PpMDHAR3 (ABA47448) from *Physcomitrella patens*; PsMDHAR (AAU11490) from *Pisum sativum*; PtrMDHAR1 (ERP59097), PtrMDHAR2 (EEE83340), and PtrMDHAR3 (ERP65334) from *Populus trichocarpa*; SlMDHAR (AAC41654) from *Solanum lycopersicum*; SoMDHAR (BAB63925) from *Spinacia oleracea*; TaMDHAR (AGX45478) and TaMDHAR4 (AFU52947) from *Triticum aestivum*; VcMDHAR (ABY49995) from *Vaccinium corymbosum*; VvMDHAR (ABQ41114) from *Vitis vinifera*; ZaMDHAR (AAD53522) from *Zantedeschia aethiopica*

In chloroplasts, as the photoreduced ferredoxin in photosystem I reduces MDHA more effectively than NADP, MDHAR barely participates in the reduction of MDA in the AsA-GSH cycle on the thylakoid membrane (Miyake and Asada 1994). Hence, MDHAR would function at a site where reduced ferredoxin cannot gain access but NAD(P)H can. Since the diffusion of ferredoxin from the photosystem I complex is most likely restricted, MDHAR might also exist in the vicinity of the thylakoid membrane (Asada 1999). In addition, flavin oxidoreductases in chloroplasts including MDHAR enhance the photoreduction of dioxygen to superoxide. The flavin oxidoreductase reduced by NAD(P)H is only auto-oxidized slowly, but the enzyme photoreduced by photosystem I in thylakoids is auto-oxidized rapidly to produce superoxide (Miyake et al. 1998). It has been supposed that the photoreduction site of dioxygen in thylakoids is FeS center X or A/B of the PSI complex (Asada et al. 1974, 1977). The flavin oxidoreductases is assumed to be photoreduced at the FeS center A/B considering their mid-point potentials (Miyake et al. 1998). MDHAR is the most likely mediator stimulating the photoreduction of dioxygen in chloroplasts for the safe dissipation of excess energy under stress conditions by producing the superoxide radical in the water-water cycle (Asada 1999).

2.3 Enzymatic Properties

MDHAR shows a specificity to MDHA although ferricyanide serves as the electron acceptor (Hossain and Asada 1985). Experimental estimation of the K_m value for MDHA is difficult, because MDHA, which is generally produced with the oxidation of AsA by AsA oxidase, spontaneously disproportionates at an appreciable rate (Bielski 1982) and its steady-state concentration cannot be maintained at a value for the saturation kinetics of many MDHARs. Under the conditions generally used for enzyme assays, the maximum concentration of MDHA is 3 μM (Sano et al. 1995). Quinones are poor electron acceptors for MDHAR, as distinct from NAD(P)H:quinone reductase which is a flavoenzyme of higher plants (Sparla et al. 1999). MDHAR cannot transfer an electron to plant-type ferredoxin from NDA(P)H, consistent with the low-level similarity of MDHAR to ferredoxin-NADP reductase (Hossain and Asada 1985).

MDHAR is capable of reducing phenoxyl radicals generated by horseradish peroxidase with hydrogen peroxide from quercetin and hydroxycinnamates, such as ferulic acid, chlorogenic acid, and coniferyl alcohol, to their respective parent phenols via a mechanism similar to the reduction of MDHA (Sakihama et al. 2000). Interestingly, MDHAR causes the generation as well as quenching of organic radicals. The one-electron reduction of TNT and 1-chloro-2,4-dinitrobenzene (CDNB) is catalyzed by MDHAR to form their nitro radicals, which would then be auto-oxidized to generate superoxide radicals (Johnston et al. 2015). The toxicity of TNT to plant may be caused by this reaction.

MDHAR shows a predilection for NADH as an electron donor to NADPH (Hossain and Asada 1985; Sano et al. 1995). The K_m value of NADH for MDHAR

ranges from 4 to 40 μM. MDHAR can be classified into two groups based on K_m values for NADPH; one is around 20–60 μM (Hossain et al. 1984; Hossain and Asada 1985; Borraccino et al. 1986; Murthy and Zilinskas 1994; De Leonardis et al. 1995) and the other is over 150 μM (Dalton et al. 1992; Sano et al. 1995, 2005b; Drew et al. 2007). Even the isoform located at chloroplasts where NADPH is more abundant than NADH shows a higher affinity for NADH than for NADPH (Sano et al. 2005b).

Dicumarol is a very strong inhibitor of menadione reductase ($K_i \approx 10$ nM) and competitive with respect to NAD(P)H (Ernster et al. 1962). MDHAR is also sensitive to dicumarol, but a much higher concentration is required for its inhibition. The K_i values of MDHAR from cucumber and spinach chloroplasts are 74 and 95 μM, respectively (Sano et al. 1995, 2005b). Moreover, dicumarol does not inhibit the reduction of FAD contained in MDHAR by NADH and appears to compete with MDHA for reduced FAD, unlike in the case of menadion reductase (Sano et al. 1995). Therefore, it is expected that dicumarol's mechanisms of MDHAR and menadione reductase inhibition are different.

The activity of MDHAR is inhibited by thiol-modifying reagents, and the inhibitory step is the reduction of enzyme FAD by NADH (Hossain and Asada 1985; Sano et al. 1995). Also, only one cysteine residue is conserved in all MDHAR isoforms. However, analyses of the mutant enzymes of MDHAR suggest that the conserved cysteine residue does not participate in the catalytic mechanism, but plays a relatively important role in maintaining MDHAR structural stability and activity (Li et al. 2010).

The reaction kinetics data suggest that the reaction of MDHAR proceeds via a ping-pong mechanism (Hossain and Asada 1985) represented as follows:

$$\text{E-FAD} + \text{NADH} + \text{H}^+ \rightarrow \text{E-FADH}_2\text{-NAD}^+ \quad \text{(Reaction 1)}$$

$$\text{E-FADH}_2\text{-NAD}^+ + \text{MDHA}^{\bullet -} \rightarrow \text{E-FAD}^\bullet\text{-NAD}^+ + \text{AsA}^- \quad \text{(Reaction 2)}$$

$$\text{E-FAD}^\bullet\text{-NAD}^+ + \text{MDHA}^{\bullet -} \rightarrow \text{E-FAD} + \text{AsA}^- + \text{NAD}^+ \quad \text{(Reaction 3)}$$

where E-FAD, E-FADH$_2$-NAD$^+$, and E-FAD$^\bullet$-NAD$^+$ represent the oxidized form, reduced charge-transfer complex, and semiquinone form of MDHAR, respectively. In the first step, the enzyme-bound FAD, E-FAD, is reduced by NADH to form a charge-transfer complex, E-FADH$_2$-NAD$^+$ (Reaction 1), which exhibits characteristic absorption at around 600 nm (Massey and Ghisla 1974). The second-order rate constant for the reduction of FAD by NADH is 1.2×10^8 M^{-1} s^{-1}, as determined by stopped-flow analysis (Sano et al. 1995). Then, the reduced enzyme donates electrons to MDHA, producing AsA through two successive one-electron transfers, possibly via a red semiquinone form of FAD, E-FAD$^\bullet$-NAD$^+$, as an intermediate (Reactions 2 and 3). These reduction processes could be directly observed using a pulse radiolysis method (Kobayashi et al. 1995). The second-order rate constant between MDHA and the fully reduced form of MDHAR at a low ratio of MDHA to enzyme concentration is 2.6×10^8 M^{-1} s^{-1}. A similar rate constant is obtained at an

excess MDHA to enzyme concentration. These results suggest that the reaction of the semiquinone form of the enzyme with MDHA occurs at the same rate or in the same rate-limiting step of oxidation of the fully reduced enzyme by MDHA.

Considering the ratio of the area of the isoalloxazine ring of FAD to the total surface of MDHAR, neither of the rate constants for the reduction of FAD by NADH nor oxidation of the reduced enzyme by MDHA, which must be diffusion controlled, can be explained simply by the bimolecular collision of the enzyme-bound FAD and substrates (Sano et al. 1995). At the pH of cells, NADH and MDHA are present in anionic forms (Lamborg et al. 1958; Bielski 1982), and the ionic interactions between the substrates and enzyme are considered to participate in these rapid reactions. The results on the rate of the entire reaction catalyzed by MDHAR, the reduction of FAD by NADH, and the oxidation of the reduced FAD by MDHA decreasing with increasing ionic strength suggest that cationic groups of amino acid residues localized near the active site of the enzyme facilitate the rapid reaction of FAD and the substrates (Hossain and Asada 1985; Sano et al. 1995; Kobayashi et al. 1995). In the reaction of the superoxide anion radical with superoxide dismutase (SOD), which is another radical-scavenging enzyme, a similar ionic strength effect was shown (Benovic et al. 1983). To achieve rapid disproportionation of the superoxide radical, SOD has evolved so that the superoxide radical is guided into the active site channel using the configuration of electrostatic charges on the enzyme surfaces (Sheng et al. 2014).

2.4 Structure

Initially, the crystal structure of MDHAR from cucumber was reported (Sano et al. 2004, 2005a). Subsequently, the structure of MDHAR from rice (OsMDHAR, PDB code 5JCI) was deposited in PDB (Do et al. 2014; Park et al. 2016). A homology search against PDB indicated that iron-sulfur protein reductases including ferredoxin reductase from *Rhodopseudomonas palustris* (PDB code 3FG2) (Xu et al. 2009), ferredoxin reductase from *Novosphingobium aromaticivorans* DSM12444 (ArR, PDB code 3LXD) (Yang et al. 2010), putidaredoxin reductase from *Pseudomonas putida* (PdR, PDB code 1Q1R and 1Q1W) (Sevrioukova et al. 2004), and ferredoxin reductase from *Acidovorax* sp. KKS102 (BphA4, PDB code 1D7Y) (Senda et al. 2000) yielded the highest homology score to MDHAR from cucumber using BLAST, exhibiting sequence identities of 30, 31, 29, and 27% with *E*-values of $1.5 \times e^{-34}$, $1.5 \times e^{-33}$, $9.5 \times e^{-29}$, and $4.7 \times e^{-24}$, respectively. The protein fold of MDHAR is similar to those of iron-sulfur protein reductases and disulfide reductases. According to the definition of domains of these enzymes, MDHAR can be divided into three domains: an FAD-binding domain, an NADH-binding domain, and a C-terminal domain (Senda et al. 2009). However, the structure of OsMDHAR shows a marked difference in the substrate-binding site from the iron-sulfur reductases (Park et al. 2016). The small iron-sulfur proteins bind to the C-terminal domain of the corresponding reductases to form an electron transfer complex. Structural

comparison of OsMDHAR with the electron transfer complexes of PdR, BphA4, and rubredoxin reductase (RdxR) (Sevrioukova et al. 2010; Senda et al. 2007; Hagelueken et al. 2007) revealed that the unique long loop of 63–80 amino acid residues of OsMDHAR occupies a part of the iron-sulfur protein in the structures of these complexes, which may be indispensable for forming the active site pocket with positively charged residues (Park et al. 2016).

The FAD molecule binds to the crevice of the FAD-binding domain through hydrogen bonds and van der Waals interactions in an extended manner. The binding of NAD to OsMDHAR caused a butterfly-like movement of the isoalloxazine ring of FAD, which was observed in BphA4 (Park et al. 2016; Senda et al. 2007). Moreover, the NAD binding to OsMDAR led to conformational shifts in Tyr174, His315, and Phe348. In the cucumber enzyme, in addition to the corresponding amino acid residues, a shift of the side chain of Ile174 was also observed (Sano et al. unpublished data). The aromatic ring of Tyr174 (Tyr173 in cucumber MDHAR), which occupies the NADH-binding site in the oxidized form of MDHAR, moves aside to allow the nicotinamide moiety to approach the isoalloxazine ring and bind in a conformation required for electron transfer. This residue functions as a "gatekeeper" that protects FAD from solvent in a ligand-free protein in several disulfide and ferredoxin reductases (Sevrioukova et al. 2004). Phe348 shifts outward to avoid conflict with Tyr174, and His315 is directed to the NAD-binding site to form hydrogen bonds with NAD (Park et al. 2016).

Glu196 of OsMDHAR forms hydrogen bonds with the 2′-hydroxy group of the ribose moiety in the adenosine component of NAD. As mentioned above, MDHAR and the bacterial iron-sulfur protein reductases show a predilection for NADH rather than NADPH as an electron donor. The sequence alignment indicates that the glutamate residue at the corresponding position is well conserved among these enzymes. The NADH-dependent BphA4 was successfully changed to the NADPH-dependent form by mutations of amino acid residues around the corresponding glutamate residue (Nishizawa et al. 2014). The pyridine nucleotide specificity of MDHAR may also be due to this hydrogen bond with NADH and the electrostatic repulsion of the corresponding glutamate residue with the negatively charged phosphate group attached to the 2′-position of the ribose moiety of NADPH. The measurement of the binding affinity of mutated OsMDHAR E196A indicated the important role of Glu196 in the specificity for pyridine nucleotides (Park et al. 2016).

From the structures of the electron transfer complexes of PdR and BphA4 with the respective iron-sulfur proteins, the conserved tryptophan residue may be capable of mediating electron transfer from the enzyme-FAD to Fe-S cluster of the iron-sulfur protein (Sevrioukova et al. 2010; Senda et al. 2007). In MDHAR, a tyrosine residue that is highly conserved corresponds to the catalytic tryptophan residue in PdR and BphA4 structurally. The reduced activity of OsMDHAR mutated on Tyr349 suggests that this conserved tyrosine is a catalytic residue (Park et al. 2016).

The structure of the OsMDHAR/NAD/AsA ternary complex shows that the active site is formed using the configuration of positive electrostatic charges, which guide the negatively charged MDHA and NADH into it. Also, in that structure,

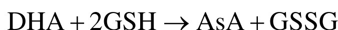
Arg320 forms a hydrogen bond with AsA and its significance for the activity of MDHAR was verified by mutational analysis (Park et al. 2016). This result is consistent with the effect of the chemical modification to arginine residues contained in MDHAR on the affinity for MDHA, not NADH (Sano et al. unpublished data).

Cysteine residues are not found around the FAD molecule in the structural model and may not be able to interact with FAD although inhibition of the reduction of the enzyme-bound FAD by NAD(P)H by thiol-modifying reagents has been reported. This suggests that cysteine residues are not directly involved in the FAD reduction, being consistent with the results of mutated MDHAR analyses (Li et al. 2010; Park et al. 2016).

3 Dehydroascorbate Reductase (DHAR)

3.1 Molecular Properties

DHA is mainly generated via the spontaneous disproportionation of MDHA that is not directly reduced to AsA. DHA reductase (DHAR, EC 1.8.5.1) participates in the regeneration of AsA from DHA (Hossain and Asada 1984). DHAR uses GSH as the electron donor, oxidizing to glutathione disulfide (GSSG):



$$\mathrm{DHA} + 2\mathrm{GSH} \rightarrow \mathrm{AsA} + \mathrm{GSSG}$$

GSH is regenerated by glutathione reductase in an NADPH-dependent reaction (Fig. 2) (Foyer and Halliwell 1976).

DHAR is a soluble monomeric enzyme and its molecular mass is around 24 kDa. The thiol group participates in the reaction catalyzed by the enzyme, indicated by the inhibition of thiol-modifying reagents (Foyer and Halliwell 1977; Hossain and Asada 1984; Dipierro and Borraccino 1991). Alignment analyses of amino acid sequences reveal that DHARs have a conserved cysteine as the catalytic residue.

A Kuniz-type trypsin inhibitor in the reduced form and thioredoxin from chloroplasts show DHAR activity (Trümper et al. 1994; Morell et al. 1997). However, their activities are low level and their amino acid sequences are distinct from that of DHAR. Furthermore, thioredoxin reductase, glutaredoxins, and protein disulfide isomerase exhibit DHAR-reducing activity (May et al. 1997; Ahn and Moss 1992; Wells et al. 1990).

DHAR has been purified from various plants including spinach leaves (Foyer and Halliwell 1977; Hossain and Asada 1984), potato tubers (Dipierro and Borraccino 1991), rice bran (Kato et al. 1997), and spinach chloroplasts (Shimaoka et al. 2000), and has been characterized enzymatically. DHAR of *Euglena* was also partially purified (Shigeoka et al. 1987). Furthermore, the GSH-dependent DHAR was purified from the rat liver (Maellaro et al. 1994), but it exhibited a markedly lower activity compared with plant enzymes. However, the cDNA of rat DHAR had been isolated (Ishikawa et al. 1998) before the genes of plant DHAR were cloned from rice (Urano et al. 2000) and spinach (Shimaoka et al. 2000). Among the DHARs

from plants, the high-level homology of the amino acid sequence has been shown, and this is also true among mammalian DHARs. However, there is not so high-level homology between plant and mammalian DHARs (Shimaoka et al. 2000; Kwon et al. 2001). To date, such GSH-dependent DHAR from animals, mainly mammals, is classified as glutathione transferase (GST) of the omega class (Board et al. 2000; Zhou et al. 2012). GST is the detoxification enzyme found mainly in the cytosol and catalyzes the conjugation of electrophilic substrate to GSH. The omega class GST (GSTO) exhibits the thioltransferase activity and characteristically catalyzes a range of reduction reactions with compounds that are not substrates for other class GSTs, such as DHA, methylated arsenic species, and *S*-phenacyl gluthationes (Board et al. 2000; Whitbread et al. 2005; Board 2011). It is suggested that GSTO may be involved in multidrug resistance and play a role in response to oxidative stress (Board 2011). The plant DHAR is also another member of the plant GST superfamily (Dixon et al. 2002). However, the plant DHAR is a monomeric enzyme although most of the other GSTs function as dimers (Dixon et al. 2002; Lan et al. 2009).

A homology search using BLAST indicates that DHAR has sequence similarity to GSTO and chloride intracellular channel (CLIC), which are present in animals. For example, pig GSTO (AAF71994) and human CLIC1 (CAG46868) exhibit sequence identities of 29 and 27% with *E*-values of $1 \times e^{-11}$ and $2 \times e^{-20}$ against DHAR from spinach (ABX26129), respectively. CLICs share weak sequence homology with proteins of the GST superfamily, and their structure suggests that CLICs are part of a family of GTSO (Dulhunty et al. 2001; Harrop et al. 2001). CLICs exist in two different forms, a soluble form and a transmembrane form. CLIC1 in its soluble form shows DHA reduction in a GSH-dependent manner (Al Khamici et al. 2015). Similarly, when DHAR from *Arabidopsis* (AtDHAR1) was expressed transiently in mammalian cells, a small portion of it was associated with the non-soluble microsomal fraction, and it endowed the cells with ion channel activity (Elter et al. 2007).

Recent studies revealed that GSTs in plants consist of 14 classes: Tau, Phi, Zeta, Theta, tetrachlorohydroquinone dehalogenase, which usually have a serine residue as a catalytic residue (Ser-GSTs); Iota, Hemerythrin, DHAR, Lambda, glutathionyl-hydroquinone reductase, microsomal prostaglandin E-synthase type 2 (mPGES-2), exhibiting a cysteine residue as a catalytic residue (Cys-GSTs); and three other classes (Lallement et al. 2014). In most DHARs, the active site motif is found around position 20, which is well conserved, being of the CPFC or CPFS form. CXXC and CXXF motifs are often conserved in thiol-disulfide oxidoreductases and involved in their redox function (Fomenko and Gladyshev 2002; Iqbalsyah et al. 2006). The similar motifs are found in Cys-GSTs such as mPGES-2 (CPFC) and CLIC (CPFS), as well as in glutaredoxins (CPFC or CPYC) (Lallement et al. 2014, 2016). It has been reported that the activity of DHAR is inhibited by thiol-modifying reagents (Hossain and Asada, 1984; Kato et al. 1997; Shimaoka et al. 2000). Furthermore, the substitution of the first positioned cysteine residue in the motif CPFC/S abolishes the DHA-reducing activity of DHAR (Dixon et al. 2002; Shimaoka et al. 2003; Lallement et al. 2016). These results suggest that this active site cysteine of DHAR forms a transient mixed disulfide with GSH, and that this reaction is involved in the catalytic function of DHAR.

Thermostable DHARs have been reported in several plants including rice (Kato et al. 1997), tropical fig (Yamasaki et al. 1999), spinach (Shimaoka et al. 2000), sesame (Chun et al. 2007), lacebark pine (Yang et al. 2009), and kiwifruit (Liu et al. 2016).

3.2 Isoforms and Localization

In plants, the activity of GSH-dependent DHAR reduction is found not only in the cytosol but also in chloroplasts (Shimaoka et al. 2000), mitochondria, and peroxisomes (Jiménez et al. 1997).

A sequence search of the *Arabidopsis* genome database identified five sequences as DHAR genes. Three sequences of the genes encoding AtDHAR1 (At1g19570), AtDHAR2 (At1g75270), and AtDHAR3 (At5g16710) are represented in the *Arabidopsis* EST data, and the activity of these three recombinant DHARs has been confirmed (Dixon et al. 2002). However, because the sequence encoding AtDHAR4 (At5g36270) contains no intron, which is possessed by other AtDHAR genes in a conserved position (Dixon et al. 2002), and its expression cannot be found in microarray expression data (Mittler et al. 2004), the gene of AtDHAR4 seems to be a pseudogene. At1g19550 is markedly smaller than other DHAR genes due to deletions in multiple regions (Gallie 2013). Confusingly, some papers adopt different numbering systems for DHARs of *Arabidopsis* (Mittler et al. 2004; Vadassery et al. 2009; Gallie 2013). AtDHAR3 contains an N-terminal extension suggested to be a transit peptide for targeting chloroplasts or mitochondria (Dixon et al. 2002; Chew et al. 2003). Its occurrence in chloroplasts has been confirmed by proteome analysis (Zybailov et al. 2008) and the localization of a GFP fusion protein (Noshi et al. 2016). AtDHAR1 and AtDHAR2 have been predicted to be cytosolic proteins as they do not exhibit clear targeting signal sequences. However, it has been reported that AtDHAR1 is found in the mitochondrial fraction (Chew et al. 2003). Thereafter, proteome and YFP fusion protein analyses identified AtDHAR1 as a peroxisome protein although this protein lacks a recognizable peroxisome targeting signal (Reumann et al. 2009; Kaur and Hu 2011). Moreover, the localization of EosFP-fused AtDHAR1 indicates that this protein is located in the cytosol (Grefen et al. 2010). Taken together, AtDHAR1 may be localized in multiple cellular compartments. On the other hand, AtDHAR2 has been suggested to be a cytosolic protein based on analysis of the cytosolic proteome (Ito et al. 2011).

In all plants that have been examined, including a bryophyte, a lycophyte, gymnosperms, and angiosperm eudicots and monocots, the numbers of DHAR genes range from two to four (Lallement et al. 2014; Zhang et al. 2015). DHARs of angiosperms can be classified into two groups: chloroplastic proteins, containing N-terminal signal peptides and the CPFC active site motif, and cytosolic proteins, containing no signal peptides and the CPFS active site motif (Lallement et al. 2014). The phylogenetic tree of DHAR also forms two main clades (Fig. 4). Indeed, analyses of the GFP fusion protein verified that some of the former and latter groups

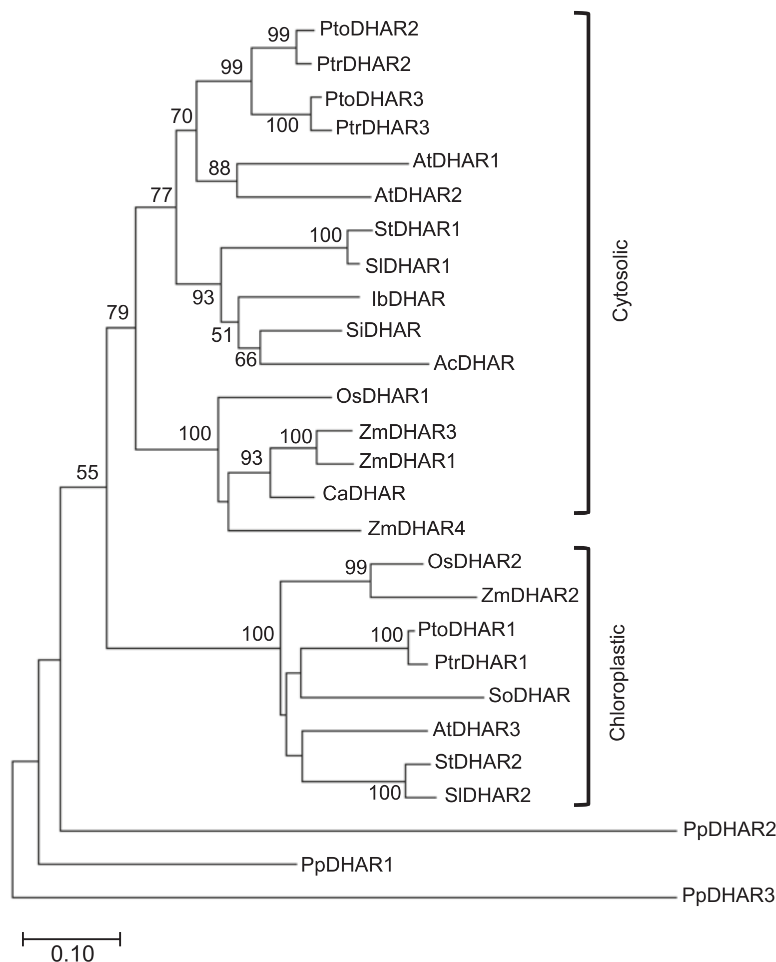


Fig. 4 Phylogenetic analysis of DHAR from plants. The phylogenetic tree was constructed with MEGA7.0 software. The scale bar on the figure shows the substitution rate against one amino acid residue. The bootstrap values calculated from 1000 replicates (only values ≥50% are indicated) are shown as percentages on branches. This phylogenetic tree was generated using the following amino acid sequences: AcDHAR (ADB85570) from *Actinidia chinensis*; AtDHAR1 (NP_173387), AtDHAR2 (NP_177662), and AtDHAR3 (NP_568336) from *Arabidopsis thaliana*; CaDHAR (AGZ80858) from *Cenchrus americanus*; IbDHAR (ALP06092) from *Ipomea batatas*; OsDHAR1 (BAA90672) and OsDHAR2 (BAD38160) from *Oryza sativa*; SoDHAR (ABX26129) from *Spinacia oleracea*; SiDHAR (ABB89210) from *Sesamum indicum*; SlDHAR1 (NP_001234822) and SlDHAR2 (NP_001234224) from *Solanum lycopersicum*; StDHAR1 (ABK32513) and StDHAR2 (ABM69253) from *Solanum tuberosum*; PpDHAR1 (AFZ39123), PpDHAR2 (AFZ39124), and PpDHAR (PpDHAR3) from *Physcomitrella patens*; PtoDHAR1 (AFS18598), PtoDHAR2, (AFS18599) and PtoDHAR3 (AFS18600) from *Populus tomentosa*; PtrDHAR1 (ADB11343), PtrDHAR2 (ADB11344), and PtrDHAR3 (ADB11345) from *Populus trichocarpa*; ZmDHAR1 (AIQ78394), ZmDHAR2 (AIQ78395), ZmDHAR3 (AIQ78396), and ZmDHAR4 (AIQ78397) from *Zea mays*

occur in chloroplasts and the cytosol, respectively (Liu et al. 2013; Tang and Yang 2013; Zhang et al. 2015; Lallement et al. 2016).

Contrary to previous reports of enzyme assays, no DHAR has been located in peroxisomes and mitochondria except AtDHAR1. ZmDHAR4, which is one of four DHAR isoforms in maize, was suggested to be a novel vacuolar DHAR based on the localization of eGFP fusion protein (Zhang et al. 2015). DHA generated in cellular compartments where DHAR is absent, including mitochondria, peroxisomes, and the apoplast, may be transported to the cytosol through membranes by facilitated diffusion or active transport systems (Horemans et al. 2000; Ishikawa et al. 2006; Miyaji et al. 2015) and reduced by DHAR in the cytosol. In transgenic tobacco plants, overexpressed cytosolic DHAR from wheat increased the AsA content of the cytosol but also of the apoplast (Chen and Gallie 2005). An *Arabidopsis* mutant completely lacking cytosolic DHAR has a decreased amount of apoplastic AsA (Yoshida et al. 2006). These results support the above-mentioned proposal regarding the regeneration of AsA in the apoplast. In addition, not only DHAR but also MDHAR is absent in the thylakoid lumen, where MDHA is also generated as described in Introduction. When the pH of the lumen is low and violaxanthin de-epoxidase functions, the MDHA in the lumen is rapidly disproportionate to DHA and AsA (Bielski 1982; Neubauer and Yamamoto 1994). The resulting DHA easily penetrates through the thylakoid membranes and is reduced by DHAR in the stroma of chloroplasts (Asada 1999; Mano et al. 2004). That might be reflected in the effect of DHAR activity in chloroplasts on inducing the non-photochemical quenching of chlorophyll fluorescence (Chen and Gallie 2008).

3.3 Enzymatic Properties

Although DHAR belongs to the GST superfamily, it shows distinct diversification in enzyme specificity from other GSTs. DHAR exhibits no detectable GSH-conjugating activity toward substrates of standard xenobiotic GST such as CDNB, 7-chloro-4-nitrobenzo-2-oxa-1,3-dibenzole (NBD-Cl), 1,2-dichloro-4-nitrobenzene (DCNB), 4-nitrobenzyl chloride (NBC), *p*-nitrophenyl acetate (4-NPA), benzyl isothiocyanate (benzyl-ITC), phenethyl-isothiocyanate (phenethyl-ITC), and ethacrynic acid (ECA), indicating that DHAR does not function as thioltransferase (Dixon et al. 2002; Lan et al. 2009; Yang et al. 2009; Liu et al. 2013; Lallement et al. 2016). DHAR exhibits no peroxidase activity with H_2O_2 and cumene hydroperoxide as Ser-GSTs, and no deglutathionylation activity can be measured using *S*-glutathionyl-phenylacetophenone and *S*-glutathionyl-menadione as other Cys-GSTs (Lallement et al. 2016). DHAR can reduce the model substrate 2-hydroxyethyl disulfide (HED) using GSH, just as any GSH-dependent oxidoreductases (Dixon et al. 2002; Lallement et al. 2016).

The apparent K_m value of DHAR for DHA varies from 0.07 to 0.50 mM, whereas the apparent K_m for GSH lies between 0.04 and 10 mM (Hossain and Asada 1984; Dipierro and Borraccino 1991; Kato et al. 1997; Shimaoka et al. 2000; Dixon et al.

2002; Amako et al. 2006; Chun et al. 2007; Jiang et al. 2008; Yang et al. 2009; Liu et al. 2013, 2016; Tang and Yang, 2013; Pandey et al. 2014; Lallement et al. 2016). In *Arabidopsis* and Chinese white poplar (*Populus trichocarpa*), the cytosolic DHAR isoform exhibits higher affinity for DHA than that of the chloroplastic isoform, while the affinities of these two isoforms for GSH are approximately the same (Dixon et al. 2002; Lallement et al. 2016). However, the chloroplastic AcDHAR1 of kiwifruit shows a higher affinity for DHA than cytosolic AcDHAR2, and the affinity for GSH of AcDHAR2 is higher than that of AcDHAR1 (Liu et al. 2016).

Kinetic analysis suggested that the reaction catalyzed by DHAR proceeds by a bi-uni-uni-uni-ping-pong mechanism, in which binding of DHA to the free, reduced form of the enzyme is followed by the binding of GSH interacting with catalytic cysteine at the first position of the active site motif CPFC/F (Shimaoka et al. 2003). The inhibition of DHAR activity by the thiol-modifying reagent iodoacetic acid is suppressed by the addition of DHA (Shimaoka et al. 2000). Accordingly, it was proposed that a cysteinyl-thiohemiketal complex is formed between DHA and the thiol group of the catalytic cysteine residue of DHAR in its reduced form (Shimaoka et al. 2003). However, no evidence supporting the formation of such an intermediate has been provided. Recently, the formation of sulfenic acid at the catalytic cysteine of DHAR with the reduction of DHA was suggested based on the crystallographic identification of hyperoxidation of the cysteine residue in rice DHAR (OsDHAR1) on crystals soaked with DHA (Do et al. 2016). This is supported by the detection of DHAR (AtDHAR2) sulfenylated at the catalytic cysteine residue in an *Arabidopsis* cell suspension under oxidative stress, which was also validated by an in vitro analysis (Waszczak et al. 2014). In general, the generation of mixed disulfide proceeds via a protein-sulfenic acid intermediate (Gupta and Carroll 2014). In the case of 2-cysteine peroxiredoxins, the thiolate anion attacks the peroxide substrate to generate cysteine-sulfenic acid at the active site, and the second free thiol attacks to form an intersubunit disulfide bond (Hall et al. 2009). The sulfenic acid at the cysteine leads to the formation of mixed disulfide with GSH and may protect DHAR against irreversible overoxidation. Consequently, a novel mechanism for DHA reduction by DHAR, generating the sulfenyl enzyme as an intermediate, has been proposed (Do et al. 2016; Bodra et al. 2017). The reaction scheme below has been rearranged so that it begins from the reduction of DHA:

$$\text{E-S}^- + \text{DHA} \rightarrow \text{E-SOH} + \text{AsA} \quad \text{(Reaction 4)}$$

$$\text{E-SOH} + \text{GSH} \rightarrow \text{E-S-SG} \quad \text{(Reaction 5)}$$

$$\text{E-S-SG} + \text{GSH} \rightarrow \text{E-S}^- + \text{GSSG} \quad \text{(Reaction 6)}$$

where E-S$^-$, E-SOH, and E-S-SG represent the reduced thiolate, sulfenylated, and *S*-glutathionylated forms of DHAR, respectively. The catalytic cysteine residue in the reduced form of DHAR, E-S$^-$, reduces DHA to AsA to form the sulfenylated DHAR, E-SOH (Reaction 4). GSH attacks the sulfenylated cysteine, generating a mixed disulfide, E-S-SG (Reaction 5). A second molecule of GSH then reacts with

E-S-SG to generate GSSG and E-S⁻ (Reaction 6). The second-order rate constant of this GSSG-forming step determined by stopped-flow analysis using AtDHAR2 is $1.3 \times 10^3\ M^{-1}\ s^{-1}$ (Bodra et al. 2017). At 4 mM GSH, which is comparable to an assumed average physiological concentration of GSH in the cytoplasm of *Arabidopsis* (Meyer and Fricker 2000), the rate of this reaction was determined to be 5.6 s^{-1}. This rate is relatively close to that of the whole reaction catalyzed by DHAR derived from steady-state kinetic analysis (3.8 s^{-1}). These rates converge further even at a GSH concentration below 4 mM (Bodra et al. 2017). This suggests that Reaction 6 may be a rate-limiting step in the reduction catalyzed by DHAR at a physiological concentration of GSH. On steady-state kinetics analysis in the presence of excess GSH, a plot of the rate of the reaction versus DHA concentration created a sigmoidal curve, suggesting a positive effect of DHA on GSH binding (Waszczak et al. 2014). Furthermore, a plot of the rate versus GSH concentration under saturated DHA conditions also showed a sigmoidal curve, suggesting a positive effect of GSH on either DHA binding or the affinity of the second molecule of GSH (Bodra et al. 2017). AtDHAR2 is the first reported DHAR behaving as an allosteric enzyme.

3.4 Structure

The first reported crystallographic study of DHAR involved a spinach chloroplastic enzyme (Mizohata et al. 2001). So far, six DHAR structures have been deposited in PDB, as follows: the crystal structures of OsDHAR1 (PDB code 5D9T) from rice (Do et al. 2016), AtDHAR1 (PDB code 5EL8) (Menault et al. unpublished), and AtDHAR2 (PDB code 5LOL) (Bodra et al. 2017) from *Arabidopsis* and PgDHAR1 (PDB code 5IQY and 5EVO) from pearl millet (*Pennisetum glaucum*) (Das et al. 2016), and the NMR solution structure of PtrDHAR3A (PDB code 2N5F) from black cottonwood (*Populus trichocarpa*) (Lallement et al. 2016). These structures show a thioredoxin-like N-terminal domain and an all-helical bundle fold C-terminal domain that are characteristic of GSTs, but they show a monomeric state unlike most GSTs (Board et al. 2000).

GSTs possess two substrate-binding sites, referred to as the G-site that binds a GSH molecule and the H-site that binds a secondary GSH and a hydrophobic electrophile molecule (Allocati et al. 2008; Zhou et al. 2012; Brock et al. 2013). The G-site is formed by highly conserved amino acid residues located within the N-terminal domain. In contrast, the H-site is formed by less-conserved residues in the C-terminal domain. In GSH-bound DHAR, GSH is at the G-site and is stabilized through non-covalent bonds (Do et al. 2016; Lallement et al. 2016; Bodra et al. 2017). According to the GSH-bound structures of PtrDHAR3A and AtDHAR2, the γ-glutamyl moiety of GSH is stabilized by hydrogen bonds with Ser73 and the salt bridge with Asp72, and the cysteinyl moiety of GSH forms a hydrogen bond with the main chain of Val60 (Lallement et al. 2016; Bodra et al. 2017). The distance between the cysteinyl sulfur of GSH and the sulfur atom of the catalytic

cysteine residue (2.0 Å at PtrDHAR3A; 2.8 Å at AtDHAR2) indicates that GSH and catalytic cysteine can form a mixed disulfide. The glycine moiety of GSH forms a single salt bridge with Lys47. The structure of the GSH-bound AtDHAR2 indicates that the γ-glutamyl moiety is also involved in van der Waals interaction with Phe22 and hydrogen bonds with Lys59 (Bodra et al. 2017). Among the GSTs, Asp72 and Sre73 are well conserved and participate in GSH binding, while Lys47 and Lys59 are less conserved, and are often substituted by equivalent charged residues (Bodra et al. 2017). The structure of OsDHAR1 suggests that both DHA and a secondary GSH can bind at the H-site of DHAR (Do et al. 2016). From the structure of AsA-bound OsDHAR1, AsA forms hydrogen bonds with Lys8, Asp19, and Lys210. The structure of PgDHAR1 bound with glycerol, which shares the backbone of AsA, also supports the contribution of Lys8 and Asp19 to DHA binding (Das et al. 2016). Asp19 has been proposed as an important residue in the binding and stabilizing of DHA in the cases of OsDHAR1, PgDHAR1, and PtrDHAR1 (Do et al. 2016; Das et al. 2016; Lallement et al. 2016). However, in AtDHAR2, the carboxyl group of its side chain is orientated to the protein interior to form a hydrogen bond with His160, complicating our understanding of its role in DHA reduction (Bodra et al. 2017). Moreover, the bound AsA in OsDHAR1 forms hydrophobic van der Waals interactions with Pro21, Phe104, and Trp207. All amino acid residues predicted to interact with AsA in the OsDHAR1 structure are conserved among DHARs (Bodra et al. 2017).

The mechanism for DHA reduction by DHAR, mentioned above, has been proposed based on the structure of OsDHAR1 (Do et al. 2016). In the first step (Reaction 4), the DHA molecule is bound to the reduced catalytic cysteine residue Cys20 and reduced to AsA. In this step, Lys8 may have a central role in the binding and protonation of DHA. In OsDHAR1 and DHAR2 of Chinese white poplar (*Populus tomentosa*) (Tang and Yang, 2013), the substitution of Lys8 to alanine significantly reduced the DHAR activity. However, although Lys8 is conserved in the amino acid sequence of AtDHAR2, it is distal to the active site, and the corresponding structural position of this residue is occupied by glycine (Bodra et al. 2017). The reduction of DHA involves nucleophilic attack by the catalytic cysteine residue Cys20 and the formation of cysteine-sulfenic acid. During this reaction, Ser23 stabilizes the reactive thiolate anion of the catalytic cysteine residue of reduced DHAR by a hydrogen bond (Das et al. 2016). However, in the chloroplastic type of DHAR containing the CPFC active site motif, this serine residue is substituted with a cysteine residue. In the second step (Reaction 5), the reactive sulfenic acid at the catalytic cysteine residue reacts with GSH bound at the G-site and forms the mixed disulfide. Subsequently, the second GSH molecule binds to the H-site and this GSH may be deprotonated to the GS^- form. The water molecule found near GSH forms hydrogen bonds with Lys8, Asp19, and His160, in the structure of the GSH-bound OsDHAR1 (Do et al. 2016). This water molecule may be positioned to deprotonate from the sulfur atom of the GSH at the H-site, generating GS^-. Then, the GSH bound with the catalytic cysteine residue is removed by the nucleophilic attack of GS^- at the H-site. As a result, a catalytic cysteine residue is reduced and one molecule of GSSG is released (Reaction 6) (Do et al. 2016).

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Regulation of Ascorbic Acid Biosynthesis in Plants

Marina Suekawa, Takayuki Kondo, Yukichi Fujikawa, and Muneharu Esaka

Abstract Plants synthesize L-ascorbic acid (AsA) by the Smirnoff-Wheeler pathway, which is considered to be the principal de novo biosynthesis pathway for AsA. In addition, three alternative biosynthesis pathways have also been proposed in plants. The AsA levels are different among organs, and they are also affected by internal factors such as growth and development as well as the environmental factors such as light, drought, salt, and extreme temperature. Among them, ascorbic acid biosynthesis in plants is mostly stimulated by light, depending on its quantity. The AsA levels are well regulated at the transcriptional and posttranscriptional levels of AsA biosynthetic enzymes. The AsA levels are considerably different among plant species. Acerola (*Malpighia glabra*), a tropical plant with a high levels of AsA in its fruits, is a powerful tool for revealing how to regulate and increase AsA levels in plants. Gene expression analysis in acerola has allowed authors to propose that high transcriptional expression level of the biosynthetic enzymes may contribute to high accumulation of AsA. The upstream regions of the genes involved in AsA biosynthesis contain the *cis*-element(s) required for specificity and high level of their gene expression. Because of its antioxidant role and nutrient value, one of the goals to understand the regulation of AsA biosynthesis is to manipulate AsA levels in plants. This chapter focuses on expression and regulation of genes involved in AsA biosynthesis of higher plants.

Keywords Acerola (*Malpighia glabra*) · Ascorbic acid biosynthesis · *cis*-Element · Fruits · Gene expression · Light · Smirnoff-Wheeler pathway

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M. Suekawa · T. Kondo · Y. Fujikawa · M. Esaka (✉)
Graduate School of Biosphere Sciences, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan
e-mail: mesaka@hiroshima-u.ac.jp

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1 Introduction

L-ascorbic acid (AsA) is one of the most abundant soluble antioxidants and enzyme cofactors involved in some important physiological roles in plants (Davey et al. 2000). In leaves, AsA mainly takes part in photosynthesis for detoxifying reactive oxygen species (ROS) such as hydrogen peroxide. Plants synthesize AsA by the Smirnoff-Wheeler pathway, which is proposed to be the principal de novo biosynthesis pathway for AsA. The crucial role of this pathway for AsA biosynthesis in plants is supported by the effect of mutations in genes involved in this pathway on AsA levels (Conklin et al. 2000). In addition to the Smirnoff-Wheeler pathway, three alternative AsA biosynthesis pathways have also been proposed. These alternative pathways may synthesize AsA through D-galacturonate (Agius et al. 2003), L-gulose (Jain and Nessler 2000; Radzio et al. 2003), and *myo*-inositol (Lorence et al. 2004), respectively (Fig. 1). Agius et al. (2003) reported that the overexpression of D-galacturonate reductase in the D-galacturonate pathway increased the AsA levels in Arabidopsis. However, little is known about the contribution of these pathways to AsA levels, compared to the Smirnoff-Wheeler pathway. In plants, AsA levels can be determined not only by AsA biosynthesis but also by its recycling. In AsA recycling, oxidized AsA (monodehydroascorbate and dehydroascorbate) are reduced back to AsA by two reductases (Chen et al. 2003; Gallie 2013). Because of the existence of its recycling, the regulation of AsA levels in plants is too complex to elucidate completely. Also, the AsA levels are considerably different among plant species, organs, and growth stages. In addition, AsA levels are sensitively regulated by environmental factors such as light, drought, salt, and extreme temperature. However, it has gradually become clear, thanks to many studies focused on the regulation of AsA levels in plants. Smirnoff and Pallanca (1996) reported that amounts of carbohydrates were a crucial factor of determination of AsA levels in plants since a close correlation between the levels of AsA and soluble carbohydrate was observed in barley seedlings. Moreover, the decrement of AsA levels responding to environmental status was minimized by feeding of sugars such as glucose and sucrose, suggesting that AsA levels were regulated by sugars. On the other hand, Tamaoki et al. (2003) mentioned that the gene expression levels of AsA biosynthetic enzymes were also essential for the determination of AsA levels in plants since such feeding of sugars could not completely compensate the decrease of AsA levels. It is considered that regulation by AsA biosynthetic enzymes at transcriptional levels is one of the crucial factors of determination of AsA levels in plants.

Humans are unable to synthesize AsA because of lack of an AsA biosynthetic enzyme catalyzing a final reaction. Therefore, fruits and vegetables are reliable sources of supplying AsA for human nutrition. Because of the antioxidant role and nutritional value of AsA, the enhancement of AsA levels in plants lead to increase in stress tolerance and nutritional value. Thus, one of the goals to understand the regulation of AsA biosynthesis is to manipulate AsA levels in plants by genetic engineering of AsA biosynthesis. This chapter mainly focuses on the regulation of AsA biosynthesis at the transcriptional levels of its biosynthetic enzymes responding to internal and environmental factors affecting AsA levels in plants. Through this knowledge, finally, we will propose one of the effective ways to manipulate AsA levels in plants.

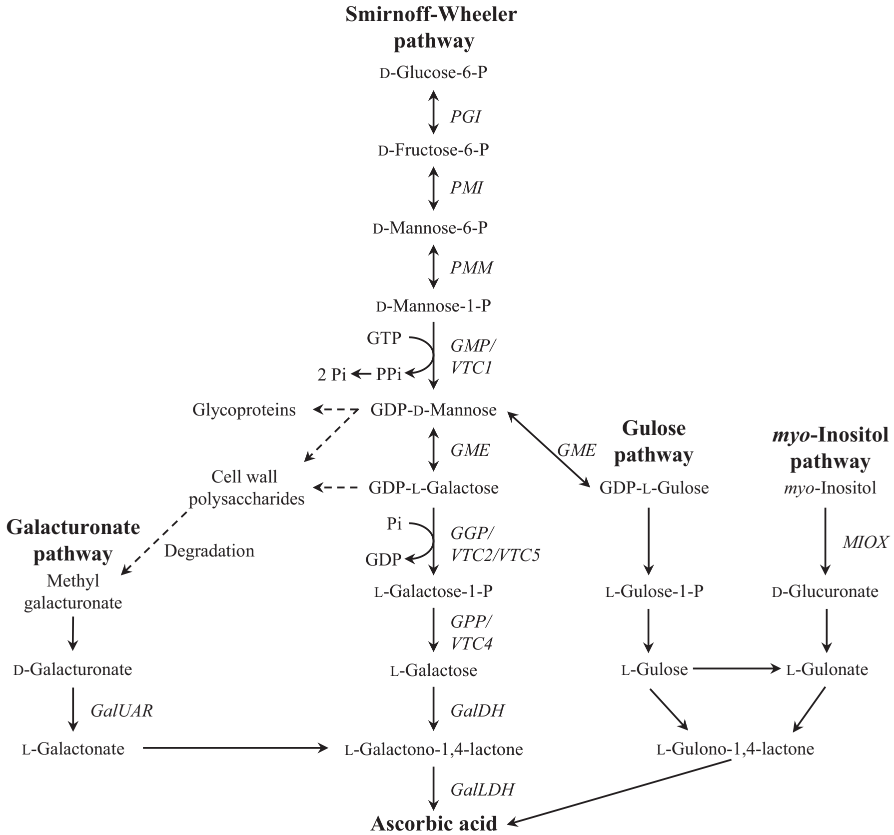

Fig. 1 Ascorbic acid biosynthesis pathways in higher plants. The Smirnoff-Wheeler pathway which is a main AsA biosynthesis pathway and alternative pathways such as galacturonate pathway, gulose pathway, and *myo*-inositol pathway are illustrated. The names of AsA biosynthesis pathway are shown in bolds, and the AsA biosynthetic enzymes are shown in italics. The abbreviations indicate as follows: *PGI* phosphoglucose isomerase; *PMI* phosphomannose isomerase; *PMM* phosphomannomutase; *GMP* GDP-D-mannose pyrophosphorylase; *GME* GDP-D-mannose 3′,5′-epimerase; *GGP* GDP-L-galactose phosphorylase; *GPP* L-galactose-1-P phosphatase; *GalDH* L-galactose dehydrogenase; *GalLDH* L-galactono-1,4-lactone dehydrogenase; *MIOX myo*-inositol oxygenase; *GalUAR* D-galacturonate reductase. The enzymes of VTCs, which were identified from the AsA-deficient *vtc* (*vitamin C*) mutant lines, are also shown

2 Ascorbic Acid Accumulation in Plants

AsA levels in plants are determined by its biosynthesis activities and metabolic turnover rate. Regarding the determination of AsA levels in plants, the activities of AsA biosynthesis may be a crucial factor because they can decide the amount of carbon flux for AsA biosynthesis. In Arabidopsis, there are some AsA-deficient *vtc* (*vitamin C*) mutant lines (Conklin et al. 2000). The *vtc* mutants seemed to be somehow involved in the regulation of AsA levels, and they played a key role as a clue to elucidate the biosynthesis pathway for AsA in plants. Four of the five *vtc* mutants

were identified as mutation of genes encoded AsA biosynthetic enzymes. Conklin et al. (1997) first genetically identified *VTC1* locus, the mutation of which resulted in ozone-sensitive phenotype and only about 30% AsA levels of the wild-type, as an AsA biosynthesis enzyme encoded a GDP-mannose pyrophosphorylase (GMP) in the Smirnoff-Wheeler pathway. Also, other mutants of the *vtc2/5* and *vtc4* mutants have been identified as lack of the genetic loci responsible for GDP-L-galactose pyrophosphorylase (GGP) and L-galactose-1-phosphate phosphatase (GPP), respectively (Conklin et al. 2006; Dowdle et al. 2007; Laing et al. 2007; Linster et al. 2007). All enzymes involved in the Smirnoff-Wheeler pathway have been already identified, and the extreme deficiency of the AsA levels in the *vtc* mutants indicates that the Smirnoff-Wheeler pathway would be a main AsA biosynthesis pathway in plants. The Smirnoff-Wheeler pathway proceeds via nine consecutive reactions including the first three reactions of glucose-6-phosphate isomerase (PGI), mannose-6-phosphate isomerase (PMI), and phosphomannomutase (PMM), and the other six steps are left after these three reactions (Fig. 1). The overexpression of *PMM* resulted in increasing AsA levels (Badejo et al. 2009a; Qian et al. 2007), nevertheless their effects on AsA biosynthesis are appeared to be limited. Considering the extreme deficiency of AsA levels in the *vtc* mutants, the other six reaction steps in the Smirnoff-Wheeler pathway by the enzymes, GMP, GDP-mannose-3′, 5′-epimerase (GME), GGP, GPP, L-galactose dehydrogenase (GalDH), and L-galactono-1,4-lactone dehydrogenase (GalLDH), can be significant to affect AsA levels in plants. GalLDH is a final enzyme of the Smirnoff-Wheeler pathway and may possibly be a candidate enzyme for catalyzing the rate-limiting step of AsA biosynthesis. However, the overexpression of *GalLDH* resulted in no drastic increment of AsA levels (Imai et al. 2009b; Landi et al. 2015; Zhang et al. 2016b). Moreover, the endogenous amount of GalLDH was proposed to be enough to convert L-galactono-1,4-lactone, a substrate of GalLDH to AsA, because L-galactono-1,4-lactone has been hardly detected in plants (Wheeler et al. 1998). Thus, other enzymes upstream of supplying L-galactono-1,4-lactone can be key enzymes to regulate AsA biosynthesis. GalDH catalyzes the oxidation of L-galactose to L-galactono-1,4-lactone. However, GalDH is not also considered to be a key enzyme because of few effects on AsA levels by the overexpression of *GalDH* (Gatzek et al. 2002). These results suggest that the endogenous activities of both GalDH and GalLDH are sufficient for conversion of L-galactose to AsA in plants under normal environmental conditions. As shown in Fig. 1, the Smirnoff-Wheeler pathway synthesizes AsA from D-glucose-6-phosphate via the intermediates, GDP-D-mannose and GDP-L-galactose, substrates of GME and GGP, respectively. These substrates are used for biosynthesis of not only AsA but also glycoprotein and cell wall polysaccharide (Lukowitz et al. 2001). Because the reactions upstream of supplying GDP-D-mannose are linked to sugar metabolism as well as AsA biosynthesis and GGP is a first committed step in AsA biosynthesis, GGP appears to be an effective enzyme to regulate AsA levels. This assumption is supported by the report that the overexpression of *GGP* substantially increased AsA levels, compared to the overexpression of *GalDH* and *GalLDH* (Bulley et al. 2009). In addition to GGP in the Smirnoff-Wheeler pathway, GMP, GME, and GPP

also can affect AsA levels. Thus, AsA biosynthesis must be controlled at multiple rate-limiting steps in the Smirnoff-Wheeler pathway. Although plants predominantly synthesize AsA by the Smirnoff-Wheeler pathway, some alternative pathways for AsA biosynthesis have also been proposed in plants. The galacturonate pathway is one of the alternative pathways in plants (Fig. 1). In this pathway, D-galacturonate derived from components of the cell wall is reduced to L-galactonate by D-galacturonate reductase (GalUAR) and then lactonized to L-galactono-1,4-lactone, which is the last intermediate in the Smirnoff-Wheeler pathway. However, aldonolactonase which catalyzes the reaction of the lactonization of L-galactonate to L-galactono-1,4-lactone has not been genetically identified yet in higher plants. Even though aldonolactonase is unidentified in higher plants, the overexpression of strawberry *GalUAR* resulted in two- to threefold increase in the AsA levels in Arabidopsis (Agius et al. 2003), tomato (Lim et al. 2016), and potato (Hemavathi et al. 2009). These studies suggest that plants can synthesize AsA through the galacturonate pathway and GalUAR is elucidated as the key enzyme of this pathway. Another alternative pathway is the *myo*-inositol pathway. This pathway synthesizes AsA via the intermediates; *myo*-inositol, D-glucuronate, L-gulonate, and L-gulono-1,4-lactone. *Myo*-inositol oxygenase (MIOX) catalyzes the conversion of *myo*-inositol to D-glucuronate, and MIOX is considered to be a key enzyme because the overexpression of *MIOX* resulted in two- to threefold increase in the AsA levels in Arabidopsis (Lorence et al. 2004). On the other hand, Endres and Tenhaken (2009) reported the overexpression of *MIOX* showed no effect on the AsA levels in Arabidopsis. There are other studies supporting the existence of *myo*-inositol pathway (Lisko et al. 2013; Toth et al. 2011); however, it is still under discussion whether *myo*-inositol pathway takes part in AsA biosynthesis in plants. The other pathway is the gulose pathway. This pathway starts with an intermediate, GDP-L-gulose to which GDP-D-mannose converted by GME involved in the Smirnoff-Wheeler pathway. GME catalyzes the reaction of GDP-D-mannose to GDP-L-gulose as well as GDP-L-galactose and plays a role in the intersection of the Smirnoff-Wheeler pathway and gulose pathway. The gulose pathway is specialized in animals, and the enzymes involved in this pathway have not been identified yet in higher plants. Nevertheless, the overexpression of rat L-gulono-1,4-lactone oxidase (*ALO*) increased the AsA levels in tobacco and lettuce (Jain and Nessler 2000). Radzio et al. (2003) reported that the overexpression of rat *ALO* in Arabidopsis *vtc* mutants fully restored the AsA levels, in spite of extreme deficiency of AsA in the mutants. It seems that the gulose pathway could have big effects on the AsA levels in plants; however, the effect of supplying L-gulono-1,4-lactone on the AsA levels in tobacco leaves was obviously limited compared to supplying L-galactono-1,4-lactone (Jain and Nessler 2000). Thus, the gulose pathway is also considered as one of the alternative pathways for AsA biosynthesis and synthesizes AsA together with other pathways in plants.

The Smirnoff-Wheeler pathway from D-glucose-6-phosphate to AsA is the major biosynthesis pathway for AsA in plants. However, AsA may be significantly biosynthesized also by other alternative pathways. The AsA levels will be mainly regulated at the transcriptional levels of their biosynthetic enzymes. Moreover, the feedback

regulations of AsA at the transcriptional, translational, and posttranslational levels have also been reported (Dowdle et al. 2007; Laing et al. 2015; Mieda et al. 2004; Tabata et al. 2002). Thus, the existence of several AsA biosynthesis pathways, their multiple rate-limiting steps, and their feedback regulations make it complex to understand the regulation of AsA biosynthesis in plants.

3 Internal Factors to Regulate Ascorbic Acid Biosynthesis

AsA levels are considerably different among various organs, such as root, stem, leaf, flower, and fruit. In tomato (Zhang et al. 2011), the AsA levels per 100 g of fresh weight (FW) of the roots and stems are about 10 mg. Moreover, those of the flowers and fruits are 10–30 mg, respectively. The leaves contain large amounts of AsA at about 80 mg. Moreover, AsA levels in organs are considerably changed during plant growth. AsA is synthesized most actively in leaves because plants use AsA for detoxifying ROS generated from the electron transport chain during photosynthesis reaction. Therefore, AsA is abundantly contained in chloroplasts at cellular level, in which the AsA levels reach to 20–50 mM (Foyer et al. 1983; Foyer and Lelandais 1996), and the AsA levels in leaves reflect those in chloroplasts (Smirnoff 2000). In general, AsA levels decreased during leaf senescence (Bartoli et al. 2000; Tamaoki et al. 2003; Zhang et al. 2009). Leaf senescence is the last phase of plant development, and it represents a process of nutrient relocation from senescing leaves to growing tissues or sink organs (Lim et al. 2007). Thus, old leaves contain less AsA than young leaves. Besides, AsA plays a role in cell expansion in cultured tobacco cells (Kato and Esaka 2000; Tabata et al. 2001), suggesting that developing tissues such as young leaves require much AsA. Hence, AsA is synthesized actively in young leaves. In Arabidopsis and potato, the AsA levels correlated with the gene expression levels of *GalLDH* in leaf senescence (Bartoli et al. 2000; Tamaoki et al. 2003). Because GalLDH is not considered to be a key regulator of AsA levels, it seemed that not only GalLDH but also other AsA biosynthetic enzymes may be involved in regulating AsA levels in leaf senescence. In Arabidopsis, AsA mannose pathway regulator 1 (*AMR1*) was identified as a negative regulator of the gene expression in the Smirnoff-Wheeler pathway (Zhang et al. 2009). In Arabidopsis, T-DNA activation-tagged and knockout lines in *AMR1* showed that the gene expression levels of *AMR1* were inversely correlated with the levels of AsA and the gene expression of biosynthetic enzymes including *GMP*, *GME*, *VTC2* (not *VTC5*), *GPP*, *GalDH*, and *GalLDH*. In these mutants, the gene expression of *MIOX* was not affected, compared to the wild-type, suggesting that AMR1 specifically and negatively regulates the gene expression in the Smirnoff-Wheeler pathway. The gene expression levels of *AMR1* gradually increased as leaf senescence progressed, along with the decrease in AsA levels. This result suggests that AMR1 is an important regulator of AsA levels in leaf senescence via regulating the gene expressions in the Smirnoff-Wheeler pathway.

Plants accumulate abundant AsA in fruits. Thus, fruits are a major source of AsA for the human diet. In general, the patterns of the AsA accumulation in fruits can be divided into two types. In tomato, grapes, and strawberries, the AsA levels increased as fruit ripening progressed (Cruz-Rus et al. 2010, 2011; Zhang et al. 2011). On the other hand, acerola, peach, and kiwifruit show the decrement of AsA levels during fruit ripening (Badejo et al. 2009b; Bulley et al. 2009; Imai et al. 2009a). However, the details of the mechanism of AsA accumulation in fruits are still unknown. Badejo et al. (2012) reported that the translocation of AsA from leaves to fruits was observed in the tomato plants treated with exogenous radiolabeled AsA. Besides, it is also reported that AsA levels were increased even in the excised tomato fruits by feeding AsA precursors. These results suggest that not only translocation but also de novo biosynthesis for AsA occurs in fruits. Because the galacturonate pathway seems to utilize D-galacturonate, a cell wall component released by breakdown of cell walls during ripening, the galacturonate pathway relatively affects AsA levels in fruits. In strawberry and grape, the gene expression levels of *GalUAR* were correlated with the AsA levels during fruit ripening (Agius et al. 2003; Cruz-Rus et al. 2010). In strawberry, the *GalUAR* promoter had high activities in its fruits, not in its leaves (Agius et al. 2005). This result suggests that the galacturonate pathway seems to work more actively in fruits than in leaves. In tomato (Badejo et al. 2012), the exogenous treatment with L-galactose lead to the increase of the AsA levels in immature and mature fruits, whereas the exogenous treatment with D-galacturonate increased the AsA levels in mature fruits, not in immature fruits. Also, the enzyme activities of GalUAR were higher in mature fruits than in immature fruits. These results suggest that the Smirnoff-Wheeler pathway synthesize AsA throughout fruit maturation, whereas the galacturonate pathway works at only ripening stage in fruit. In apple, the exogenous treatment with D-galacturonate increased the AsA levels in old leaves, not in young leaves (Li et al. 2010a). As described above, the galacturonate pathway seems to constitute the metabolism of the breakdown products of cell walls. D-Galacturonate might be preferred to use for AsA biosynthesis compared with intermediates of the Smirnoff-Wheeler pathway because the AsA synthesis via the Smirnoff-Wheeler pathway might compete to use its substrates with the synthesis of other compounds, such as cell wall polysaccharides and glycoproteins. Thus, in AsA biosynthesis in fruits, the galacturonate pathway may work complementary with the Smirnoff-Wheeler pathway, according to their substrate availabilities.

Roots contain AsA at low levels, compared with leaves and fruits because of its effect on root morphology (Xu et al. 2013), and little is known about AsA biosynthesis in roots. The gene expressions of GGP and GPP which are suggested to be key enzymes for the AsA levels in leaves were hardly detected in the roots of radish (Xu et al. 2013). In Arabidopsis, two loci of *VTC2* and *VTC5* encode GGP (Dowdle et al. 2007; Laing et al. 2007; Linster et al. 2007), and the gene expressions of *VTC2* and *VTC5* were dominant in leaves and roots, respectively, suggesting that VTC5 is a crucial regulator of AsA levels in the roots of Arabidopsis (Dowdle et al. 2007). In rice, there are three GMP isozymes, VTC1-1, -3 and -8 (Qin et al. 2016). *VTC1-3* was specifically expressed in roots, in contrast to the abundant expression of *VTC1-1* in leaves. The suppression of *VTC1-1* and *VTC1-3* resulted in the

decrement of the AsA levels in leaves and roots, respectively. The organ-specific gene expression might be responsible for regulation of AsA levels in roots.

The AsA levels are significantly different among plant species. Acerola is a tropical plant, originally come from South America, and it is known as one of the plants containing much higher AsA in fruits (Badejo et al. 2009b; Davey et al. 2000). The AsA levels are about 1300 and 330 mg/100 gFW in fruits and leaves, respectively. The AsA levels in acerola leaves are about eightfold higher than those in Arabidopsis leaves. Therefore, acerola can provide valuable information how plants accumulate much higher AsA. As a result of the gene expressions analysis in acerola fruits (Badejo et al. 2009b), the gene expressions in the Smirnoff-Wheeler pathway were correlated with the AsA levels during fruit ripening. Also, as shown in Table 1, the gene expressions were much higher in acerola leaves than in Arabidopsis leaves. The gene expressions of acerola *GMP*, *GME*, *GGP*, *GalDH*, and *GalLDH* were 100-, 30-, 700-, 30-, and 5-fold higher than those of Arabidopsis, respectively. On the other hand, the gene expression of *PMM* was also correlated with the AsA levels in fruits and leaves of acerola; nevertheless, the gene expression levels of *PMM* in acerola was little different from those in Arabidopsis and tomato. In acerola, PMM would be involved in regulation of AsA levels but might not be a critical factor for high accumulation of AsA (Badejo et al. 2009a). The high gene expressions of several AsA biosynthetic enzymes, especially *GMP*, *GME*, and *GGP* can be responsible for higher AsA accumulation in acerola. It was also report that in the fruits of *Ziziphus jujube* Mill. (Zhang et al. 2016a), which contains approximately 1000 mg/100 gFW of AsA levels, *GMP*, *GME*, and *GGP* were highly expressed. In contrast, only *GGP* was highly expressed in fruits of tomato with low AsA levels (Ioannidi et al. 2009). The high expression of genes encoding AsA biosynthetic enzymes in the Smirnoff-Wheeler pathway in acerola would be due to their high promoter activities. The promoter activity of acerola *GMP* was considerably higher than that of the Arabidopsis *GMP* (Fig. 2). As shown in Fig. 3, a lot of well-known consensus sequences were found in the promoter region of acerola *GMP*. The deletion analysis suggested that a critical novel *cis*-element (GAAGT) responsible for high promoter activity is localized between −1087 and −1083 bp of acerola *GMP* promoter (Kondo et al. 2017). In acerola, the high-level expressions of enzymes in the Smirnoff-Wheeler pathway were shown to be regulated at transcriptional levels of AsA biosynthetic enzymes. Furthermore, novel *cis*-element(s) localized in their promoter regions was suggested to be responsible for high gene expressions of enzymes in the Smirnoff-Wheeler pathway.

4 Environmental Factors to Regulate Ascorbic Acid Biosynthesis

AsA levels in plants change drastically responding to environmental status, such as light, temperature, and water (Grace and Logan 1996; Moran et al. 1994; Seminario et al. 2017; Smirnoff and Pallanca 1996; Yungyuen et al. 2017). This fluctuation of

Table 1 Comparison of the ascorbic acid levels and mRNA abundance of the Smirnoff-Wheeler pathway genes in the leaves of Arabidopsis and acerola.

Plant species	Ascorbic acid level (mg/100 gFW)	Relative mRNA abundance				
		GMP	GME	GGP	GalDH	GalLDH
Arabidopsis	44.03 ± 7.05	16.43 ± 0.75	50.80 ± 2.00	12.00 ± 1.15	27.32 ± 0.82	19.10 ± 0.66
Acerola	332.16 ± 16.73	1724 ± 38	1535 ± 70	7840 ± 388	701.7 ± 9.7	80.43 ± 11.28

Adapted from Badejo et al. (2009b)

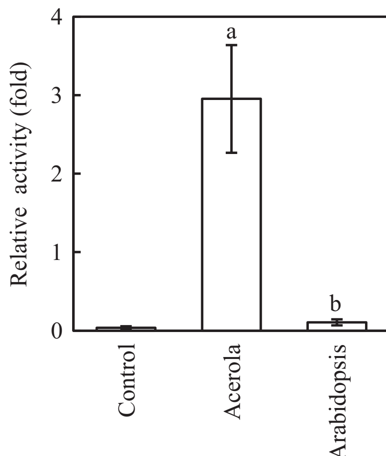

Fig. 2 Promoter analysis of *GMP* from acerola and Arabidopsis. The DNA fragments of the 5′-upstream region of acerola *GMP* (−1185 to +60 bp) and Arabidopsis *GMP* (−1200 to +27 bp) were subcloned the upstream of the Renilla luciferase reporter gene in experimental vectors. The vector containing no promoter was used as a control. Arabidopsis protoplasts were transformed with a Renilla luciferase experimental vector and the pMONT vector containing beetle red luciferase under control of the *cauliflower mosaic virus* (CaMV) 35S promoter as an internal control vector. After transfection, protoplasts were incubated overnight, then luminescence signals were measured as promoter activities. Promoter activities are represented as relative activities dividing the relative light units of Renilla luciferase by that of beetle red luciferase. Bars represent means ± SE ($n = 3$). Datasets with the different letter indicate significant differences ($p < 0.05$). Adapted from Kondo et al. (2017)

AsA levels by environmental factors will occur more rapidly than those by internal factors, such as growth and development. Among of environmental factors, ascorbic acid biosynthesis in plants is mostly stimulated by light, depending on its quantity. Plants cannot survive under no light condition because they need light energy to produce carbohydrates including sucrose and starch by photosynthesis. However, ROS are inevitably produced as unfavorable byproducts through photosynthesis reactions. To detoxify ROS generated in photosynthesis, plants require antioxidants such as AsA under the light condition. Logan et al. (1996) reported that plants accumulated more AsA in sun-acclimated tissues than shade-acclimated tissues. Also, AsA levels were increased in the high-light intensity in *Vinca major* and *Schefflera arboricola* (Grace and Logan 1996). In leaves, AsA appear to be predominantly synthesized via the Smirnoff-Wheeler pathway. In the Smirnoff-Wheeler pathway, several steps were reported to be stimulated by light. Maruta et al. (2008) indicated that the gene expression level and enzyme activity of PMI were positively correlated with the AsA levels in Arabidopsis leaves under the light condition. Moreover, it was also reported that the gene expressions of *GMP*, *GPP*, and *VTC2* were up-regulated, whereas the gene expressions of *GME* and *GalDH* were not affected by light (Yabuta et al. 2007). These results indicated that the light-inducible AsA accumulation is regulated at the transcriptional levels of the AsA biosynthetic enzymes in the

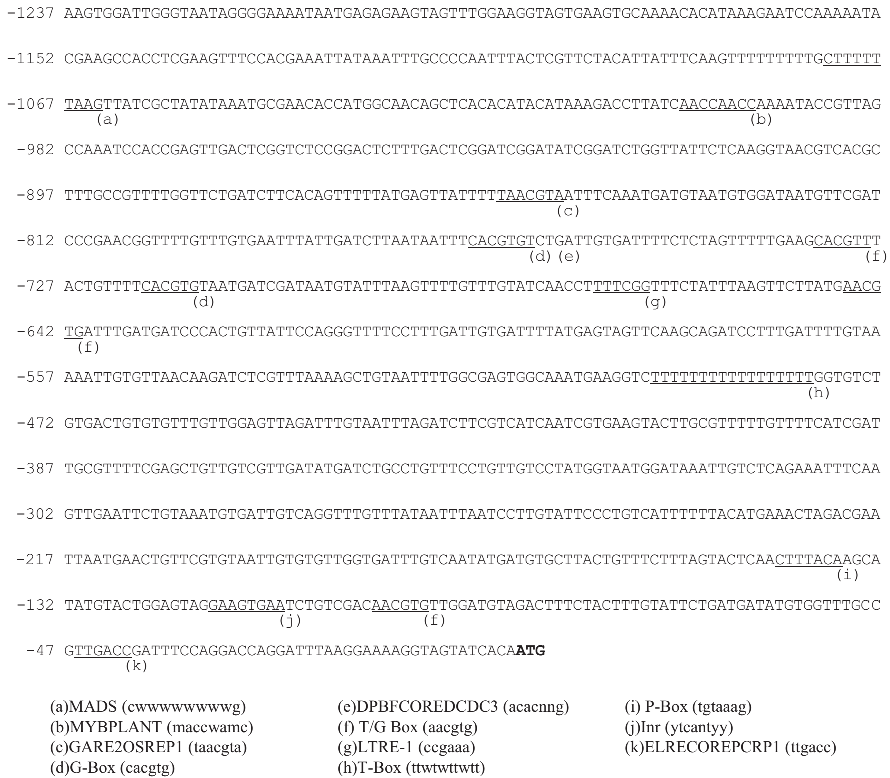

Fig. 3 Promoter sequence and putative cis-elements of acerola *GMP*. The sequence of 5′-upstream region of acerola *GMP* (−1237 to +3 bp) is shown with the initiation codon (ATG) in bold type. The putative *cis*-elements were analyzed using the PLACE program (Higo et al. 1998) and similar sequences to *cis*-elements reported previously are underlined. Adapted from Badejo et al. (2008)

Smirnoff-Wheeler pathway. The AMR1 is a negative regulator of the Smirnoff-Wheeler pathway, and the gene expression of *AMR1* was down-regulated under the high-light intensity (Zhang et al. 2009). It suggests that AMR1 controls the AsA levels under the light condition in Arabidopsis.

Under the light condition, the sugar levels in plants also increase because photosynthesis reaction is activated. It is an important issue whether the gene expressions in the Smirnoff-Wheeler pathway under the light condition is affected by light itself or sugars generated by photosynthesis. It was reported that the gene expression of *GalLDH* in broccoli florets was down-regulated by removal of broccoli leaves (Nishikawa et al. 2005). This down-regulation of the *GalLDH* expression in the florets seems to be caused by the stagnation of sucrose supply from leaves because the exogenous sucrose induced the gene expression of *GalLDH*. However, it was also mentioned that the decline of the AsA levels in the post-harvest broccoli florets could not be completely compensated by exogenous sucrose. In Arabidopsis, it was found that the exogenous sucrose slightly induced the gene expression levels of

VTC2 and *GalLDH* although light induced those of not only *VTC2* and *GalLDH* but also *GMP* and *GPP* (Yabuta et al. 2007). These results suggest that the gene expression levels in the Smirnoff-Wheeler pathway are controlled by not only sugars but also light itself, leading to the fluctuation of AsA levels.

The promoter regions of AsA biosynthetic enzymes have *cis*-acting sequences responding to light. Gao et al. (2011) reported that the Arabidopsis *VTC2* promoter had a novel *cis*-element(s), responsible for light-inducible expression, in between −70 and −40 bp of its promoter region. In kiwifruit leaves, the promoter activities of *GGP* were affected by light, and the G-box in the promoter would be responsible for light responses (Li et al. 2013). Moreover, it was reported that the promoters of rice *GPP* and *GalLDH* showed higher promoter activities under the light condition than the dark condition in Arabidopsis leaves (Fukunaga et al. 2010). The GT1 BOX and TGACG motif in their promoters may contribute to light-inducible expression. In contrast to the up-regulation under the light condition, the AsA biosynthesis is down-regulated under the dark condition. It was reported that the gene expression levels of *GMP* and *GalLDH* were decreased in the shaded tobacco leaves, compared with the unshaded tobacco leaves (Tabata et al. 2002). In Arabidopsis, the gene expression levels of *GMP*, *GPP*, *VTC2*, and *GalLDH* were slightly decreased under dark condition (Yabuta et al. 2007). In addition to the AsA biosynthesis regulation at transcriptional levels, Wang et al. (2013) reported that the posttranscriptional regulation of AsA biosynthetic enzymes occurred under dark condition. The COP9 signalosome (CSN) constitutes a multisubunit complex and plays a role in plant photomorphogenesis through the regulation of protein ubiquitination and degradation (Schwechheimer 2004). They reported that one of the subunits, CSN5B, interacted with VTC1 under dark condition, and subsequently VTC1 was degraded by the 26S proteasome. Another protein of CSN complex, CSN8, affects AsA levels as well as CSN5B. The *csn8* mutants contain AsA at the high level, compared to wild-type. These results suggest that CSNs negatively regulate AsA biosynthesis at the posttranscriptional level.

The AsA levels in fruits are also influenced by light conditions. As mentioned above, the AsA levels in fruits are affected by two factors, translocation from source organ and de novo biosynthesis for AsA in fruits (Badejo et al. 2012). It is possible that the translocation rate of AsA from leaves is changed by light because the AsA levels in leaves are considerably affected by light conditions (Tabata et al. 2002; Yabuta et al. 2007). However, there are some reports that light can affect the de novo AsA biosynthesis in fruits of kiwifruit, tomato, apple, and citrus (Lado et al. 2015; Li et al. 2009, 2010b; Massot et al. 2012). The AsA levels in apple fruits, especially in its peels, were decreased by shading fruits along with the reduction in the gene expressions of *GalDH* and *GalLDH* (Li et al. 2009). Moreover, in tomato fruits under dark condition, the down-regulated gene expression of *GPP1* and *VTC2* might be responsible for the decrement of AsA levels (Massot et al. 2012). These results suggest that in both fruits, AsA biosynthesis via the Smirnoff-Wheeler pathway is regulated by light. On the other hand, the decrease in the gene expression of *GalUAR* led to the decrement of AsA levels in shaded citrus fruits (Lado et al. 2015). Also in fruits of grapes, the gene

expression levels of *GalUAR* are positively correlated with the AsA levels under low- and high-light intensities (Cruz-Rus et al. 2010). These results suggest that light would affect the AsA biosynthesis in fruits not only by the Smirnoff-Wheeler pathway but also by the galacturonate pathway. Agius et al. (2005) reported that the promoter of strawberry *GalUAR* had activity in strawberry fruits under light condition but not under dark condition, and the G-box like elements in the promoter region of strawberry *GalUAR* might be responsible for the light-inducible activity. The existence of *cis*-elements being responsible for light-inducible expression supports the opinion that the light-responsible change of AsA levels in fruits is led by the fluctuation of the gene expressions of AsA biosynthetic enzymes, not by the change of translocation rate.

Plants cannot avoid oxidative stress by ROS generated from photosynthesis. Moreover, plants suffer from oxidative damage caused by various environmental stress conditions, such as drought and salt (Akram et al. 2017; Tripathy and Oelmuller 2012). Thus, to mitigate oxidative damage, plants need more AsA under stress conditions. However, under stress conditions, AsA biosynthesis is up-regulated or down-regulated depending on the variation and intensities of environmental stresses. In soybean, AsA levels were decreased under drought stress along with the decrement of the gene expression of *VTC1* as well as the depression of GalLDH activities (Seminario et al. 2017). In tomato leaves, the gene expression of *GalUAR* was remarkably induced by salt and oxidative stresses, whereas AsA levels were decreased (Suekawa et al. 2016). *GPP* was only AsA biosynthesis enzyme induced by cold stress in tomato fruits (Ioannidi et al. 2009). Because of such differences, the regulation of AsA biosynthesis responding to environmental stress remains unclear so far. In Arabidopsis, there is a possibility that one of the ethylene-responsive factors, ERF98, regulates AsA biosynthesis under stress conditions (Zhang et al. 2012). *ERF98* was expressed under salt stress and affected on the AsA levels as well as expressions of *VTC1* and *VTC2* in Arabidopsis. The study also revealed that Arabidopsis ERF98 bound to the promoter of *VTC1*, suggesting that Arabidopsis ERF98 could regulate the gene expression of *VTC1* and possibly *VTC2* under salt stress. Especially, Arabidopsis ERF98 may interact with the dehydration-responsive element (DRE) in the promoter region of *VTC1*, resulting in the up-regulation of the gene expression of *VTC1* under salt stress. In tomato, it was also reported that one of the HD-Zip family transcription factors, SlHZ24, bound to the promoter of *GMP3* and then activated its gene expression (Hu et al. 2016). In addition to *GMP3*, the gene expressions of *PMI*, *GME1*, *GGP*, *GPP1*, *GPP2*, and *GalDH* were also affected by the fluctuation of *SlHZ24* gene expression, suggesting that SlHZ24 could activate most of enzymes for the Smirnoff-Wheeler pathway. The gene expression of *SlHZ24* was induced by oxidative stress. Therefore, SlHZ24 should be involved in the regulation of the AsA levels under oxidative stress in tomato. In kiwifruit (Li et al. 2013), the gene expression of *GGP* was induced by plant hormones, abscisic acid (ABA) and salicylic acid (SA), and the promoter region of *GGP* has hormone-responsive elements such as ABA and methyl jasmonate (MeJA)-responsive elements as well as stress-responsive elements such as hypoxia- and heat stress-

responsive element, and MYB binding site. Besides, its promoter activities increased in response to ABA and SA. This study suggests that *GPP* expression is regulated at transcriptional level by salt stress in kiwifruit.

5 Conclusion and Future Perspectives

AsA levels are highly and complexly regulated in plants, and they are significantly affected by internal factors such as growth and development as well as the environmental factors such as light, salt, drought, and extreme temperature. Light sensitively regulates AsA biosynthesis in plants. Several AsA biosynthesis pathways have been proposed in plants. The Smirnoff-Wheeler pathway predominantly works for AsA biosynthesis, especially in leaves. In fruits, the galacturonate pathway would be preferred for AsA biosynthesis because of the abundance of the AsA precursor in ripen fruits. These AsA biosynthesis pathways would be regulated at both transcriptional and posttranscriptional levels of biosynthetic enzymes by the internal and environmental factors.

Enhancing AsA levels in plants, especially in crops, has been a big subject for improving stress tolerance and the nutritional value for the human diet. In acerola, multiple AsA biosynthetic enzymes in the Smirnoff-Wheeler pathway were highly expressed compared with Arabidopsis (Badejo et al. 2009b). As shown in Fig. 4, the overexpression of acerola *GMP* in the transgenic tobacco increased the AsA levels by only twofold, compared to the wild-type (Badejo et al. 2008). These results suggest that the overexpression of the single gene is not sufficient for the substantial increment of the AsA levels. In acerola, high gene expressions of almost all AsA biosynthetic enzymes in the Smirnoff-Wheeler pathway could highly activate the flux of the Smirnoff-Wheeler pathway and result in high AsA accumulation. This assumption is supported by the study of Bulley et al. (2009). The transient overexpression of kiwifruit *GGP* alone resulted in fourfold increase of the AsA levels in tobacco leaves. On the other hand, the co-overexpression with both kiwifruit *GGP* and *GME* increased the AsA levels by ninefold. The overexpressions of multiple AsA biosynthetic enzymes are necessary to dramatically increase AsA levels in crops; however, it is not easy to enhance gene expressions of all AsA biosynthetic enzymes in the Smirnoff-Wheeler pathway. From the result of the gene expression analysis of acerola, GMP, GME, and GGP might be necessary to increase AsA levels effectively, probably because the steps of GMP, GME, and GGP are intersections of biosynthesis of AsA and other compounds such as cell wall polysaccharides and glycoproteins. Therefore, simultaneous overexpression of these enzymes can activate the flux of the AsA biosynthesis effectively. There are two possible ways to overexpress multiple AsA biosynthetic enzymes; one is overexpressing AsA biosynthetic enzymes themselves, the other is overexpressing regulator(s) for the gene expressions of AsA biosynthetic enzymes. However, it was reported that the overexpression of *SlHZ24*, which is a positive regulator of the Smirnoff-Wheeler pathway, resulted in only 1.5- and 1.3-fold increase in the AsA levels in tomato leaves and

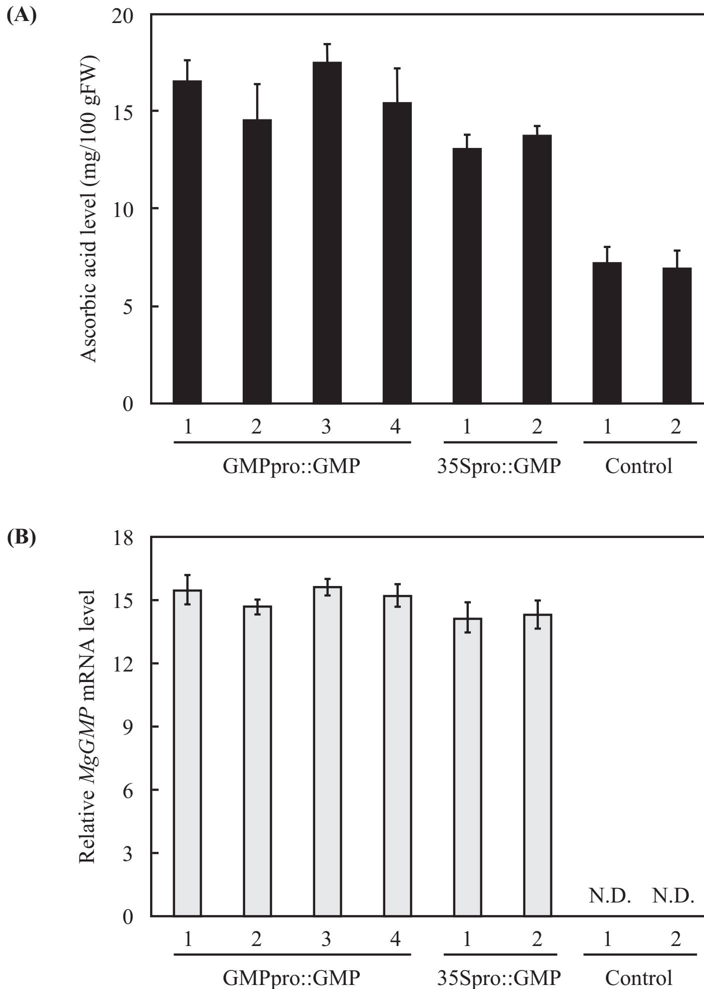

Fig. 4 The AsA levels in tobacco over expressing acerola *GMP*. The AsA level and relative acerola *GMP* mRNA expression in transformed and untransformed leaves of tobacco were analyzed. (**A**) AsA levels were measured in lines transformed with plant expression vectors containing acerola *GMP* under control of the acerola *GMP* promoter (*GMP*pro::*GMP*) and the CaMV 35S promoter (35Spro::*GMP*). Untransformed plants were used as control. Data represent the mean ± SE. (**B**) The relative acerola *GMP* mRNA levels of transformed lines described above were evaluated by real-time RT-PCR. The results were normalized to the expression of actin mRNA. Data represent the mean ± SE. Adapted from Badejo et al. (2008)

fruits, respectively (Hu et al. 2016). Thus, a novel regulator might be necessary to powerfully enhance AsA levels in plants.

In cultured tobacco cells, the gene expressions of *GMP* and *GalLDH* were decreased by feeding AsA (Tabata et al. 2002). Also, the transcription of *VTC2* was inhibited by exogenous AsA in Arabidopsis (Dowdle et al. 2007). It was reported

that the upstream open reading frame of *GGP* took part in the suppression of *GGP* at the translational level under high AsA levels in Arabidopsis (Laing et al. 2015). Moreover, the enzyme activities of spinach GalDH were inhibited in vitro by AsA (Mieda et al. 2004). Considering the feedback regulations of AsA biosynthesis, in plants such as acerola with marked high AsA levels, AsA biosynthesis may be unaffected by the feedback regulation of AsA. Thus, the utilization of highly active promoters of AsA biosynthesis genes from high AsA accumulating plants might be effective for manipulating AsA levels in crops.

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Ascorbate-Glutathione Cycle and Abiotic Stress Tolerance in Plants

Carlos G. Bartoli, Agustina Buet, Gustavo Gergoff Grozeff, Andrea Galatro, and Marcela Simontacchi

Abstract Plants confront fluctuating and in some cases intense environmental conditions, such as changes in irradiation, water availability, extreme temperatures, mineral nutrient accessibility, and air pollutants exposition among others. In order to face abiotic stress situations, the redox buffer capacity, mainly represented by ascorbic acid (AsA) and glutathione (GSH) pools, is involved in growth–stress responses crossroad. These compounds are associated in a set of reactions known as AsA-GSH cycle. The main function of the AsA-GSH cycle originally observed was the detoxification of reactive oxygen species (ROS) in different subcellular compartments such as chloroplast, mitochondria, or cytosol. More recently, the crucial participation of the AsA-GSH cycle in the optimization of photosynthesis was established. In addition, these antioxidants are considered essential components of cell signaling pathways triggering adaptive plant responses. The role of AsA-GSH cycle is analyzed regarding the ability of plants to overcome some selected abiotic stress situations.

Keywords Abiotic stress · Ascorbate · Glutathione · Reactive oxygen species

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C. G. Bartoli (✉)
Instituto de Fisiología Vegetal, CCT CONICET La Plata—UNLP, La Plata, Argentina

Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata,
La Plata, Argentina
e-mail: carlos.bartoli@agro.unlp.edu.ar

A. Buet · G. Gergoff Grozeff · M. Simontacchi
Instituto de Fisiología Vegetal, CCT CONICET La Plata—UNLP, La Plata, Argentina

Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata,
La Plata, Argentina

A. Galatro
Instituto de Fisiología Vegetal, CCT CONICET La Plata—UNLP, La Plata, Argentina

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1 Introduction

Life is supported through a continuous movement of electrons in mitochondria and chloroplasts allowing energy production. These electron flows affect the equilibrium of interconvertible redox couples such as NAD^+/NADH and $NADP^+$/NADPH and in turn impacts cellular redox status (Foyer and Noctor 2009). The balance between oxidized and reduced forms of glutathione (GSSG/2GSH couple) in the cell is considered to be the major thiol-disulfide cellular redox buffer and could be used as an indicator for the redox environment of the cell. Taking into consideration its redox potential and concentration, ascorbic acid (AsA) acts as the final antioxidant and its recovery depends on GSH, thus both species are interconnected (Foyer and Noctor 2011). The subcellular localization, transport, and participation of both redox couples in physiological processes, and their contribution to adaptive responses of plants under abiotic stress situations will be discussed in this chapter.

2 AsA and GSH Functions

2.1 As Antioxidants

The formation of reactive oxygen species (ROS) is continuous and constitutes an integrated part of the metabolism (Table 1). Although ROS are often associated with stressful situations that can lead to cell death, they also have an important role transmitting information allowing appropriate cellular responses to developmental and environmental changes (Foyer and Noctor 2005). Plant cells are endowed with antioxidant systems to prevent the extension of the oxidative reactions by ROS and other oxidative species formed mainly across mitochondrial and chloroplastic electron transport. These systems include enzymatic and non-enzymatic detoxifying mechanisms. GSH (L-γ-glutamyl-L-cysteinyl-glycine) and AsA are the most abundant non-enzymatic antioxidants; they can reach intracellular concentrations from 5 to 20 mM, respectively (Asada and Takahashi 1987). Even though both species have been historically known as antioxidants, their functions in living organisms greatly exceed this role. GSH is the most abundant low molecular thiol in plants, discovered in 1926 (Hunter and Eagles 1926), and later associated with powerful antioxidant functions against lipid peroxides and ROS in general. AsA, in turn, was discovered by Szent-Gyorgyi by the same time (Buettner and Schafer 2006) and later identified as vitamin C. It is considered the terminal small molecule antioxidant in biological systems (Sharma and Buettner 1993), acting as a natural reductant of free radical species.

In addition, AsA and GSH are closely related by the so-called AsA-GSH cycle (Fig. 1). When H_2O_2 is generated by the metabolic pathways mainly in chloroplasts, peroxisomes, and mitochondria, AsA is oxidized to the radical monodehydroascorbate (MDHA) by the reaction catalyzed by ascorbate peroxidase (APX, EC 1.11.1.7)

Table 1 Reactive oxygen and nitrogen species

Reactive species			Sources
Superoxide anion	$O_2^{\bullet -}$	Free radical	Photosystem I and II, mitochondrial electron transport, plasma membrane NADPH oxidase, xanthine oxidase in peroxisomes, peroxidases in apoplast
Hydroxyl radical	$HO^{\bullet}$	Free radical	From H_2O_2 in the Fe catalyzed Fenton reaction
Singlet oxygen[a]	$^1O_2$	Non-radical	Electronically excited chlorophyll
Hydrogen peroxide	H_2O_2	Non-radical	Glycolate oxidase activity and fatty acid β-oxidation in peroxisomes. Oxalate oxidase, amine oxidase, and peroxidases in apoplast. Superoxide dismutase
Nitric oxide	NO	Free radical	Apoplast, mitochondria, chloroplasts, peroxisomes[b]
Peroxynitrite	$ONOO^-$	Non-radical	From the reaction between NO and $O_2^{\bullet -}$

Reactive oxygen species (ROS) are partially reduced or activated forms of oxygen (O_2) produced during aerobic metabolism. Free radical species contains one or more unpaired electrons and are capable of independent existence. Adapted from Mittler (2002)

[a]The most stable form is mentioned ($^1\Delta g$)

[b]Mechanisms of NO synthesis in plants remain to be fully characterized (Santolini et al. 2017)

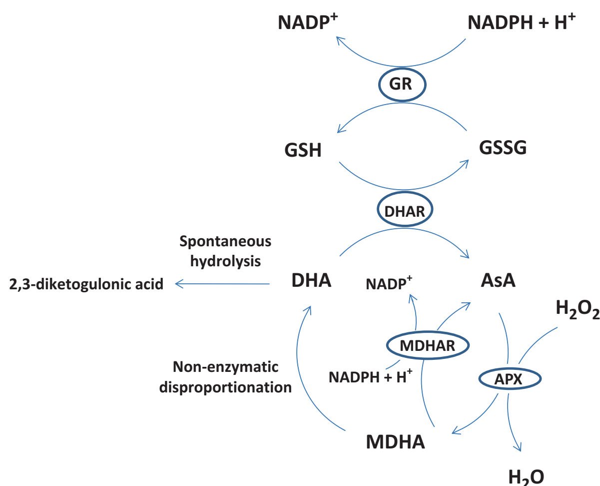

Fig. 1 Scheme showing the consumption and regeneration of AsA and GSH, and the enzymes involved in ascorbate-glutathione (AsA-GSH) cycle. Reactive oxygen species are generated in chloroplasts, mitochondria, peroxisomes, cytosol, and apoplast (Table 1). *APX* Ascorbate peroxidase, *DHAR* dehydroascorbate reductase, *MDHAR* monodehydroascorbate reductase, *GR* glutathione reductase

in order to transform this reactive species into water. The radical MDHA is less reactive than other free radicals and disproportionates spontaneously to dehydroascorbate (DHA) and AsA. Then, AsA is regenerated from DHA by the action of the enzyme DHA reductase (DHAR, EC 1.8.5.1) that requires GSH. The GSSG thus formed is then reconverted to GSH by the action of the enzyme glutathione reductase (GR, EC 1.6.4.2) that uses NADPH as cofactor (Fig. 1). GR is in charge to maintain high GSH/GSSG ratio in close relationship with $NADPH/NADP^+$ ratio (Diaz-Vivancos et al. 2015).

In addition, the enzyme MDHA reductase (MDHAR, EC 1.6.5.4) catalyzes the reduction of MDHA to AsA, requiring NAD(P)H. This enzyme has different isoforms present in cytosol, chloroplasts, mitochondria, and peroxisomes and is associated with plasma membrane too.

It has been demonstrated that the AsA-GSH cycle takes place in mitochondria, chloroplasts, peroxisomes, and cytosol (Jiménez et al. 1997) since the enzymes mentioned above are present in these subcellular compartments. APX has multiple isoforms that are found in the chloroplast stroma (Nakano and Asada 1981), associated to chloroplast thylakoids (close to PSI) (Groden and Beck 1979; Miyake and Asada 1992), cytosol, mitochondria, and peroxisomes (Jiménez et al. 1997). In leaves about 70–80% of the AsA-dependent H_2O_2 scavenging enzymes are located in the chloroplasts. NADPH-dependent GR is present in the chloroplast stroma (Foyer and Halliwell 1976) as in other compartments. GR activity inclines the couple GSH/GSSG towards GSSG reduction in thermodynamic equilibrium with $NADPH/NADP^+$ ratio (Jocelyn 1972). As a consequence, GSH is mostly in the reduced form and reaches concentration of 3.5 mM in chloroplasts (Foyer and Halliwell 1976). This allows keeping a redox environment suitable for the activation and stabilization of Calvin cycle and the reduction of DHA to AsA. The GSH:GSSG ratio is usually in 20:1 (Mhamdi et al. 2010), with variations in specific compartments, being higher in cytosol and lower in vacuole (Meyer et al. 2007; Queval et al. 2011).

Besides the enzymatic reduction of DHA to AsA by DHAR, it is worth to mention that GSH is able to reduce DHA non-enzymatically at pH higher than 7 (Foyer and Halliwell 1976), conditions that can be reached in the stroma of illuminated chloroplasts. On the other hand, some of the AsA-GSH cycle enzymes were found to be active in the apoplast (Podgórska et al. 2017).

2.2 *Other Functions of AsA and GSH*

Being GSH the major reduced thiol, there is a close relationship between sulfur plant status and GSH content. GSH acts as the main long-distance transport form of reduced sulfur from leaves to organs with high demand for protein synthesis, and it is also proposed that GSH acts as a signal transported via phloem from shoot, inhibiting sulfate uptake in roots, thus regulating plant sulfur homeostasis (Rennenberg and Herschbach 2014). Both, external sulfate uptake and assimilation, are inhibited by GSH through effects on specific transporters and enzymes (Buchner et al. 2004).

GSH acts as the electron donor in the reduction of adenosine 5′-phosphosulfate to sulfite in plastids; however, in a complex relationship the same step is also inhibited by GSH decreasing adenosine 5′-phosphosulfate reductase (APR) activity, at protein and mRNA level (Vauclare et al. 2002).

GSH participates in heavy metal and xenobiotic detoxification. Conjugation of GSH to a number of xenobiotics is catalyzed by glutathione *S*-transferase enzymes. A variety of hydrophobic compounds, such as herbicides, become more water soluble and less toxic than the original compounds after GSH conjugation, being the mechanism of detoxification and the basis of resistance of some plant species to certain herbicides (e.g., chloroacetanilides and triazines) (Labrou et al. 2005). GSH is also the precursor in the synthesis of phytochelatins [(γ-Glu-Cys)n-Gly, $n = 2–11$], which are important in detoxifying certain heavy metals such as copper (Cu), cadmium (Cd), and zinc (Zn), since after chelation they are accumulated in the vacuole. Plant cells respond to high concentrations of heavy metals by increasing the synthesis of phytochelatins and decreasing their GSH levels (Tukendorf and Rauser 1990; Dixon et al. 1998; Edwards et al. 2000; Sheehan et al. 2001).

GSH forms part of a complex regulatory network underlying growth, stress tolerance, and it is also necessary for certain developmental steps, such as gametogenesis, seed development, and postembryonic growth, evidenced through the use of *Arabidopsis thaliana* mutants in which the absence of GSH causes embryo lethality (Cairns et al. 2006). These results point out that GSH is essential for normal plant development.

GSH is required in the regulation of the plant cell cycle through the control of the G1–S transition. The recruitment of GSH into the nucleus in the G1 phase affects the redox state of the cytoplasm, where GSH concentration decreases, modifying the expression of transcripts associated with proteins involved in the oxidative signaling and stress tolerance (Diaz-Vivancos et al. 2010). GSH movement between cellular compartments has a profound effect on localized redox state, affecting the redox-sensitive motifs (i.e., cysteine residues, metal cofactors) of cell cycle-regulatory proteins. Thus, it has been suggested that the balance between ROS production and subsequent removal through the action of antioxidants impacts on cell proliferation (Menon and Goswami 2007). Similarly, *vtc2* mutants with low AsA show increased level of oxidation in the nuclei and a delay in the progress of the cell cycle in the proliferation zone of embryonic roots (de Simone et al. 2017).

GSH is also required for the synthesis of pathogen defense-related molecules and disease resistance, protecting against biotic stress (Noctor et al. 2012). In addition, GSH is a source of sulfur for the synthesis of secondary metabolites, and it is found in high concentrations in nitrogen-fixing nodules, where GSH or homoglutathione, have proved to be essential for proper development of the root nodules during the interaction of legumes and rhizobia (Frendo et al. 2005). GSH protects against a broad spectrum of abiotic stress in addition to heavy metal, including drought, salinity, UV radiation, cold, and heat (Srivalli and Khanna-Chopra 2008).

One of the most important protein modification, known as protein thiolation, is produced through the redox signaling pathway mediated by dithiol/disulfide transitions carried out by the activity of thioredoxins (TRX), and participates in plant cell

death-mediated defense responses (Kuźniak et al. 2013). One specific form of protein thiolation that depends on the presence of GSH is called *S*-glutathionylation, considered an important redox post-translational mechanism regulating protein activity. In addition, an important interaction of GSH consists in the reaction with nitric oxide (NO) leading to the formation of *S*-nitrosoglutathione (GSNO) which constitutes a form to transport the bioactive molecule NO and also involves the modulation of protein activity through the post-translational pathway known as *S*-nitrosylation (Lyndermayr et al. 2005).

Besides its antioxidant function, AsA participates in many physiological processes such as photosynthesis, hormone biosynthesis, growth and development regulation; and in plant acclimation responses to adverse environmental conditions. The wide collection of AsA mutants has facilitated the identification and description of the processes in which AsA is involved.

In the photosynthetic apparatus, AsA plays an important role in photoprotection either as a direct antioxidant, scavenging ROS, or as a cofactor of enzymes of the xanthophyll cycle and electron donor. The xanthophyll cycle constitutes the non-photochemical quenching (NPQ), an essential process by which excess light energy is dissipated in order to avoid photoinhibition. When plants are exposed to high light intensity, light harvesting complexes receive more energy that could be driven to photosynthesis through electron transport chain. In other words, to prevent photooxidative damage mainly of photosystem II, zeaxanthin, located at antenna complexes, is able, in conjunction with low pH, to dissipate energy safely as heat and drive excited chlorophyll to its basal state (Demmig-Adams and Adams 1996; Jahns and Holzwarth 2012). Zeaxanthin is synthesized by the reaction catalyzed by violaxanthin de-epoxidase. This thylakoid lumen located enzyme requires AsA as cofactor and low pH to be active (Hager and Holocher 1994; Bratt et al. 1995). In addition, AsA can act as an alternative photosystem II electron donor and thus, support electron transport when oxygen-evolving complexes are inactivated (Tóth et al. 2009).

AsA is also involved in biosynthetic pathways including dioxygenases in their steps. These enzymes require Fe^{2+} and AsA as cofactors (Prescott and John 1996). One example is the 1-aminocyclopropane-1-carboxylate oxidase which catalyzes the final step of ethylene synthesis (Smith et al. 1992). Other dioxygenases participate in gibberellin (GA) biosynthesis. Gibberellin 20-oxidase and GA_{20}-3β-hydroxylase use AsA as co-substrate (Lange 1994; Smith et al. 1990). Dioxygenases are also present in secondary plant metabolism pathways as flavonoids and alkaloids biosynthesis. Flavanone 3-hydroxylase catalyzes the formation of dihydroflavonols which are intermediates in the biosynthesis of many secondary metabolites such as flavonols, anthocyanidins, and proanthocyanidins, and it requires AsA (Britsch and Grisebach 1986). AsA is also needed for the activity of anthocyanidin synthase, flavone synthase I, and flavonol synthase (Saito et al. 1999; Britsch 1990; Britsch et al. 1981).

Moreover, AsA plays an important role in plant growth and stress responses through hormone signaling. It has been reported that *A. thaliana* plants deficient in AsA synthesis (*vtc1*) showed increased abscisic acid (ABA) levels (Pastori et al. 2003; Kerchev et al. 2011). This leads to plant growth arrest. In leaves, water deficit

leads to an increase in ABA levels, triggering H_2O_2 accumulation and the subsequent stomatal closure (Zhang et al. 2001). In this process, the AsA levels and its redox status are crucial in order to reach the H_2O_2 level needed to trigger stomatal movement (Chen and Gallie 2004).

Apoplastic AsA participates in cell growth and cell signaling (Horemans et al. 1998). Shoot growth in response to auxin involves the AsA redox state; when reduced apoplastic AsA is diminished, there is a loss of response to auxin (Pignocchi et al. 2006). It is worth to mention here that the AsA oxidase is involved in regulating the apoplastic AsA redox state by catalyzing its oxidation to MDHA (Pignocchi et al. 2003). AsA influences cell-wall composition and mechanical characteristics as cofactor of prolyl hydroxylases enzymes that catalyze the formation of hydroxyproline-rich glycoproteins (HRGPs) present in the cell wall (De Tullio et al. 1999).

3 Subcellular Localization and Transport of AsA and GSH

The first step of GSH synthesis occurs in plastids; cysteine and glutamate are converted in γ-glutamylcysteine (γ-EC) by the enzyme γ-EC synthetase (Wachter et al. 2005). The second and final step adds glycine and converts γ-EC in GSH through the activity of glutathione synthetase (GSHS). Both γ-EC and GSH can be transported outside plastids; in fact, it is assumed that GSH synthesis occurred mostly in the cytosol where GSHS is also present (Noctor et al. 2012; Lim et al. 2014). Cytosol and plastids have similar GSH concentration, around 3.2–3.5 mM (Krueger et al. 2010), and GSH concentration is clearly higher in leaves than in roots (Noctor et al. 2002). In agreement, sulfate reduction is several times higher in green leaves than in other plant organs, and the reaction is strongly stimulated by light (Fankhauser and Brunold 1978). This light enhancement is to be expected because of the requirement for GSH and ferredoxin as reductants for adenosine-5′-phosphosulfate and sulfite, respectively.

In some plant species, glycine is replaced by alanine or serine in the γ-glutamylcysteinylglycine tripeptide, as in homo-GSH (γ-Glu-Cys-β-Ala), found in many legumes or hydroxymethyl GSH (γ-Glu-Cys-Ser) present in cereals, with similar functions to GSH (Marschner 2012).

Compartment-specific alterations in GSH levels impact on metabolism and defenses, and it is worth to note that a measurement of total content of GSH and GSSG in tissues does not necessarily reflect the redox environment in subcellular compartments such as the nucleus, mitochondria, or chloroplasts (Diaz-Vivancos et al. 2015). As an example, GSH content found in trichomes of *Arabidopsis* resulted two or three times higher than in basal and epidermal cells (Gutierrez-Alcala et al. 2000). GSH concentration in different compartments or cell types is controlled through its biosynthesis, redox state, use, degradation, and transport (Foyer et al. 2001). Long-distance transport of GSH occurs since it has been found in xylem and phloem (Schneider et al. 1994), being the most abundant thiol in phloem, and exchange between phloem and xylem sap also takes place (Mendoza-Cózatl et al. 2011).

Plasma-membrane transport systems for GSH and GSSG are important in maintaining adequate levels in vacuole and apoplast. In particular, in apoplast GSH reaches low concentration with a probable role in defense against pathogens (Vanacker et al. 1998). If GSSG is generated in apoplast, it should be reduced after re-entering to the cytosol since there is no enzyme for GSH recovery in apoplast. It was also demonstrated a recycling of GSH and its efflux from the root cells with a net increase in the external medium; however, the presence of GSH transporter in the plasma membrane has not been demonstrated (Ferretti et al. 2009). Efflux of GSH from cells probably occurs through MRPs (multidrug-resistance-associated protein) belonging to the ATP binding cassette (ABC) protein family, known as ATP-driven pumps for GS-X and GSH across membranes in animals (Foyer et al. 2001). GSH uptake through high and low affinity components was characterized in several plant species, and also a specific symport system exists for GSSG uptake (Foyer et al. 2001). Chloroplasts are able to uptake GSH, in addition to synthesize it (Noctor et al. 2002), while GS-X and GSSG are transported into the vacuole detoxifying conjugated xenobiotics from the cytosol. Enzymatic activities acts recycling GSH/GSSG to amino acids in apoplast and vacuole, both compartments with low GSH:GSSG ratios (Foyer et al. 2001).

In mammalians, it has been suggested a role for GSH in the export of iron from cells, mainly through the formation of GSH-Fe-NO complexes that could be transported by MRP system (Richardson and Lok 2008). Similar compounds, with the structure $(NO)_2$-Fe-GSH have shown adequate membrane permeability (Ueno et al. 1999), and could have a role delivering a safe form of Fe in plants (Buet and Simontacchi 2015; Ramirez et al. 2011).

AsA is a ubiquitous molecule, it is found in all cellular compartments, cytosol, mitochondria, chloroplasts, vacuole, peroxisomes, and apoplast. Its concentration ranges from 2 mM in the vacuole to 20 mM in cytosol. In spite of its abundance and its presence in most cell compartments, it took almost 50 years to elucidate its synthesis pathway in plants. Nowadays, this biosynthetic pathway is known as the Smirnoff-Wheeler pathway (Please see Chap. 6) (Fig. 1).

Since the final step of AsA synthesis takes place in mitochondria, it needs to be transported to the rest of plant cell compartments. AsA is capable of crossing the plasma membrane through AsA/DHA-specific transporters/exchangers that showed a greater affinity for DHA (Horemans et al. 1997). The AsA/DHA exchanger in the plasma membrane is particularly important given that the DHAR and the enzymes of the AsA-GSH cycle are not present in the apoplast. This exchanger uptakes DHA into the cytosol and translocate AsA to the apoplast, and it is essential to maintain AsA levels and redox homeostasis owing to the mentioned processes where it is involved. In this regard, it has been identified high redox potential b-type cytochrome (known as cytb_{561}) that is present in the plasma membrane of higher plants (Asard et al. 2001; Nanasato et al. 2005). It is able to operate such as transmembrane MDHAR, using cytoplasmic AsA as the electron donor and apoplastic MDHA as the electron acceptor (Nanasato et al. 2005; Horemans et al. 1994). Another isoforms of this cytochrome are located in the tonoplast (Griesen et al. 2004). In chloroplasts, AsA is taken up as the monoanionic form through a saturable carrier in the

chloroplast envelope that presents a relatively low affinity (5 mM) (Foyer and Lelandais 1996). Recently, Miyaji and collaborators (2015) showed that the phosphate transporter AtPHT4;4 functions as an AsA transporter in the chloroplast envelop enabling the AsA entrance from the cytosol. A small fraction (10–20% of chloroplastic AsA) reaches the thylakoid lumen through a fast process but none transporter has been described.

It has been reported that AsA can be transported over long distances from source tissues to newly and non-photosynthetic tissues. It can be taken up by roots and transported by xylem and translocated from leaves via phloem (Mozafar and Oertli 1993; Franceschi and Tarlyn 2002; Tedone et al. 2004). In phloem they are also present the enzymes that participate in AsA biosynthesis with the exception of L-galactono-γ-lactone dehydrogenase (Hancock et al. 2003). This could be relevant in the supply of AsA precursors to sink tissues, but this contribution remains to be elucidated.

4 Role of AsA-GSH Cycle Under Stress Conditions

4.1 General Considerations

Plants are subjected to continuous changes of the environment. Normal fluctuations of irradiance, temperature, and other external factors affect processes such as photosynthesis or respiration. Small or large changes in the physiological status may occur during the day, as for example, the reduction in the water status of the leaf cells as a result of increasing evaporation conditions at midday due to temperature and light variations. Additionally, plants may be affected in the long term by the restriction or excess of resources such as water or mineral elements. Beyond the extension and kind of environmental changes, physiological disorders provoke a common feature: the increase in ROS generation (Miller et al. 2010). Under conditions where the amount of photons reaching the leaves is in excess compared to that required for CO_2 fixation, the ROS production increases (Asada 2006). This involves that part of the reducing equivalents getting in excess into the photosynthetic electron transport chain may be derived to the univalent reduction of O_2 forming ROS (Asada 2006). Consequently, ROS steady-state concentration may be continuously changing in chloroplasts (but also in other subcellular compartments). AsA-GSH cycle links plant metabolism with ROS production through the detoxification of H_2O_2 in chloroplast, mitochondria, or peroxisomes (Foyer and Halliwell 1976; Asada 1999). This formation of ROS can be modulated under harmful surrounding conditions by AsA-GSH cycle channeling the excess of reducing power to the safety formation of water (Asada 1999). Oxidative modifications may take place as a consequence of increased level of ROS especially under extreme environmental conditions. Increases in the oxidized state of antioxidants, oxidative damage to macromolecules or ROS steady-state levels were originally considered as detrimental for different physiological processes but now arise a new role as signaling

function for them in plants (Foyer et al. 2017). These changes in the antioxidant-ROS network can be conceived as signals defining the fate of plant cells.

Foyer and Noctor (2016) proposed that the antioxidant defense is designed to modulate the accumulation of ROS instead of completely abolishing them. This concept is particularly important for physiological processes requiring a redox stimulus. Sensing mechanisms of ROS production may control plant growth and development. Physiological processes such as abscission zone formation, root hair growth or stomata closure depend on the ROS generation mediated by NADPH oxidases (Sakamoto et al. 2008; Jones et al. 2007; Kwak et al. 2003).

This association of plant metabolism with the steady-state levels of ROS (or eventually changes in the redox state of antioxidants or in the increment of oxidative damage to macromolecules) may represent integrators of signals from the environment with plant functions (e.g., hormone-related processes). An interaction of redox signaling with hormones such as auxins for the control of plant development and growth has been proposed (Schippers et al. 2016). Depending on the developmental stage, this network defines plant physiological status such as growth or dormancy establishment. This is illustrated in tree buds presenting high AsA content in a reduced state during active growth in spring/summer and a low concentration of AsA in a highly oxidized state during the rest period in autumn/winter (Gergoff Grozeff and Bartoli 2014).

Researchers asked themselves what are the protective processes exerted by antioxidants in this scenario. It is suggested that the antioxidant network may regulate ROS production keeping a steady state of ROS high enough to trigger cell protecting mechanisms (e.g., hormone action, gene expression, or others) (Noctor et al. 2017). Alternatively, it may control the extension of oxidative damage to macromolecules that would participate as specific signals depending on the subcellular compartment where they are generated (Møller et al. 2007). In addition, these reactions produce changes in the oxidized/reduced ratio of AsA or GSH that may be good indicators/sensors of redox status of plant cells (Noctor et al. 2016). All this components may act in conjunction for triggering adaptive responses of plant metabolism to a challenging environment.

4.2 The Role of AsA-GSH Cycle Under Different Abiotic Stress

4.2.1 Drought

Since ROS and antioxidants (enzymatic and non-enzymatic components) form a network that may be conceived as a sensor of environmental conditions, the contribution of AsA-GSH cycle on plant tolerance was largely studied under challenging environments in several species. The closure of stomata aiming at water loss avoidance under drought conditions entails lowering CO_2 uptake limiting Calvin cycle, leading to a typical increase in ROS production in plant cells (Noctor et al. 2014).

The participation of AsA and GSH in both antioxidant and signaling functions has been demonstrated by several experimental evidences. Leaves of a wheat cultivar with high concentration of AsA show lower oxidative damage compared with leaves of a cultivar with low AsA under drought conditions (Bartoli et al. 2004). Plants with increased AsA content display higher photochemical quenching and non-photochemical quenching than plants with lower AsA (Tambussi et al. 2000) demonstrating the role of AsA-GSH cycle ameliorating photosynthetic activity under water stress. The GSH/GSSG ratio can be determined by the use of the redox-sensitive GFP. It was reported that this antioxidant becomes more oxidized during drought (Jubany-Mari et al. 2010) suggesting a signaling function of this redox couple during abiotic stress conditions (Noctor et al. 2014).

4.2.2 Salinity

Salinity in plants induces nutritional alterations as a consequence of ion toxicity as well as osmotic stress. The increments in Na^+ concentrations are toxic, producing a decrease in the uptake and concentration of K^+ due to the chemical similarity of Na^+ and K^+, leading to a metabolic disorder (Acosta-Motos et al. 2017). Besides ROS production as by-products of plant metabolism, abiotic stresses induce the activity of the apoplastic NADPH oxidase catalyzing O_2^- formation that then originates H_2O_2. Evidences demonstrate that this NADPH oxidase-dependent synthesis of ROS is crucial for the adaptive responses of plants to salinity such as regulation of Na^+/K^+ balance (Ma et al. 2012). NADPH oxidase inhibitors avoid the induction of antioxidant enzymes, particularly APX and GR under saline conditions (Ben Rejeb et al. 2015). Furthermore, the expression of genes involved in the early response of plants to salt stress is also induced by H_2O_2 treatment (Schmidt et al. 2013). These experiments, among many others, demonstrate the active role of AsA and GSH in the response of plants to saline stress.

4.2.3 Light

Chloroplasts constitute the main source of ROS in leaves derived from photosynthetic activity (Foyer and Noctor 2016). AsA-GSH cycle has a crucial role keeping compatible steady-state levels of ROS with plant metabolism under increasing irradiance conditions. Tomato plants with very low AsA present a severe oxidative damage when they are moved from a low to a high light environment (Baldet et al. 2013). Mutant plants lacking stromal and thylakoidal APX are prone to photooxidative damage under high light conditions (Kangasjärvi et al. 2008). Furthermore, distinct gene expression and enzyme activity are observed after high irradiance treatments in these mutants compared with wild type plants.

Besides the amount of light, the red/far red light ratio (R/FR) indicates the quality of light reaching the surface of the leaves. High or low R/FR represents a signal of sunny or shadow conditions, respectively, and determines the accumulation of

both high or low AsA and GSH concentrations (Bartoli et al. 2009). Increments of both antioxidants under high R/FR illustrate a plant response to avoid the potential risk of oxidative damage under a sunny light environment.

These results demonstrate the involvement of APX and antioxidants in both photoprotection and redox signaling functions as integrants of the AsA-GSH cycle under changing light conditions.

4.2.4 Chilling

Low temperatures induce photooxidative stress in plants, especially in those species adapted to a warm climate. Light-dependent increments in ROS production and reductions in antioxidant (i.e., AsA and GSH) concentrations have been observed under chilling conditions (Wise and Naylor 1987). Plants with lower capacity to synthesize AsA (Wang et al. 2013; Yang et al. 2017) or with decreased activity of thylakoid APX (Duan et al. 2012) or GR (Shu et al. 2011) are more susceptible to chilling stress. On the other hand, H_2O_2 treatments improve the tolerance of plants exposed to subsequent low temperatures giving an evidence of signaling participation of ROS in the adaptive response (Yu et al. 2003; Wang et al. 2010a). Furthermore, plants with reduced thylakoidal APX activity show increased expression of stress related genes showing compensatory increments of other antioxidants (Duan et al. 2012). As previously mentioned for other abiotic stresses, the antioxidant-ROS network has protective and signaling functions in plants under low temperatures.

4.2.5 Heat Shock

Heat shock induces oxidative stress in plants (Larkindale and Knight 2002). Mustard plant seedlings exposed to an elevated temperature treatment show increments in H_2O_2 production followed by increments in the AsA and GSH concentration and activities of antioxidant enzymes such as APX and GR (Dat et al. 1998a, b). This exposition to high temperature induces thermotolerance (Dat et al. 1998a, b). Furthermore, non-lethal heat-shock treatments are applied to increase abiotic stress tolerance and have been proved to be useful to extend the postharvest life of different plant organs. For example, mild heat treatment (40 °C for 3.5 min) produces an increase in mitochondrial H_2O_2 in spinach leaves keeping high reduced/oxidized ratio of AsA and GSH, and extends their storage in the dark after detachment (Gómez et al. 2008). However, heat-shock treatment at higher temperatures (55 °C for 10 min) provokes reduction in the concentrations of AsA and GSH and a high Fe-dependent ROS production (non-mitochondrial source) triggering root cell death (Distéfano et al. 2017). Notably, this cell death is prevented with external GSH supplementation (Distéfano et al. 2017). These results suggest that the extension of the abiotic stress (and hence, oxidative stress) determines the recovery or death of plant cells.

5 Manipulating AsA and GSH Levels in Plants: A Promise to Deal with Abiotic Stress Improving Yield and Health Properties of Fruits and Vegetables

In recent years, there has been an increasing concern in consuming fruits and vegetables due to the importance for human nutrition and health. Fruits and vegetables, the main source of antioxidants in the diet, are associated with a lower risk of degenerative diseases, being a great opportunity improving health by increasing consumption (Ames et al. 1993). Due to the significance of antioxidants for plant productivity and stress tolerance, and also for human health, the manipulation of AsA and GSH metabolism in plants has become an important issue of research.

Under this scenario, genetic engineering strategies may provide a promising tool to optimize tolerance traits (Cushman and Bohnert 2000). Genetic engineering may increase the quantity of certain compound (or group of compounds) changing the expression of a gene (or genes), overcoming rate-limiting enzymatic steps in the biosynthetic pathways (Verpoorte and Memelink 2002). The recent advances regarding AsA synthesis pathways and biotechnology make feasible the possibility to improve AsA levels in plants (Zhang et al. 2007). Bulley et al. (2009) employing expression studies in kiwifruit, and gene over expression in *Arabidopsis,* reported that GDP-L-galactose guanyltransferase is a major regulating point in AsA biosynthesis through the L-galactose pathway (Fig. 2). These studies highlighted the potential importance of this rate-limiting step for breeding experiments, and showed the necessity to combine expression studies with other physiological experiments, since gene expression may not reflect enzyme activity. Baldet et al. (2013) also described that several tomato mutant lines defective in genes encoding enzymes from the AsA biosynthetic pathway (GDP-D-mannose pyrophosphorylase and the GDP-L-galactose phosphorylase genes) showed reduced AsA content and suffered from severe bleaching upon exposure to high light intensity.

The main efforts to enhance AsA content in plants have been focused in increasing not only the biosynthetic pathway enzymes, but also in improving the regeneration of the oxidized forms of this antioxidant (Lorence and Nessler 2007; Zhang et al. 2007). In addition, other strategies are destined to inhibiting degradation, introducing alternative pathways [by exogenous novel gene(s)], or switching the localization of key enzymes [summarized by Zhang et al. (2007)].

Regarding GSH, as its synthesis involves three amino acids and two enzymes in two steps ATP-dependent reactions (Fig. 2), the manipulation of GSH metabolism may be suitable also for crop species. However, manipulation of GSH partitioning seems difficult due to the limitation on the GSH high affinity transporters identification (Maughan and Foyer 2006). Also, being cysteine supply the key limiting factor for GSH synthesis, it should be essential to take into account solutions that also modulate sulfur assimilation for further enhance the capacity of plants to synthesize GSH (Maughan and Foyer 2006).

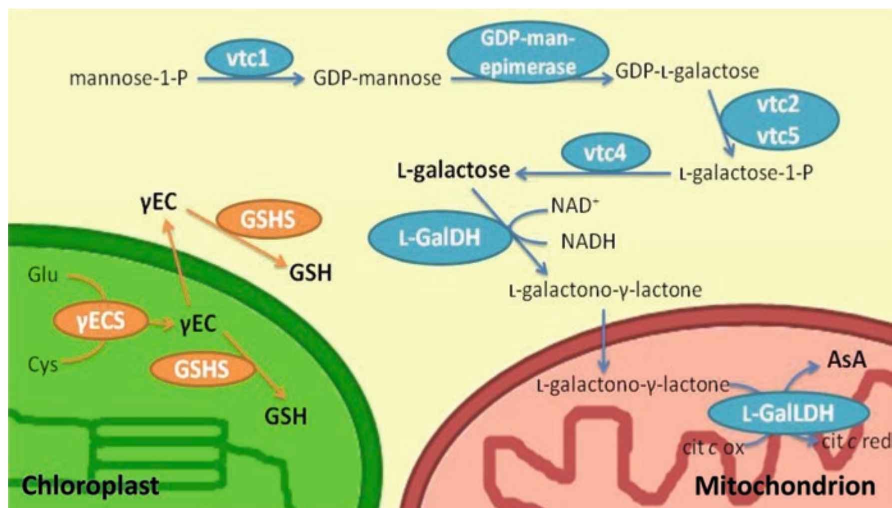

Fig. 2 Biosynthetic pathways for GSH and AsA. The GSH synthesis pathway is outlined in orange. ECS corresponds to the γ-EC synthetase and GSHS, to the GSH synthetase. This latter enzyme is present in chloroplasts and cytosol. The main AsA synthesis pathway in leaves is outlined in blue. VTC1 corresponds to the GDP-mannose pyrophosphorylase VTC2 and VTC5 to GDP-L-galactose phosphorylase, VTC4 to L-galactose-1-phosphate phosphatase, L-GalDH to L-galactose dehydrogenase, and L-GalLDH, to the L-galactono-γ-lactone dehydrogenase. This latter enzyme is located in the inner mitochondrial membrane and delivers electrons to the mitochondrial electron transport chain

In order to achieve successful strategies, information at multiple levels including genes, enzymes, compartmentalization, transport, and accumulation should be encouraged (Verpoorte and Memelink 2002). Also, the regulation of multiple genes or the combination of more than one strategy would be better than the manipulation of a single gene or enzyme (Zhang et al. 2007). This could be particularly interesting in the case of AsA and GSH due to their connected metabolism. In this sense, Le Martret et al. (2011) have observed that the simultaneous expression of DHAR and GR led to a more pronounced reduction of H_2O_2 levels in leaf discs, than in lines expressing DHAR alone, improving chilling tolerance. Expression of both enzymes also conferred methyl viologen-induced oxidative stress tolerance (Le Martret et al. 2011).

The potential to counteract environmental stress became relevant due to the concern about climatic change. Plant productivity is limited by different kind of stresses. Most of the limitations can be attributed to the abiotic stresses that can appear during the lifetime of the plants, such as drought, salinity, extreme temperatures, light, UV radiation, heavy metals, nutrient deficiency, air pollution, herbicides, and many others (Boyer 1982; Bhattachrjee 2005). The understanding of the processes involved in the stressed plant physiology can lead to the development of new tools to manage stress situations and the design of strategies to overcome or prevent the damage (Nelson et al. 1998). In this context, some genetic strategies developed to manipulate AsA and GSH concentrations in plants leading to increased tolerance to abiotic stress are summarized in Table 2.

Table 2 Increased abiotic stress tolerance and antioxidant concentration in plants genetically modified in genes related with the synthesis and regeneration of ascorbic acid (AsA) and glutathione (GSH)

Gene expression	Abiotic stress	AsA	GSH	Plant species	Reference
MDHAR (from *Malpighia glabra*)	NaCl	↑	nd	*Nicotiana tabacum*	Eltelib et al. (2012)
MDHAR (from *Avicennia marina*)	NaCl	↑	nd	*Nicotiana tabacum*	Kavitha et al. (2010)
DHAR (from rice)	NaCl cold	↑	↑	*Nicotiana tabacum*	Le Martret et al. (2011)
DHAR:GR (from rice and *E. coli*)	Paraquat	↑	↑	*Nicotiana tabacum*	Le Martret et al. (2011)
DHAR (from wheat)	Ozone	↑	↑	*Nicotiana tabacum*	Chen et al. (2003)
				Zea mays	Chen and Gallie (2005)
DHAR (from human)	Cold NaCl paraquat	↑	↓ (and ↑GSSG)	*Nicotiana tabacum*	Kwon et al. (2003)
DHAR (from rice)	NaCl	↑ slight	nd	*Arabidopsis thaliana*	Ushimaru et al. (2006)
DHAR (from *Arabidopsis*)	High light, high temperature, paraquat	↑	↑	*Arabidopsis thaliana*	Wang et al. (2010b)
GR (from *E. coli*)	Paraquat and SO_2	nd	↑	*Nicotiana tabacum*	Aono et al. (1993)
GR (from *E. coli*)	Paraquat	nd	↑	*Nicotiana tabacum*	Aono et al. (1995)
GR (from *E. coli*)	Photoinhibition	nd	↑	*Populus tremula x alba*	Foyer et al. (1995)
GR (from *E. coli*)	Paraquat	↓ slight	Without change	*Nicotiana tabacum*	Foyer et al. (1991)
GSHS (from *E. coli*)	Heavy metal (Cd)	nd	↑ under Cd exposure	*Brassica juncea*	Zhu et al. (1999)
GalUR (from strawberry)	NaCl	↑	↑	*Solanum tuberosum*	Upadhyaya et al. (2011)
GalLDH (from *Rosa roxburghii*)	NaCl and paraquat	↑	nd	*Nicotiana tabacum*	Liu et al. (2013)

DHAR dehydroascorbate reductase, *MDHAR* monodehydroascorbate reductase, *GR* glutathione reductase, *GSHS* glutathione synthetase, *GalUR* D-galacturonic acid reductase, *GalLDH* L-galactono-1,4-lactone dehydrogenase, *nd* non determined

Abiotic stress studies also comprise an interesting source of information to develop new tools to manipulate vegetables and fruits during postharvest, specially related to oxidative stress (Cisneros-Zevallos 2003; Toivonen 2003a, b). The analysis of the reports about pre- and postharvest biology field in fruits and vegetables shows several works dealing with the effect of abiotic stress in perishables (Pedreschi and Lurie 2015). Fruit ripening involves the coordinated expression of hundreds of genes, as it can be seen in peach (The International Peach Genome

Initiative 2013) or in tomato (The Tomato Genome Consortium 2012). To understand several processes involved in growth and development, the abiotic stress physiology can give us some new insights of complex processes such as fruit ripening (Gapper et al. 2013). Actually, some strategies designed to improve the content of antioxidant compounds in plants may involve the establishment of stress conditions increasing plant defense responses, raising the content of specific compounds. These strategies may involve, in some cases, the employment of a low (or sublethal) dose of an agent capable of inducing a physical or chemical stress (Costa et al. 2006). Postharvest treatments with UV radiation increased AsA content in some fruits like tomato (Jagadeesh et al. 2011) and apple (Hagen et al. 2007). UV-C treatments in strawberry fruits enhanced the activities of GR, MDHAR, and DHAR enzymes, increasing GSH and GSSG, and delaying the fruit decay comparing to control (Erkan et al. 2008).

Overall, the knowledge of plant responses to abiotic stress at molecular and metabolic level should converge to improve plant breeding strategies leading to crops best adapted to fluctuating environmental conditions. However, not only genetic engineering approaches, but also combined postharvest strategies should converge to obtain optimal antioxidant levels improving nutritional and health properties of fruits and vegetables.

6 Conclusions

Plant growth, productivity, and nutritional quality are affected by constant challenges from the surrounding. ROS-antioxidant networks are integrated to cell metabolism, and constitute crucial signals for important plant decisions, including developmental and adaptive processes. In this sense, plants show a tight interaction of ROS and antioxidants with hormonal signaling.

Evidence from the last years shows that enhancement of the own antioxidant defenses through the modulation of genes affecting the AsA-GSH cycle improves the performance of plants when they face a broad spectrum of environmental stressful conditions. This points out a positive association between the redox state of antioxidants with the capacity to withstand oxidative stress.

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Ascorbate-Glutathione Cycle and Biotic Stress Tolerance in Plants

Elżbieta Kuźniak, Tomasz Kopczewski, and Joanna Chojak-Koźniewska

Abstract The ascorbate-glutathione cycle (AsA-GSH cycle) is a central pathway of the plant cells linking the H_2O_2-scavenging activity to redox signalling. Here, we summarize the most recent advances in our understanding of the role of AsA-GSH cycle in plant–pathogen interactions. Special attention is paid to the regulatory functions of the AsA-GSH cycle components in plant defence against pathogens, their cross talk with other stress signalling pathways and the functional differences between the cellular compartments in relation to the ascorbate and glutathione-dependent protective systems. As under field conditions, different stresses are likely to occur simultaneously, the involvement of AsA-GSH cycle in the signalling network that regulates the response of plants to a combination of pathogen infection and abiotic stress is also addressed.

Keywords Ascorbate-glutathione cycle · Plant–pathogen interactions · Redox signalling · Compartmentation of stress responses · Stress combinations

1 Introduction

Plants are continuously exposed to a broad range of stress factors which substantially limit crop productivity worldwide. In the nearest future, environmental stresses are predicted to become more severe and widespread owing to the climate change. Under natural conditions, combinations of two or more stress factors,

E. Kuźniak (✉) · T. Kopczewski
Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland
e-mail: elzbieta.kuzniak@biol.uni.lodz.pl

J. Chojak-Koźniewska
Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

Genetically Modified Organisms Controlling Laboratory, Plant Breeding and Acclimatization Institute – National Research Institute, Błonie, Poland

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acting simultaneously or sequentially, are common to many agricultural areas. The global climate change is leading to the emergence of new and complex stress combinations, and there is growing concern about their impact on crop production. One of the major threats of global warming is the establishment of “new” pests and pathogens extending their geographic range as climatic conditions become more favourable to them (Luck et al. 2011; Atkinson and Urwin 2012; Suzuki et al. 2014). Thus, a detailed understanding of how plants respond to environmental stress factors is not only of fundamental, but also of practical importance since it is necessary to sustain crop productivity and to further support programmes aimed at improving stress tolerance of crop plants.

Environmental stresses alter the production of reactive oxygen species (ROS) in plant cells and influence the interplay between ROS generation and scavenging mechanisms (Muneé-Bosch et al. 2013). A key role in the antioxidant system is attributed to the ascorbate-glutathione cycle (AsA-GSH cycle) which components operate in all compartments of the plant cell. The AsA-GSH cycle is considered to control ROS level through its scavenging activity as well as to contribute to redox sensing and signalling (Foyer and Noctor 2003). The AsA-GSH cycle activity strongly influences the steady-state level of ROS in cells as well as the duration, localization, and amplitude of ROS signals, referred to as ROS signature, which determines the specificity of ROS signalling (Foyer and Noctor 2009; Kuźniak 2010; Shigeoka and Maruta 2014). As ascorbate and glutathione, the main redox buffers of the plant cell, interact with numerous compounds, the ROS-induced changes in their pools can be sensed and transduced to other redox-sensitive signalling pathways, e.g. those mediated by phytohormones such as salicylic acid (SA) and abscisic acid (ABA). Thus, these antioxidants constitute a part of a much complex signalling network that regulates plant growth and stress responses. Traditionally, the imbalance between ROS generation and detoxification was regarded as a major cause of the oxidative damage under stressful conditions and plant resistance was positively linked to the capacity of the antioxidant system. In a wider context, however, the AsA-GSH cycle plays a key role in the plant redox signalling network which controls almost all aspects of plant biology, including defence responses to biotic stress (Foyer and Noctor 2011; Noctor et al. 2017).

2 The Ascorbate and Glutathione-Related Redox Modules

The redox environment of a cell is defined by the redox potential of each redox couple, the pH, and the concentrations of the oxidized and reduced forms of the species comprising redox couples (Schafer and Buettner 2001). The overall redox environment of cells is determined by the equilibrium between ROS and the antioxidant system. The AsA-GSH cycle occurring throughout the plant cell, recycles AsA and GSH and detoxifies H_2O_2. The reduced forms of ascorbate and glutathione are regenerated by the cycle of enzymatic reactions which uses NADPH as the reducing power. Thus, the key redox couples of plant cells, ascorbic acid/

dehydroascorbic acid (AsA/DHA), reduced glutathione/glutathione disulphide (GSH/GSSG) and NADPH/NADP are linked through the AsA-GSH cycle (Noctor 2006). It acts as the main ROS-scavenging and signalling pathway, and ascorbate and glutathione, remain one of the major redox managers in the plant cell and key factors in tolerance against stress. Ascorbate and glutathione can either react with redox active compounds or donate electrons or reducing equivalents to enzymatic reactions (Foyer and Noctor 2011). The metabolic pools of ascorbate and glutathione are tightly linked by the action of enzymes constituting the AsA-GSH cycle, i.e. ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). In the AsA-GSH cycle, APX utilizes two molecules of ascorbic acid (AsA) to reduce H_2O_2 to water with concomitant production of monodehydroascorbic acid (MDHA). APX is a widely distributed antioxidant enzyme, as five different isoforms have been detected at distinct subcellular localizations, namely in the cytosol, chloroplast stroma and thylakoids, peroxisomes, and mitochondria. It has higher affinity to H_2O_2 than catalase. The Km value for catalase is 20–25 mM H_2O_2, while the Km values of chloroplast APX are in the micromolar range; for stromal and thylakoid isoforms, they are 80 and 23 μM, respectively (Miyake and Asada 1992). This makes APX an efficient scavenger of H_2O_2 involved in the subtle regulation of its level. MDHA, an APX-produced radical with a short lifetime, either disproportionates spontaneously to DHA and AsA or is reduced to AsA by a NADPH-dependent enzyme, MDHAR. The latter is the only known enzyme which uses an organic radical as a substrate. In chloroplasts, the reduction of MDHA can occur enzymatically, by MDHAR or directly by ferredoxin. The ferredoxin-mediated reduction of MDHA competes with the photosynthetic electron transfer to $NADP^+$ and prevents NADPH formation. The rate of MDHA reduction by ferredoxin is 30 times higher than that of $NADP^+$ (Miyake and Asada 1992), and it seems that ferredoxin-mediated ascorbate regeneration in chloroplasts has priority over reduction of CO_2 in the Calvin cycle (Backhausen et al. 2000). DHA, a short-lived chemical, is reduced by DHAR using GSH as an electron donor. Otherwise, at pH values greater than 6.0, DHA is easily decomposed to tartare and oxalate. GSH can also reduce DHA non-enzymatically. This reaction occurs at pH values greater than 7 and at GSH concentration higher than 1 mM; hence, it may be important in chloroplasts (Sharma et al. 2012). GSSG produced in this cycle is then converted back to GSH by the action of GR, at the expense of NADPH. As NADPH is consumed in the regeneration process of oxidized ascorbate, reductions in assimilate supply to the pentose phosphate pathway which occur under stress, may affect the antioxidant potential of the AsA-GSH cycle. Moreover, this process competes with other NADPH-dependent reactions that contribute to the plant defence against pathogens such as membrane repair processes and biosynthesis of secondary metabolites, e.g. phenols, lignin, and phytoalexins (Doubnerová and Ryšlavá 2011).

Ascorbate and glutathione are the main hydrophilic antioxidants and redox buffers in plant cells and their buffering capacity maintains the overall cellular homeostasis. Ascorbate is present in millimolar concentrations and the GSH content is usually about tenfold lower (Noctor 2006). In plant cells, the ascorbate and glutathi-

one pools are highly reduced in the cytosol, chloroplasts, and mitochondria. They are oxidized in the apoplast and vacuoles, organelles lacking efficient mechanisms to regenerate AsA and GSH from their oxidized forms (Foyer and Noctor 2011; Shigeoka and Maruta 2014; Noctor and Foyer 2016). The levels and redox ratios of ascorbate and glutathione depend on the balance between the rates of their biosynthesis and turnover mediated by the enzymes of the AsA-GSH cycle. The repeated redox-cycling of these antioxidants allows to regulate the cellular redox state which is particularly important for plant survival strategies under stress (Paciolla et al. 2016; Noctor et al. 2017).

Ascorbate and glutathione are found in all cellular compartments and cell types. More than 90% of ascorbate is located in the cytosol, determining the redox buffering capacity of this compartment. A considerable part of its pool is exported to the apoplast, where it is found in millimolar concentration (Conklin 2001; Arrigoni and De Tullio 2002; Foyer and Noctor 2011). At this location, ascorbate is considered to be the only significant redox buffer (Pignocchi and Foyer 2003). In *Arabidopsis*, the immunocytochemical labelling revealed the highest levels of ascorbate in peroxisomes and the cytosol and the lowest one in vacuoles. The intermediate labelling was found in nuclei, mitochondria, and plastids (Zechmann et al. 2011). Under high light conditions, however, a significant part of the ascorbate pool is located in the vacuoles where it helps to reduce the phenoxyl radicals produced by H_2O_2-mediated oxidation of phenols (Takahama 2004). The content of glutathione in *Arabidopsis* cells was found to be highest in mitochondria followed by nuclei, the cytosol, peroxisomes, plastids, and vacuoles (Zechmann et al. 2008; Zechmann 2014).

The biological role of ascorbate and glutathione in plants has been intensively studied; however, their functions in defence against pathogens seem to be underexplored. The cellular ascorbate and glutathione contents and their redox ratios have been showed to contribute in different ways to pathogen defence. Owing to their antioxidant properties, they can protect cell compartments from oxidative damage. Ascorbic acid scavenges H_2O_2, superoxide anion (O_2^-), and singlet oxygen ($^1O_2$) in the hydrophilic environments of the cell. The broad antioxidant function of AsA is related to its ability to donate electrons in many non-enzymatic and enzymatic reactions. It is a co-substrate for 2-oxoacid-dependent dioxygenases; thus, it is involved in the biosynthesis of ethylene, flavonoids, anthocyanins as well as hydroxyproline-rich proteins which are involved in the cross-linking of the cell wall after pathogen attack (Zhang 2013). Ascorbate regulates the accumulation of mRNA for enzymes involved in anthocyanin and flavonol synthesis (Page et al. 2012). In the chloroplasts, it is a cofactor for violaxanthin de-epoxidase in the xanthophyll cycle which dissipates excess light energy. It also maintains α-tocopherol in the reduced state. Moreover, by keeping the prosthetic metal ions in reduced forms, AsA maintains the activity of antioxidant enzymes (Arrigoni and De Tullio 2002; Zhang 2013). The studies of Monteiro et al. (2007) revealed a previously uncharacterized antioxidant function of AsA. The authors showed that 1-Cys peroxiredoxin from *A. thaliana* uses ascorbate as reductant although peroxiredoxins have been described to be strictly dependent on thiols. They also suggested that the

ascorbate-dependent peroxidase activity of 1-Cys peroxiredoxin can be relevant for cellular redox processes in plants due to high concentration of AsA in all compartments and the role of peroxiredoxins in redox signalling.

The antioxidant role of glutathione is related to the reduction of ROS and indirectly, to its involvement via the AsA-GSH cycle in reduction of DHA to AsA and in maintaining zeaxanthin and α-tocopherol in the reduced states. It is also a substrate for glutathione-S-transferases (GST) and glutathione peroxidases, enzymes which participate in the xenobiotic detoxification, reduction of lipid hydroperoxides, and in ROS removal. The involvement of GSH in the antioxidant defence mechanisms is also related to its interactions in a reversible manner with protein cysteinyl thiols, a process known as glutathionylation. The binding of a glutathione to a thiol group protects the protein from irreversible inactivation or regulates protein activity (Rouhier et al. 2008). It has been shown that GST from *Arabidopsis* undergoing glutathionylation possesses DHAR activity (Dixon et al. 2005).

Several studies have shown that there is a close relationship between increased level of H_2O_2 in plant tissues and the glutathione status (Willekens et al. 1997; Rizhsky et al. 2002; Queval et al. 2009). Thus, the status of glutathione pool is now recognized as a marker of oxidative stress triggered by overaccumulation of H_2O_2 and other peroxides which is a hallmark of biotic stress-induced changes in plants (Wojtaszek 1997; Muckenschnabel et al. 2001; Chojak-Koźniewska et al. 2017). The major pathways of H_2O_2 metabolism mediated by glutathione which could be important for plant defence against pathogens involve three distinct types of peroxidases, namely APX, thiol peroxidases such as peroxiredoxins and GST. APX is functionally linked to glutathione through the AsA-GSH cycle. Peroxidases from the peroxiredoxins family can use GSH alone or via the action of glutaredoxin (GRX) and some GSTs possess a GSH-dependent peroxidase activity (Rouhier et al. 2008). These two pathways of glutathione peroxidation are independent of the AsA-GSH cycle per se; however, their activities depend on the availability of GSH regenerated via the cycle and they are involved in defence against pathogens. Transcripts of certain GSTs appear to be useful indicators of the level of intracellular H_2O_2 generated during oxidative stress and they can be induced by SA, a known mediator of plant response to biotrophic pathogens (Queval et al. 2009; Sappl et al. 2009).

The AsA-GSH cycle is an antioxidant pathway but it also regulates the signalling potential of ascorbate and glutathione. They are sensors of the environmental changes transducing them into redox signals and co-ordinating the response. To date, photosynthesis is the best understood metabolic pathway in plant cells governed by redox control. However, it is becoming increasingly evident that many other cellular processes in plants are subject to redox regulation which is a universal, fine-tuning mechanism involved in adjusting the whole-cell metabolism to the constantly changing environment (Fedoroff 2006).

The most studied redox system in photosynthetic organisms is the thioredoxin system; however, emerging evidence indicates that GRXs which sense the GSH/GSSG ratio could also be important in redox signalling in plants (Rouhier et al. 2008). Thioredoxins interact with numerous proteins involved in a broad range of

physiological processes and regulate their activity. Two enzymes of the AsA-GSH cycle, APX and DHAR, have been identified as targets of thioredoxins (Dos Santos and Rey 2006). The chloroplast thioredoxins are linked to ferredoxin but the cytosolic ones are reduced by NADPH; thus, they are associated with the NADP-glutathione-ascorbate network. The NADP-dependent thioredoxin reductases transfer the reducing power of NADPH to the thioredoxin/peroxiredoxin system for scavenging ROS (Cha et al. 2015). Their functional involvements under stress conditions, however, have not been elucidated. In *Arabidopsis*, the cytosolic thioredoxin *AtTRXh5* gene was found to be up-regulated during incompatible interactions with the bacterial pathogen *Pseudomonas syringae*, indicating its possible implication in response to pathogens (Laloi et al. 2004).

There are two classical, well-characterized examples of the involvement of redox reactions in plant defence against pathogens, namely programmed cell death (PCD) manifested by the hypersensitive response (HR) and the SA-regulated pathogenesis-related (PR)-1 gene expression.

PCD is induced by pathogens at the sites of infection through a mechanism known as HR, and is one of the best-known defence responses mediated by redox signals. HR is assumed the most common defence reaction in resistance against avirulent pathogens with an obligatory biotrophic lifestyle. It results in rapid killing of infected plant cells and the neighbouring, non-infected ones, ultimately leading to death of the invading pathogen which requires living host cells for growth and reproduction. Moreover, HR induces signals that spread into more distant tissues and trigger defence mechanisms (Greenberg 1997; Kombrink and Schmelzer 2001; Greenberg and Yao 2004). Several necrotrophic fungi, however, induce PCD as an invading strategy to kill the plant cells and feed on the dead tissues (Govrin and Levine 2000).

ROS, especially H_2O_2, act as key signals in plant PCD. The generation of ROS during the HR follows a biphasic pattern. The low-amplitude first phase of ROS production is induced by PAMPs (Pathogen-Associated Molecular Patterns) and the second phase with massive accumulation of ROS results from the interaction between the pathogen *avr* gene products and the plant *R* genes (Torres et al. 2006). The kinetics and amplitude of the second rise of ROS, which depends on the balance between ROS production and scavenging, influence the rate of plant cell death and the defence reactions (Mur et al. 2000). Thus, the interplay between ROS and antioxidants determines the redox environment suitable for PCD. A pivotal role in promoting the oxidative conditions needed for HR has been reported for cytosolic APX. Tobacco plants infected with tobacco mosaic virus and *P. syringae* pv *phaseolicola* activated PCD through a signalling pathway involving post-transcriptional down-regulation of cytosolic APX, a key H_2O_2-detoxifying enzyme (Mittler et al. 1998).

Several studies have linked the oxidative cell death mechanism with the contents and redox status of ascorbate and glutathione. The level of GSH has been shown to increase during HR and the glutathione pool in the apoplast, an interface which mediates the first cross talk between host and pathogen, was maintained in the oxidized state (May et al. 1996; Vanacker et al. 1998). Moreover, the HR was

suppressed when bacterial pathogens were applied together with GSH (Mur et al. 2005). Glutathione deficiency of the *Arabidopsis pad2-1* mutant impaired H_2O_2 production in response to the oomycete *Phytophthora brassicae* and correlated with reduced HR (Dubreuil-Maurizi et al. 2011).

The role of glutathione in the regulation of PR1 expression is related to changes in redox state of the NPR1 (Non-expressor of Pathogenesis-Related 1) protein and its translocation from the cytosol to the nucleus to induce SA-dependent transcription of PR proteins (Mou et al. 2003). NPR1 protein is a redox-sensitive, transcriptional cofactor which mediates the transmission of SA signal in plant defence responses. The output of the defence response was found to be determined by the interplay between H_2O_2, SA, and GSH. The SA-regulated transcriptional regulation of defence genes has been found to be a biphasic process. The first, oxidative phase, manifested by increased accumulation of ROS, especially H_2O_2, and decreased GSH-dependent reducing power, is followed by a reductive phase characterized by enhanced GSH content and reducing capacity. These temporal redox changes regulate the conformation of NPR1, a master regulator of SA-mediated defence genes (Mou et al. 2003; Wu et al. 2012). The SA-mediated monomerization of NPR1, conditioning its transfer to the nucleus, is catalysed by thioredoxins (Tada et al. 2008). The NPR1-dependent plant defence signal transduction pathway exemplifies a mechanism by which nuclear NPR1 level is regulated by SA that mirrors the cellular redox state represented by the glutathione redox ratio. The synergism between GSH and SA was shown in transgenic tobacco with increased GSH level which synthesized more SA, was resistant to *P. syringae,* and expressed genes of the NPR1-mediated SA signalling pathway (Ghanta et al. 2011).

PCD is also influenced by ascorbate-dependent redox regulations. The ascorbate-deficient *Arabidopsis* mutants, *vtc1* and *vtc2*, activated localized PCD and had enhanced basal resistance to *P. syringae*. The *vtc1* and *vtc2* mutations enhanced GSH/GSSG ratio and led to constitutive expression of pathogenesis-related (PR) defence genes. The adjustments in the glutathione pool resulting from ascorbate deficiency in the *vt*c mutants, namely the increased GSH/GSSG ratio together with low redox buffering capacity, favour the monomerization and nuclear translocation of NPR1 and facilitate systemic acquired resistance (SAR) responses, for which NPR1 is a master regulator (Pavet et al. 2005).

Interestingly, some natural compounds that induce resistance by a priming mechanism have been shown to control the cellular redox environment after infection (Aranega-Bou et al. 2014). For example, hexanoic acid protected tomato plants against *Botrytis cinerea* by activating a set of detoxifying and redox balance-related genes, including several glutathione transferases, peroxidases, and GR as well as by increasing the ascorbate and glutathione-reduced/-oxidized ratios (Finiti et al. 2014). Thus, hexanoic acid treatment provided a less oxidized environment after infection which might be critical for limiting the growth of necrotrophic pathogens which are known to stimulate ROS generation to their own benefit (Govrin and Levine 2000). In the interactions with necrotrophic pathogens, the reduction of antioxidant capacity followed by increased oxidation and cell death may contribute to susceptibility. Other priming agents, such as thiamine, riboflavin, and chitosan can

also modulate the cellular redox status to protect plants against pathogens (Aranega-Bou et al. 2014).

The ROS signals resulting from the ROS/antioxidants interplay can be transmitted locally, from cell to cell, and spread systemically, throughout the plant in the form of the autopropagating ROS wave. The autopropagating nature of the ROS wave signal means that each cell along its path independently activates its own RBOH (Respiratory Burst Oxidase Homologue) enzyme at the plasma membrane and generates ROS in the apoplast. The ROS wave may therefore spread a stress signal from its initiation site to the systemic tissues (Mittler et al. 2011; Gilroy et al. 2014).

An important aspect of the signalling function of glutathione is related to its involvement in thiol–disulphide interactions in many processes, including gene expression. These interactions can be mediated by GRXs, which reduce disulphide bridges with the help of two glutathione molecules and are key enzymes for the plant response to environmental constraints (Rouhier et al. 2008). It has been shown that GRXs regulate the activity of basic leucine zipper-type transcription factors called TGA which interact with NPR1 and are essential for the regulation of many SA-responsive genes, such as the PR1 gene. In *Arabidopsis*, Ndamukong et al. (2007) identified GRX interacting with TGA factors which was transcriptionally activated when SA content was elevated. Zhou et al. (2000) found that mutations in NPR1 which impaired SA signalling in *Arabidopsis* inhibited the interaction of NPR1 with two transcription factors from the TGA family, TGA2 and TGA3. As these factors were also shown to bind the SA-responsive element of the *Arabidopsis* PR-1 promoter, the results directly link NPR1 to SA-induced PR-1 expression through TGA transcription factors.

3 The Ascorbate-Glutathione Cycle in Plants Under Biotic Stress

The ROS-antioxidant perturbations constitute one of the first responses of plants to infection and antioxidants have been shown to have an underlying influence on plant defence mechanisms against a variety of pathogens. Changes in the cellular contents of ascorbate and glutathione as well as the activities of AsA-GSH cycle enzymes have been reported in numerous studies. Early reports showed that the induced resistance of melon and tomato plants against *Fusarium oxysporum* was accompanied by an increase in the content of GSH (Bolter et al. 1993) and the incompatible tomato–*Cladosporium fulvum* interaction was characterized by a marked accumulation of glutathione, especially in the form of GSSG (May et al. 1996). In the barley-powdery mildew pathosystem, the accumulation of GSH and the activation of some AsA-GSH cycle enzymes were observed during the incompatible interaction and not the compatible one, whereas the content of AsA decreased in both (El-Zahaby et al. 1995). In maize genotypes resistant to *Fusarium,* the activities of the AsA-GSH cycle enzymes were higher than in the susceptible

ones (Lanubile et al. 2012), confirming that the susceptibility to pathogens of different cultivars of the same plant species correlate with the activity of the AsA-GSH cycle.

Changes in the total contents of ascorbate and glutathione as well as in their redox states depending on the type of the plant–pathogen interaction have been also reported in tomato plants infected with *P. syringae* pv *tomato*. A sustained reduction in GSH pool size and redox state concomitant with slight AsA content increase were observed in the susceptible tomato line. In interaction with the resistant cultivar, the glutathione pool homeostasis was maintained and no effect on ascorbate was observed (Kuźniak and Skłodowska 2004b). Similarly, a progressive decrease in the GSH content accompanied by a relative stability in AsA concentration was observed in *B. cinerea*-infected tomato leaves (Kuźniak and Skłodowska 1999). The authors suggested that the shortage of GSH supply could be a limiting factor for operation of the AsA-GSH cycle at the advanced stage of this interaction, when the disease symptoms were the severest, indicating the breakdown of defence mechanisms. In general, the accumulation of GSH is considered to occur in incompatible (plant resistance to pathogen invasion) interactions of plants with pathogens (Gullner and Kömives 2001). As incompatible interactions are usually accompanied by a marked oxidative burst (Wojtaszek 1997), the increased GSH level could reflect an enhanced demand for antioxidant protection or be related to the involvement of GSH in the redox signalling network underlying plant defence responses (Dubreuil-Maurizi and Poinssot 2012; Noctor et al. 2012). In contrast, decreased concentration of AsA, at least transient, appears to be necessary for the elicitation of defence reactions (De Gara et al. 2003; Foyer and Noctor 2005). In a model interaction of *Lotus japonicus* with a non-pathogenic *P. syringae* strain, reduction of ascorbate concentration was suggested to play an important role in plant defence by favouring ROS accumulation. The concentration of glutathione, however, was maintained high and could protect from ROS toxicity. Under biotic stress, this ascorbate-glutathione interplay could serve to fine-tune the content of ROS (Bordenave et al. 2013). This may be necessary to initiate and regulate the downstream defence mechanisms mediated by ROS.

Within the context of a close relationship between ascorbate and glutathione, compensatory responses between AsA and GSH have been reported during some plant–pathogen interactions. In the susceptible interaction of *Eucalyptus sieberi* with *P. cinnamomi,* under intense oxidative stress, the increase in AsA content compensated for the decrease in GSH (Dempsey et al., 2012). Similarly, in the interaction of *B. cinerea* with common ice plant (*Mesembryanthemum crystallinum*), a model intermediate C3-CAM (Crassulacean Acid Metabolism) plant, the pathogen-induced changes in the AsA and GSH contents and APX activity indicate that some compensatory ascorbate-related mechanisms could exist to ensure efficient antioxidant protection in the case of glutathione deficiency and vice versa (Gabara et al. 2012). The antioxidative compensatory mechanisms involved in the regulation of ascorbate- and glutathione-dependent defence described for ice plant–*B. cinerea* interaction were not found in tomato infected with this fungal pathogen (Kuźniak and Skłodowska 2005b). The latter supports the observation that the antioxidants are not interchangeable and ascorbate and glutathione can affect

different aspects of plant response to pathogens. Ascorbic acid is involved in the synthesis of hydroxyproline-rich glycoproteins and hormones as well as in gene expression (Zhang 2013). GSH is likely to exert a more general role in regulation of gene expression (Noctor et al. 2012; Schnaubelt et al. 2015). Different patterns of *B. cinerea*-induced ascorbate- and glutathione-related changes found in the resistant (ice plant) and susceptible (tomato) interaction indicate that besides a general conceptual framework of the role of these antioxidants in plant defence against pathogens, it has some elements specifically tailored for individual pathosystems.

Although the involvement of ascorbate and glutathione in plant responses to pathogens has been unambiguously shown, underlying molecular mechanisms remain poorly characterized. Ascorbate and glutathione have been repeatedly reported to play a complex role in plant resistance which is far beyond the ROS detoxification activity (Zhang 2013; Noctor et al. 2012). For example, early and high-dose ascorbate treatment as well as artificial glutathione enhancement by L-2-oxothiazolidine-4-carboxylic acid application alleviated the symptoms of virus infection in plants, whereas ROS eliminators such as dimethylthiourea and tiron, did not (Zechmann et al. 2007; Wang et al. 2011). These results point to a signalling role of these antioxidants in plant resistance to pathogens, also reported in other studies. The antioxidant capacity and redox state of the ascorbate and glutathione pools have been shown to regulate defence responses such as induction of PR proteins and phytoalexins (De Gara et al. 2003; Pastori et al. 2003). The ROS-antioxidant perturbations can initiate signalling pathways leading to further changes in the hormonal balance and gene expression (Foyer and Noctor 2011; Denancé et al. 2013). In this context, the AsA-GSH cycle is an antioxidant pathway but it also regulates the signalling potential of ascorbate and glutathione.

Studies on the interactions of glutathione-deficient *pad2* and *cad2* mutants of *Arabidopsis* with pathogens revealed that a certain level of glutathione, specific for a given pathosystem, is required for resistance (Ball et al. 2004; Parisy et al. 2007). The link between GSH synthesis and defence against pathogens was supported by the fact that the *Arabidopsis pad2* mutant containing only 30% of the wild type glutathione and decreased indole phytoalexin, camalexin level, was impaired in resistance to *P. brassicae* and *P. syringae* and lost the ability to induce HR response. The *pad2* mutation is localized in the GLUTAMATE-CYSTEINE LIGASE gene coding for the first enzyme of glutathione biosynthesis (Parisy et al. 2007). As the *pad2* mutant was also hyper-susceptible to other pathogens, these results point to the importance of glutathione in disease resistance of *Arabidopsis*. The glutathione-deficient *pad2 Arabidopsis* mutant also showed decreased insect resistance due to reduced accumulation of glucosinolates (Schlaeppi et al. 2008). In this mutant, the mechanisms of chemical defence were ineffective because the biosynthesis of camalexin and glucosinolates depends on glutathione acting as a S atom donor (Parisy et al. 2007; Schlaeppi et al. 2008).

Surprisingly, ascorbate deficiency has been shown to have opposite effects on plant resistance to pathogens. Ascorbate-deficient *Arabidopsis* mutants showed increased SA level and enhanced resistance to *P. syringae* whereas at high ascorbate

content the expression of PR genes was suppressed (Pastori et al. 2003; Pavet et al. 2005). These data suggest that ascorbate and glutathione, although tightly linked in the defence system, could have some specific functions in plant disease resistance to pathogens.

4 The Role of Compartment-Specific Changes in the AsA-GSH Cycle Activity in Biotic Stress Signalling

Biotic stress results in compartment-dependent ROS and redox signatures that could determine the specificity of plant response. In plant cells, each compartment contains its own set of ROS-generating and ROS-processing mechanisms; thus, the ROS and redox state of each compartment are maintained at different levels in accordance with its specific metabolic requirements. This gives rise to unique ROS and redox signatures generated under stress at the different compartments of the cell which contribute to the specific functions attributed to ROS and redox signals (Vanacker et al. 1998; Kuźniak and Skłodowska 2004a; Kuźniak and Skłodowska 2005a; Großkinsky et al. 2012).

The outcome of ROS-mediated signalling depends not only on the ROS signature but also on the site of ROS production. The interaction between ROS and related redox signals generated in different compartments is essential for stress signalling. Although considerable advances have recently been made concerning the ROS/redox signalling (Heyneke et al. 2013; Luschin-Ebengreuth and Zechmann 2016), the compartment-specific role of the AsA-GSH cycle during plant–pathogen interaction are still a matter of debate.

The oxidative effects of infection have been proved to affect all organelles, so the compartment-specific changes of ROS production and activity of the antioxidant system could be valuable indicators of stress (Kuźniak and Skłodowska 2005b). As these changes are masked when whole-cell extracts are analysed, their biological relevance might be underestimated. Observations on crude tissue extracts may provide only partial insight into the redox-dependent regulations of plant defence responses. The coordination of many ROS/redox signalling pathways between cellular compartments is necessary for maintaining energy and metabolic fluxes as well as for activating an adequate defence response (Fig. 1).

The apoplast is the first plant compartment where plant–pathogen interactions occur. It is therefore essential for pathogen recognition and the establishment of specific defence response (Qi et al. 2017). Apoplast is the major site of rapid ROS generation in plants infected by pathogens, the so-called oxidative burst. ROS produced mainly by RBOH, cell wall peroxidases and amine oxidases are released into the apoplast. At this location, they can directly affect the invading pathogen, serve as substrates in oxidative cross-linking of lignin precursors and of cell wall proteins, and regulate callose deposition at the cell wall, all being important features of plant resistance to pathogens (Bolwell et al. 2001; Li et al. 2017).

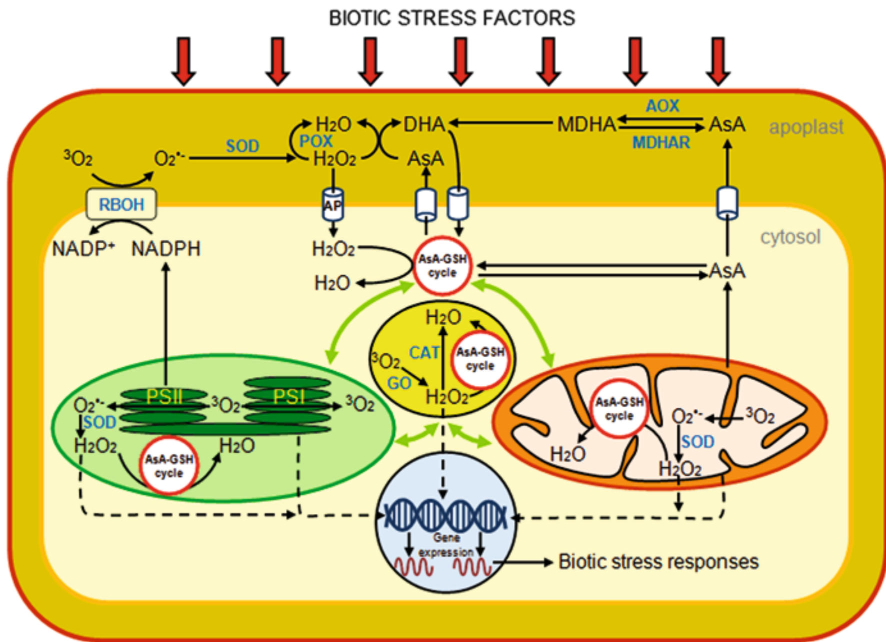

Fig. 1 Compartmentation of the ascorbate-glutathione (AsA-GSH) cycle in the plant cell and its role in biotic stress response. The AsA-GSH cycle is localized in several compartments of the plant cell, e.g. chloroplasts, mitochondria, peroxisomes, and the cytosol. The co-ordinated work of the cycle in different cellular compartments modulates the level of ROS, preventing cellular damage and regulating ROS signalling. Upon pathogen attack, O_2^- produced in the apoplast by the action of RBOH is converted by SOD to H_2O_2 which can be metabolized in the apoplast space either enzymatically by POX or non-enzymatically by AsA, or transmitted through the AP into the cytosol. The apoplastic H_2O_2- and ascorbate-dependent redox signals interfere with the AsA-GSH cycle components in other cellular compartments. Moreover, biotic stress induces disturbances in the electron fluxes in chloroplasts and mitochondria as well as in the peroxisomal metabolism leading to overproduction of H_2O_2 which is scavenged in the AsA-GSH cycle in these organelles or diffuses to the cytosol. Redox imbalances which occur in chloroplasts and mitochondria could participate to retrograde signalling. The signalling effects of H_2O_2 can be transduced by an increase in H_2O_2 itself and by perception of perturbed redox state via redox-sensitive proteins. It results in redox-dependent reprogramming of gene expression which shapes the plant defence response. *AsA* ascorbic acid (reduced), *AOX* ascorbate oxidase, *AP* aquaporin, *CAT* catalase, *DHA* dehydroascorbate, *GO* glycolate oxidase, *MDHA* monodehydroascorbate, *MDHAR* monodehydroascorbate reductase, *POX* cell wall peroxidase, *PSI/II* photosystems I and II, *RBOH* the respiratory burst NADPH oxidase homologue, *SOD* superoxide dismutase

Moreover, the apoplast O_2^-/H_2O_2 producing and scavenging mechanisms contribute to hormone-mediated stomata immunity (Baxter et al. 2014; Xia et al. 2015). Stomata function as part of the plant defence mechanisms against pathogens (Arnaud and Hwang 2015). Many bacteria and fungi with different lifestyles enter plants only through stomata while they are open, thus stomata closure prevents penetration through these pores and the subsequent colonization of the host tissues. Upon contact with pathogens and the perception of pathogen-associated molecular

patterns (PAMPs), plants activate a signalling cascade mediated by ROS and phytohormones, i.e. ABA and SA, which triggers stomata closure. Conversely, pathogens have evolved virulence factors which inhibit stomata closure or induce stomata reopening to counteract stomatal immunity. Some pathogens, e.g. *P. syringae*, can modify ABA signalling in plants and this hormone functions as a virulence factor for them (de Torres-Zabala et al. 2007). Interestingly, ABA and SA induce stomata closure through different ROS pools generated in the apoplast. ABA exerts its role via ROS produced by RBOH, whereas SA signalling is mediated by ROS generated by the cell wall peroxidases (Acharya and Assmann 2009; Miura et al. 2013).

Enzymatic and non-enzymatic AsA oxidation is an essential factor creating the apoplast redox status (Paciolla et al. 2016) and contributing to redox stress signalling. A potentially important role in the regulation of stomata aperture and stress signalling was suggested for the apoplastic DHA. In the leaf apoplast of tobacco plants overexpressing ascorbate oxidase, DHA resulting from oxidation of AsA that typically occurs under stress, has been reported to elicit stomata closure (Fotopoulos et al. 2008). These results emphasize the role of apoplastic AsA in the perception of environmental factors and the function of DHA as a potential stress signalling agent which can modulate plant responses to stress. The latter could be related to the ability of DHA to directly interact with GSH and with regulatory proteins containing cysteine residues (Morell et al. 1997). The ROS-induced modifications of these proteins are of physiological importance in regulating both plant metabolism and gene expression under stress (Dietz 2008; Gadjev et al. 2006).

The apoplastic ROS signals can also be transmitted across the plasma membrane and sensed in the cytoplasm, chloroplasts, and the nucleus resulting in changes in gene expression (Fig. 1). H_2O_2 can either enter the cell through specialized aquaporins, peroxiporins (Bienert et al. 2007; Hooijmaijers et al. 2012; Tian et al. 2016), or react with extracellular/transmembrane redox-sensitive proteins (Wrzaczek et al. 2010). Antioxidants located in the apoplast determine the lifetime and specificity of apoplastic ROS signalling. Oxidation of the apoplastic ascorbate and glutathione pools can also be involved in transmitting the apoplast-born redox signals to the cytosol and chloroplasts (Foyer and Noctor 2011). The redox states of ascorbate and glutathione redox couples provide information for acclimation and defence at the gene-expression level (Mou et al. 2003; Pastori et al. 2003; Zhang 2013).

In plant cells, the role of chloroplasts is far beyond the photosynthetic function of these organelles, as they are also involved in many other processes such as nitrogen and sulphur assimilation, production of secondary metabolites and phytohormones. Chloroplasts are sensitive sensors of environmental changes and their redox balance is easily perturbed (Kopczewski and Kuźniak 2013). The apoplastic ROS signal is transmitted via cytosolic signalling pathways to the chloroplasts, where a secondary ROS generation could be initiated (Shapiguzov et al. 2012). The accumulation of apoplastic H_2O_2 is also involved in the induction of the chloroplastic and cytosolic antioxidant enzymes, APX and GR (Hu et al. 2005). In the photosynthesizing plant cells, chloroplasts are the major sites of ROS production. They are, simultaneously equipped with complex antioxi-

dant systems, of which the AsA-GSH cycle has been most extensively studied (Asada 1999; Foyer and Noctor 2009). The chloroplasts in mesophyll cells contain 20–40% of the ascorbate and 10–50% of the leaf glutathione (Foyer and Noctor 2009). It has also been estimated that 65% of the total leaf DHAR activity and 70% of the total GR activity were localized in the chloroplasts of the mesophyll cells (Gillham and Dodge 1986). Changes in the chloroplast antioxidant system are part of the signalling pathways responsible not only for the optimization of photosynthesis and other metabolic processes, but also for stress signalling (Foyer and Noctor 2009). Redox signals arising from chloroplasts make an important contribution to immunity towards pathogens (Han et al. 2013). It has been suggested that enhanced H_2O_2 production in the light reflects a transient inactivation of the antioxidant system rather than an increased H_2O_2 production, possibly to trigger specific ROS-dependent defence responses (Trotta et al. 2014). This mechanism was likely to operate in chloroplasts of tomato leaves infected with *B. cinerea*, as the activity of APX and the contents of AsA and GSH were decreased by the pathogen. This was accompanied by the decrease in the ascorbate and glutathione redox ratios (Fig. 2, Kuźniak and Skłodowska 2001). Interestingly, the main H_2O_2 scavengers in the chloroplasts, APX, AsA, and GSH were down-regulated by the necrotrophic fungal pathogen and not by the biotrophic *P. syringae* pv *lachrymans*. In cucumber chloroplasts, the activity of APX and the content of AsA were only transiently decreased after *P. syringae* pv *lach-*

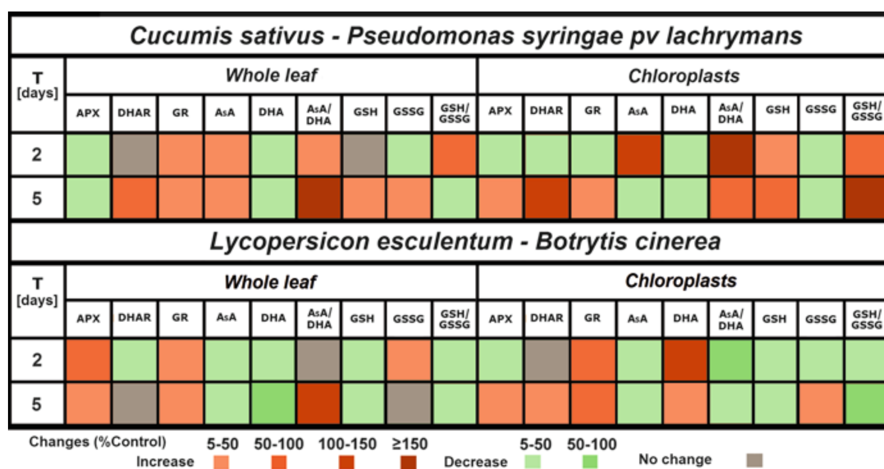

Fig. 2 Changes in the ascorbate-glutathione cycle activity in chloroplasts and whole-leaf extracts of cucumber (*Cucumis sativus*) infected with *Pseudomonas syringae* pv *lachrymans* and tomato (*Lycopersicon esculentum*) infected with *Botrytis cinerea.* Analyses were performed in whole-leaf extracts and chloroplasts 2 and 5 days after infection. The figure was drawn using mean values from control (non-infected) plants as 100% (Kuźniak and Skłodowska 1999, 2001; Kuźniak et al. 2016; Kopczewski, unpublished data). *AsA* ascorbic acid (reduced), *APX* ascorbate peroxidase, *DHA* dehydroascorbate, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase, *GSH* glutathione (reduced), *GSSG* glutathione disulphide

rymans infection whereas the GSH content and the ascorbate and glutathione redox ratios were significantly increased (Fig. 2; Kuźniak et al. 2016). The uncoupling of the ascorbate- and glutathione-related redox system could reflect the specificity of the AsA-GSH cycle functioning in different compartments. The model study of Polle (2001) showed redox uncoupling of these two antioxidant pools in chloroplasts due to low rates of DHA formation owing to high MDHAR activity. In general, these results confirm that the AsA-GSH cycle-related antioxidant machinery in chloroplasts is targeted during pathogenesis although the pathogen-induced oxidative stress is generated mainly extracellularly, and the effect depends on the nature of pathogen. As biotrophs colonize the apoplastic space and necrotrophs immediately enter the plant cell, the diversity of responses could be related to the mode of action of the necrotrophic and biotrophic pathogens and based on how defence against these pathogens is controlled (Glazebrook 2005). For example, the defence mechanisms related to RBOH-mediated ROS production in the apoplast seem to be preferentially effective against biotrophic pathogens as *Arabidopsis rboh* mutants were resistant to the necrotrophic *Alternaria brassiciccola* (Pogány et al. 2009).

The involvement of chloroplasts in plant immunity is further supported by the observation that the pathogen resistance of plants differs between light and dark, and in plants resisting pathogen attack the development of the HR requires light (Kuźniak et al. 2010). The fact that functional chloroplasts are required for the HR (Mateo et al. 2004) links plant immunity with light perception through their metabolism, including the chloroplastic AsA-GSH cycle. For example, chloroplast ROS and redox status were essential for the progress of HR during tobacco–*Xanthomonas campestris* pv *vesicatoria* interaction, but did not contribute to the induction of pathogenesis-related genes (Zurbriggen et al. 2009). The contribution of chloroplasts to plant immunity is broadened by hosting the biosynthetic pathways of SA and jasmonic acid which could regulate defence gene expression (Kangasjärvi et al. 2012). In the interaction of *B. cinerea* with common ice plant, the co-regulation of photosynthesis, antioxidant defence and immunity was also dependent on the metabolic type of photosynthesis. An infection-induced decrease in photochemical activity was found only in plants performing CAM metabolism (Gabara et al. 2012) and shortly after inoculation the activity of antioxidant enzymes was significantly affected by the redox state of plastoquinone (Nosek et al. 2015). Considering the timing of these changes, the results suggest that plants induced the resistant response in the form of HR-like lesions only when the redox state of the plastoquinone pool signalled high light conditions. Light and the redox state of photosynthetic electron transport were shown to play an important role in the conversion of L-galactono-1,4-lactone to AsA in the mitochondria, which determines the rate of AsA synthesis and influences its cellular level (Yabuta et al. 2008). Moreover, in CAM plants the steady-state and infection-induced AsA content and APX activity were significantly higher than in C3 plants, indicating an increased ascorbate-related antioxidant potential. A reverse relationship occurred for GSH which was higher in C3 plants (Gabara et al. 2012). Generally, these studies indicate that in C3 and CAM plants of *M. crystallinum*, distinct mechanisms were involved in the successful defence

against *B. cinerea* infection. They include specific co-regulation of the immune response, the photochemical activity of photosystem II and the antioxidant capacity. The activity of the AsA-GSH cycle in chloroplasts, the main regulator of the H_2O_2 content in these organelles, could also be implicated in the regulation of defence gene expression via the chloroplast-to-nucleus signalling (Fig. 1; Blanco et al. 2014).

As to the coordination of the ascorbate- and glutathione-dependent antioxidant defence in different compartments, it is worth noting that the AsA-GSH cycle enzymes, APX, MDHAR, and GR, are dual targeted to chloroplasts and mitochondria. Thus, the AsA-GSH cycle-dependent antioxidant defence is interrelated already at the gene level (Chew et al. 2003). Moreover, APX, MDHAR, and GR are also targets for post-translational modifications. They were differentially modified by nitration and S-nitrosylation mediated by NO-derived molecules. These processes led to irreversible inhibition and increase of cytosolic APX activity, respectively. The peroxisomal MDHAR was inhibited by these post-translational modifications whereas the chloroplastic and cytosolic GR were not affected (Begara-Morales et al. 2016). The complex interrelationship between cellular compartments was also exemplified by the study of Davletova et al. (2005), showing that in the absence of the cytosolic APX1, the chloroplast H_2O_2-scavenging system in *Arabidopsis* plants collapsed, leading to H_2O_2 accumulation and protein oxidation. Moreover, transgenic tobacco plants with suppressed cytosolic APX1 were hyperresponsive to pathogen infection, activating PCD in response to lower pathogen load than control plants (Mittler et al. 1999).

Although the diffusion of H_2O_2 from chloroplasts has been demonstrated in vitro (Mubarakshina et al. 2010) and aquaporins in the chloroplast envelope have been suggested to be involved in this process (Borisova et al. 2012), it seems that H_2O_2 itself is not the signal triggering nuclear gene expression. It rather acts through compartment-specific redox-sensitive elements which can transmit the signal to the nucleus (Petrov and Van Breusegem 2012; Kopczewski and Kuźniak 2013). In plants, hundreds of redox-target proteins with regulative functions have been identified, including transcription factors (Dietz 2008; Gadjev et al. 2006). This transmission, however, may be hindered by the redox buffering capacity of the cytoplasm. In tomato leaf cells infected with *B. cinerea*, the redox state of the cytoplasm was maintained on a significantly higher level than in the remaining cellular compartments. The cytoplasm was also better protected by the AsA-GSH cycle from the pathogen-induced oxidative stress than mitochondria and peroxisomes (Kuźniak and Skłodowska 2005b). This was proposed to be related to the necessity to withstand massive influx of DHA and GSSG from the apoplast during the oxidative burst (Horemans et al. 2000; Foyer et al. 2001).

Recently, a new scenario has been proposed according to which the fluctuating environmental conditions usually change the redox balance and induce moderate metabolic changes in the chloroplasts, signalled out of these organelles for acclimation, but ROS are kept under the intraorganellar control. However, under extreme stress, ROS accumulate to high levels, the chloroplast loses its integrity and H_2O_2 is released to the cytosol where it feeds into the MAPK (Mitogen-Activated

Protein Kinase) signalling pathway which controls defence gene expression and the HR (Dietz et al. 2016). The latter mechanism is likely to be activated in plants infected by pathogens or exposed to a combined action of abiotic and biotic stressors, which enhance lipid peroxidation leading to membrane permeabilization (Chojak et al. 2012).

The ROS/AsA-GSH cycle interaction produces organelle-specific redox signature which, similarly to ROS themselves, is critical for the retrograde signalling among different cellular compartments. This retrograde signalling has been extensively studied for chloroplasts and mitochondria (Blanco et al. 2014). Recently, it has been proposed that the WHIRLY1 protein perceives the redox changes in chloroplasts and is monomerized and translocated to the nucleus leading to acclimation and immunity responses. This mechanism, although analogous to the regulation of NPR1, could act as a NPR1-independent signalling pathway (Foyer et al. 2014).

A role in retrograde signalling has also been suggested for peroxisomes (Nyathi and Baker 2006; Corpas et al. 2017). In peroxisomes, which capacity to produce ROS is greater than in other compartments due to their essentially oxidative type of metabolism, the level of H_2O_2 is controlled by catalase and APX. However, under stress, which usually leads to overproduction of H_2O_2, it could be released into the cytosol through aquaporins in the peroxisomal membrane and initiate signalling cascades with different biological outcome (Corpas et al. 2017). Peroxisomal metabolism also results in generation of redox signals related to the ascorbate and glutathione redox state, as all components of the AsA-GSH cycle operate in these organelles (Kuźniak and Skłodowska 2005a; Nyathi and Baker 2006). In the tomato–*B. cinerea* interaction, the activities of all AsA-GSH cycle enzymes as well as ascorbate and glutathione concentrations and redox ratios in leaf peroxisomes were significantly decreased concomitant with disease symptom development. It indicated a collapse of the antioxidant protective system in peroxisomes at advanced stage of infection (Kuźniak and Skłodowska 2005a). At the early stage of fungal infection, however, peroxisomes preferentially accumulate at the sites where pathogen hyphae penetrate the plant cells (Koh et al. 2005). These results point to an important role of peroxisomes under infection, possibly related to the involvement of oxidative processes in preventing fungal penetration into plant epidermal cells (Mellersh et al. 2002).

Oxidative stress induced by infection triggers changes in the concentration and redox state of ascorbate and glutathione, but also in their subcellular distribution. In catalase-deficient *cat2 Arabidopsis* mutant exposed to oxidative stress, the concentration of GSSG in the vacuole increased from 5% to 25% of the cell glutathione. An enhanced accumulation of GSSG was also observed in chloroplasts where it may have consequences for redox-based reactions, including glutathionylation of thioredoxins (Queval et al. 2011; Noctor et al. 2013). The oxidation of GSH leading to the production of GSSG can be mediated by ROS and DHA. The reaction of GSH with H_2O_2 and O_2^- is slow whereas this with DHA occurs at a high rate and is accelerated by DHAR. Thus, it could be more important for the close collaboration of ascorbate and glutathione (Mhamdi et al. 2013). The

sequestration of GSSG in vacuoles and other compartments such as apoplast (Vanacker et al. 1998) and peroxisomes (Großkinsky et al. 2012) is an important element of the redox homeostasis and signalling through the glutathione-mediated system. Under control conditions, it serves to clear the cytosol of an active redox signal whereas under stress it helps to avoid excess stimulation and enables termination of stress signalling (Noctor et al. 2013).

The importance of the subcellular compartmentation in the AsA-GSH cycle-related antioxidant defence against pathogens has been evidenced in several plant–pathogen interactions (El-Zahaby et al. 1995; Vanacker et al. 1998; Kuźniak and Skłodowska 2005b). However, the patterns of spatiotemporal changes in the AsA-GSH cycle activity seem to be related to the plant–pathogen interaction to ensure an adequate defence response (Fig. 2; Großkinsky et al. 2012; Kuźniak and Skłodowska 2005b). The up-regulation of the AsA-GSH cycle, seen as plant's general response to oxidative stress imposed by pathogens is accompanied by diverse responses specifically tailored to a given host–pathogen interaction and related rather to its functions in multiple nodes of the signalling network. In the tomato–*B. cinerea* pathosystem, a general shift of the cellular redox balance towards oxidative state was found in the whole-leaf extracts as well as in chloroplasts, mitochondria, and peroxisomes (Kuźniak and Skłodowska 2005b). The timing and intensity of changes were compartment dependent and the pro-oxidative effect was more pronounced in peroxisomes and mitochondria when compared with chloroplasts and the cytosol. It was manifested by the decrease in the contents and redox ratios of the ascorbate and glutathione pools as well as by the insufficient activities of MDHAR, DHAR, and GR, reductases required for the recycling of AsA and GSH. This indicated that the cellular compartments were differentially protected by the AsA-GSH cycle. These compartment-specific changes could have implications for the redox signalling and the coordination of defence mechanisms (Kopczewski and Kuźniak 2013).

5 The Ascorbate- and Glutathione-Dependent Mechanisms in Plant Response to Abiotic and Biotic Stress Combinations

Among multiple combinations of environmental stress factors which can affect plants under natural conditions, those between abiotic stresses and pathogens have been shown to be important growth- and yield-limiting interactions (Mittler 2006; Suzuki et al. 2014). The plant response to stress combination is dependent on the type of stress factors, the initial threshold of stress, and the individual plant's stress history (Rasmussen et al. 2013; Pandey et al. 2015). Many evidence from field and laboratory studies suggests that plants respond to a specific combination of stresses in a non-additive manner, showing changes on metabolic, physiological, and molecular levels which cannot be predicted from the knowledge of single stress effects (Atkinson and Urwin 2012). For example, under combined stress conditions, changes of the levels of ROS, the expression of ROS-processing

enzymes and antioxidants differ from those induced by stress factors applied individually (Sewelam et al. 2016). Maintaining the redox balance is critical for the normal plant growth and performance (Ellouzi et al. 2014) and tight coordination of ROS production and processing mechanisms during combined biotic and abiotic stress is particularly important. Transcriptional, quantitative genetic, and agronomic studies showed that plants respond to multiple stresses using similar defence signalling pathways mediated by ROS, redox signals, and phytohormones. ROS-mediated mechanisms, which involve disturbances in the cellular ROS homeostasis followed by changed detoxification capacity, link abiotic and biotic stress defence (Perez and Brown 2014).

Recently, the concept of priming has gained considerable attention as a potent mechanism of producing multiple stress-resistant crops. It is well known that after a mild, primary stress which promotes a plant to a "primed" state, it shows tolerance to a second, strong stress (Wiese et al. 2004; Conrath et al. 2015). This mechanism allows plants to adjust to multiple stresses. Shared transcriptional response and ROS signalling have been proposed to play a key role in inducing this process (Perez and Brown 2014), and there are several reports on the abiotic stress factors, mainly salinity and drought, affecting plant resistance to pathogens. Most of them indicate that abiotic stress compromised the defence response to pathogens (You et al. 2011; Chojak et al. 2012; Ramegowda and Senthil-Kumar 2015; Chojak-Koźniewska et al. 2017). However, there are no reports concerning the involvement of AsA-GSH cycle in plant tolerance to abiotic and biotic stresses acting in combination. The available reports refer mainly to the activity/concentration changes of some AsA-GSH cycle components in plants exposed to metal stress and infected. For example, Nenova and Bogoeva (2014) focused only on APX activity changes in response to copper and *Fusarium culmorum* in wheat leaves and roots. They found organ-specific and copper dose-dependent trend in decreasing of APX activity when copper and *F. culmorum* acted together, while in plants treated with copper individually APX activity was up-regulated. Another study revealed that Al treatment increased APX activity in *Cajanus cajan*, whereas Al in combination with *F. incarnatum* down-regulated it (Satapathy et al. 2012). In cucumber pre-treated with 100 mM NaCl for 7 days and then infected with *P. syringae* pv *lachrymans*, differences in timing and intensity of H_2O_2 and O_2^- accumulation between plants exposed to combined stress and to individual action of salinity and the pathogen were observed. Infection-induced oxidative stress was stronger when cucumber plants were pre-treated with NaCl, as shown not only by enhanced ROS accumulation but also by an increase in lipid peroxidation (Chojak et al. 2012). Moreover, salinity intensified disease symptoms on cucumber leaves, resulting in more extensive necrosis with chlorotic halos, and increased bacteria proliferation at the late stage of infection, compared to plants infected without NaCl pretreatment (Chojak-Koźniewska et al. 2017). This was accompanied by modifications of the antioxidant system response, exemplified by the induction of FeSOD, a chloroplastic SOD isoform found in cucumber plants under combined stress at early stage of pathogenesis (Chojak-Koźniewska et al. 2017). Activation of FeSOD indicated that enhanced antioxidant protection of these organelles was necessary under multiple

stress conditions (Mittler 2017). Moreover, H_2O_2 generated by FeSOD could impact the AsA-GSH cycle and SA signalling, as chloroplasts are also sites of SA synthesis which is essential in plant immunity to biotrophic pathogens (Dempsey et al. 2011; Suzuki 2016). In *Arabidopsis* mutants lacking FeSOD, down-regulation of chloroplastic genes and impaired chloroplast development was observed (Pilon et al. 2011). Thus, the increased activity of FeSOD could be important to sustain the role of chloroplasts in plant defence against pathogens.

The AsA-GSH cycle, which is a major component of the antioxidant system, is also one of the most important mechanisms controlling oxidative balance of the plant cell under stress (Foyer and Noctor 2011). Mutants impaired in the AsA-GSH cycle showed high ROS levels and increased sensitivity to stress (Huang et al. 2005). The AsA-GSH cycle is integrated in response to multiple stress by transcription factors. In *Arabidopsis*, zinc-finger transcription factor ZAT12, which responses to high H_2O_2 content, is induced by several abiotic and biotic stress factors. It induces the expression of APX genes resulting in tolerance to oxidative stress (Atkinson and Urwin 2012).

In cucumber plants, all AsA-GSH cycle components were affected under combined salt and biotic stress (Fig. 3). This combination strongly modified the antioxidant system response compared to individual stressors, indicating that plant cells exposed to a combination of abiotic and biotic stresses require specific antioxidant protection. In cucumber leaves under combined stress, APX activity was significantly decreased compared to plants infected with the bacterial pathogen without exposition to NaCl (Fig. 3). Changes in APX activity are common stress markers (AbdElgawad et al. 2016). Moreover, APX, especially its cytosolic and chloroplastic isoforms, is a key element of stress signalling (Shigeoka et al. 2002). The decreased APX activity, besides being the effect of AsA depletion, could be related to SA signalling, as one of the proposed models of SA action is the inhibition of APX (Durner and Klessig 1995) and SA biosynthesis takes place in chloroplasts (Dempsey et al. 2011).

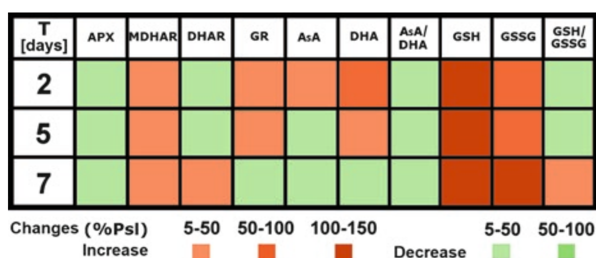

Fig. 3 The effect of NaCl pretreatment on the ascorbate-glutathione cycle activity in leaves of cucumber plants (*Cucumis sativus*) after *Pseudomonas syringae* pv *lachrymans* infection. Cucumber plants were pre-treated for 7 days with 100 mM NaCl and then infected with *P. syringae* pv *lachrymans*. Analyses were performed 2, 5, and 7 days after inoculation. The figure was drawn using mean values from plants infected without salt treatment as 100% (Chojak-Koźniewska 2017). *AsA* ascorbic acid (reduced), *APX* ascorbate peroxidase, *DHA* dehydroascorbate, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase, *GSH* glutathione (reduced), *GSSG* glutathione disulphide, *MDHAR* monodehydroascorbate reductase

The activity of DHAR was mostly down-regulated which indicates lowered capacity to regenerate AsA under combined stress, as confirmed by DHA accumulation. MDHAR activity was increased suggesting that AsA regeneration is mediated mainly by this enzyme, but only at the early stage of infection (Chojak-Koźniewska 2017). Results for cultivated tomato *Lycopersicon esculentum* (*Solanum lycopersicum*) and its wild salt-tolerant relative *L. pennellii* confirmed the role of MDHAR in AsA regeneration (Mittova et al. 2000). However, at the advanced stage of pathogenesis in cucumber plants pre-treated with NaCl, ascorbate recycling via MDHAR appeared to be ineffective as AsA content decreased. The decreased APX activity under combined stress, accompanied by increased ROS generation and lipid peroxidation as well as lowered AsA content and DHA accumulation, indicated an enhanced oxidative burden imposed by multiple stress (Fig. 3; Chojak et al. 2012; Chojak-Koźniewska et al. 2017). Salt stress and *Pseudoperonospora cubensis* infection applied sequentially on cucumber plants induced oxidative stress and increased the activities of APX and GR, but it was insufficient enough for plants to be protected from oxidative stress (Nostar et al. 2013).

In cucumber leaves, when NaCl and the pathogen acted together, the activity of GR increased at the early stage of infection, compared to the infected plants (Fig. 3). It could indicate an increased demand for GSH being a key signalling molecule under biotic stress, at the time points when plant defence mechanisms may be mobilized (Noctor et al. 2012). Changes in the AsA-GSH cycle enzyme activities observed in cucumber (Fig. 3) indicated enhanced cycling of AsA and GSH which may contribute to the coordination of multifactorial response.

Changes in the total concentration of ascorbate and glutathione, their redox forms as well as changes in AsA/DHA and GSH/GSSH redox ratios under stress conditions provide information about cell redox homeostasis which is crucial for cell metabolism regulation, growth processes, and activation of plant defence responses (Zechmann 2014; Noctor and Foyer 2016). Increasing the contents of AsA and GSH through ascorbate/glutathione recycling has been found to be advantageous for plants under stress because it can limit the deleterious effects of oxidative stress and reset the redox homeostasis. Changes in the AsA and GSH pools in cucumber leaves occurred both after bacterial infection acting alone (data not shown) and in combination with NaCl and were dependent on the stage of infection development. Under combined stress, however, the tendency to decrease AsA and DHA accumulation compared to the plants infected without NaCl pretreatment prevailed. Moreover, NaCl intensified the post-infection accumulation of GSH and GSSG in the leaves (Fig. 3). Increased GSH content, not accompanied by an increase in AsA, may indicate a substantial change in the intracellular redox state. The changes in the ascorbate pool could have important consequences for plant response to multiple stress as AsA serves as the cofactor for enzymes involved in ABA biosynthesis, is involved in phytohormone-mediated signalling and low ascorbate content enhances SA and ABA signalling pathways (Pastori et al. 2003). According to Foyer and Noctor (2011), changes in AsA and DHA levels are difficult to unambiguous interpretation because much of the DHA pool is stored in apoplast and spatially separated from the ascorbate intracellular pool. It is therefore possible

that the overall intracellular ascorbate pool is maintained in a reduced state, even during oxidative stress, due to the redox buffer capacity of the cytosol. The cytosolic environment in the photosynthesizing plant cells is usually highly reducing due to a significant excess of the reduced over the oxidized forms of ascorbate and glutathione (Fedoroff 2006). Moreover, maintaining the low apoplast AsA/DHA redox ratio is extremely important for stomatal movement control and response to stress (Chen and Gallie 2004). In cucumber plants treated with NaCl and infected with *P. syringae* pv *lachrymans* (Fig. 3), changes in ascorbate and glutathione pools were nonparallel. The uncoupling of these redox pairs could be due to limitations in DHAR activity (Fig. 3). In cucumber plants exposed to salt stress and then infected, the AsA/DHA redox ratio decreased compared to plants infected without NaCl treatment, whereas the GSH/GSSG ratio was increased at the late stage of pathogenesis. Moreover, changes in AsA/DHA and GSH/GSSH were much stronger than in cucumber plants treated with these stressors individually (Chojak-Koźniewska 2017). These results support the concept that AsA and GSH perform specific functions in plant cells and should not be regarded as equally valued antioxidants.

The regeneration of AsA and GSH via the AsA-GSH cycle depends on NADPH which supply can be limited under stress. The acclimatization to abiotic stresses and defence reactions to pathogens, which require energy inputs and diversion of carbon metabolites to anabolic pathways, are associated with reprogramming of the primary metabolism. Thus, the NADPH generating dehydrogenases, such as NADP-malic enzyme (NADP-ME) and NADP-isocitrate dehydrogenase (NADP-ICDH) could be important to support the AsA-GSH cycle action under stress (Noctor 2006; Leterrier et al. 2012). In *Fragaria vesca*, NADP-ME has been shown to deliver NADPH for regenerating GSH via GR action (Blanch et al. 2013). NADPH production via NADP-ICDH has been shown to be important in promoting glutathione-dependent redox signalling and maintaining redox stability in response to post-infection oxidative stress (Mhamdi et al. 2010).

The reaction of tomato plants exposed to salt stress and *P. syringae* pv *lachrymans* infection confirmed that combination stress generates unique ROS and AsA-GSH cycle-related redox signatures (Fig. 3). The response to the combined stress resulted in novel interactions between the AsA-GSH cycle components, especially ascorbate and glutathione. This implies specific adjustments of the AsA-GSH cycle components to other elements of the stress signalling network, e.g. ROS and phytohormones such as SA or ABA (Mhamdi et al. 2013; Baxter et al. 2014; Xia et al. 2015). ROS and SA act in a regulatory loop wherein ROS induce SA accumulation and SA enhances their production which finally activate antioxidants (Khan et al. 2015). Combined stress changed the SA/ABA balance which hindered the PR1 gene expression, known to be regulated by the glutathione redox status (Noctor et al. 2012). Salt stress through ABA up-regulation could have antagonistic effects on SA-mediated signalling and compromises the defence against *P. syringae* pv *lachrymans*, a biotrophic pathogen (Chojak-Koźniewska et al. 2017). In the interplay between ABA, SA, and ROS signalling pathways, ABA has been suggested to be positioned between ROS and SA (Kissoudis et al. 2014).

6 Conclusions

Biotic stressors affect the redox balance of plant cells and the AsA-GSH cycle has an important role in managing the ROS generated in response to pathogens. In addition to its direct involvement in ROS processing, the AsA-GSH cycle may protect against biotic stress by activating defence mechanisms through redox signalling, and ascorbate and glutathione seem to fulfil a central role in this process. Ascorbate and glutathione are the key players in redox regulatory systems and they function in multiple nodes of the defence signalling network. The versatile cross-communication of glutathione and ascorbate with other signalling molecules in the plant defence network is crucial for conditioning the response to abiotic and biotic stress combinations which are common to many agricultural areas. At the subcellular level, the interplay between ROS and the AsA-GSH cycle components generates compartment-specific stress response signature which plays important role in sensing, signalling, and activating plant defence.

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Exogenous Ascorbic Acid Mediated Abiotic Stress Tolerance in Plants

Yi Xu and Bingru Huang

Abstract Ascorbic acid (AsA) plays various roles in regulating plant responses or tolerance to environmental stresses. The positive effects of exogenous AsA application are well documented, ranging from seed germination to biomass or yield production, particularly under various abiotic stress conditions. AsA is well known for its roles in ameliorating oxidative damages on plant growth, physiological and metabolic processes by activating nonenzymatic and enzymatic components of antioxidant metabolism. Exogenous AsA application also mitigates stress-inhibition of photosynthesis, delays leaf senescence or loss of chlorophyll, and helps maintain cell membrane stability. In addition, AsA also facilitates the maintenance of ion homeostasis (i.e., Na^+, K^+, Mg^{2+}, Ca^{2+}) in plants exposed to abiotic stress. Interactions between AsA, phytohormones, and other metabolites also play roles in regulating plant growth and stress adaptation. This chapter provides an overview of physiological, biochemical, and molecular effects of exogenous application of AsA for various plant species under abiotic stresses. Future research addressing transcriptomic, proteomic, and metabolomic profiles will provide further insights into mechanisms of improved growth and stress tolerance brought by exogenous application of AsA.

Keywords Abiotic stress · Antioxidant system · Ascorbic acid · Growth · Photosynthesis · Reactive oxygen species

1 Introduction

Plants in natural environments are subject to various environmental stresses, such as extreme temperatures (heat or cold), drought, salinity, and metal toxicity in soils, which adversely affect plant growth and development. Various physiological, biochemical, and metabolic processes or components are altered by abiotic

Y. Xu · B. Huang (✉)
Department of Plant Biology, Rutgers University, New Brunswick, NJ, USA
e-mail: huang@sebs.rutgers.edu

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stresses associated with stress damages or adaptation, such as antioxidant metabolism, photosynthetic processes, cell membranes, osmotic adjustment, ion homeostasis, and hormone metabolism (Hirt and Shinozaki 2004; Rao et al. 2006). One of the most common plant responses to abiotic stresses is the production of reactive oxygen species (ROS), which cause oxidative damages to cellular constituents, such as proteins, DNA, and lipids, and deteriorate cellular structures (Gill and Tuteja 2010). The hallmark of membrane lipid damages by ROS is lipid peroxidation leading to the production of malondialdehyde (MDA), which disrupts membrane integrity (Smirnoff 1998). In order to detoxify ROS during stress adaptation, plants have developed enzymatic and nonenzymatic antioxidant mechanisms, including the accumulation of antioxidant compounds, such as ascorbic acid (AsA), as well as the activation of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX); however, the natural defense ability can be undermined by overproduced ROS (Mittler 2002). Exogenous application of antioxidants has been found to be an effective approach to strengthen ROS-scavenging capacity, thus protecting plants from oxidative damages under various abiotic stress conditions (Shalata and Neumann 2001; Hoque et al. 2007; Chen and Murata 2011; Akram et al. 2017; Kobayakawa and Imai 2017; Wang et al. 2017). Improving plant abiotic stress tolerance by effective utilization of organic compounds with important physiological functions is one of the major goals for modern agriculture, which will not only benefit crop species and secure human food source, but will also have practical meaning for non-crop species to adapt to both global and local climate change.

Ascorbic acid is naturally synthesized in actively growing plants and is present in high quantities in mature leaves, where fully developed chloroplasts contain about 30–40% of plant AsA and stromal concentration is as high as 50 mM (Foyer and Noctor 2003). Ascorbic acid is also localized in the apoplastic space of the cell, taking up to 10% of AsA pool (Noctor and Foyer 1998) or 2 mM assuming that 10% of fresh weight belongs to the apoplast (Burkey et al. 2003). AsA synthesis, however, can be inhibited by abiotic stress, causing a decline in endogenous AsA content in plant tissues (Mukherjee and Choudhuri 1983; Sairam et al. 2005; Xu et al. 2006). Ascorbic acid is a small water-soluble molecule, which can be absorbed into plants and can lead to an increase in endogenous AsA content. The increase of endogenous AsA content following exogenous application of AsA was found in various plant species exposed to different types of abiotic stresses, including salinity (Athar et al. 2009; Younis et al. 2010; Billah et al. 2017), heat (Kumar et al. 2011), drought (Farooq et al. 2013), osmotic (Alam et al. 2014; Terzi et al. 2015), ozone (Kobayakawa and Imai 2017), and cadmium stresses (Wang et al. 2017). The increased pool of AsA available in plant cells provides protective roles for plants against those abiotic stresses. It is also essential that AsA is maintained in a high reduced ascorbate versus oxidized dehydroascorbate (DHA) state to be effective, and the redox state (AsA/DHA ratio) could serve as an important indicator for plant abiotic stress tolerance, either between tolerant and sensitive cultivars (Burkey et al. 2003), or between transgenic and wild-type plants (Kwon et al. 2003). Alam et al. (2014) reported that exogenous application

of AsA significantly increased the AsA/DHA ratio in three *Brassica* species, which resulted in improved osmotic stress tolerance. It is currently not clear which of these, endogenous AsA content or AsA/DHA ratio, takes the primary role in AsA-mediated abiotic stress tolerance. Perhaps both of them benefit the plant in a synergistic way, but how plants regulate each other and determine the timing and scale for their respective effects remains unknown.

Ascorbic acid is one of the most extensively studied antioxidants that has been detected in almost all plant tissue types (Aro and Ohad 2003; Borland et al. 2005), which plays pivotal roles in antioxidant potential, balancing and scavenging ROS production (Noctor and Foyer 1998; Muller-Moule et al. 2004). Ascorbic acid is also involved in the regulation of many other processes, such as cell division, cell expansion, osmotic adjustment, and other metabolic processes (Blokhina et al. 2003; Pignocchi and Foyer 2003). Ascorbic acid has been widely used as a plant growth regulator to enhance plant abiotic stress tolerance, and has been involved in different stress types, application methods, AsA concentrations, and treatment durations for many plant species and cultivars (Table 1).The damages caused by abiotic stress could be mitigated by exogenous application of AsA in various plant species. Understanding the effects of AsA regulating antioxidant metabolism and other physiological or biochemical processes is of great significance for developing effective practices to improve plant tolerance to various abiotic stresses. This chapter focuses on the discussion of exogenous application of AsA on the following aspects: (1) antioxidative mechanism, (2) cell membrane stability, (3) photosynthesis, (4) ion homeostasis, (5) interactions with phytohormones and other metabolites, and (6) plant growth and yield. Finally, we also propose future research perspectives for enhancing our understanding of the manner in which exogenous application of AsA improves plant abiotic tolerance.

2 Antioxidative Mechanism

When plants are subject to abiotic stresses, ROS, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$), are produced in excessive amounts, which may exceed the antioxidative capacity of the cell and cause cellular damages (Iturbe-Ormaetxe et al. 1998; Gill and Tuteja 2010). On the other hand, ROS are also signaling molecules and are present under both ambient environmental conditions and abiotic stress. Therefore, the delicate balance of ROS regulation is very important for plant survival. Plants have evolved several antioxidative strategies to counteract oxidative stress, including the use of enzymatic and nonenzymatic antioxidants (Posmyk et al. 2009). Several antioxidants, such as AsA and glutathione (GSH), are involved in direct ROS scavenging, while ROS-scavenging enzymes, such as SOD, CAT, peroxidase (POD), APX, monodehydroascorbate reductase (MR), dehydroascorbate reductase (DR), and glutathione reductase (GR), mediate the biochemical conversion of O_2^- and H_2O_2 to water. The induction of these enzyme activities is

Table 1 Summary of publications related to exogenous application of AsA for plants under abiotic stress

Species	Stress type	Stress parameter	Stress duration	AsA application type	AsA concentration	AsA application time	References
Barley (*Hordeum vulgare* L.)	Cadmium	100, 300, 500, 700 μM	15 days	Irrigation	200 mg/L	15 days	Ullah et al. (2016)
Wheat (*Triticum aestivum* L.)	Cadmium	100 μM	24 h	Seed priming	1 mM	6 h	Wang et al. (2017)
Maize (*Zea mays* L.)	Chilling	–	36 days	Foliar spray	20, 40 mg/L	Once	Ahmad et al. (2013)
Maize	Drought	–	7 days	Irrigation	75, 150 ppm	7 days	Darvishan et al. (2013)
Maize	Drought	−1.3 MPa	8 days	Foliar spray	50, 100, 150 ppm	1 days	Dolatabadian et al. (2009)
Wheat	Drought	35, 70% water-holding capacity	28 days	Seed priming	2 mM	10 h	Farooq et al. (2013)
Wheat	Drought	–	From the end of stem elongation stage till physiological maturity	Foliar spray	200 mg/L	Twice	Hafez and Gharib (2016)
Sunflower (*Helianthus annuus* L.)	Drought	40, 80% soil field capacity	30 days	Seed priming	0.5 mM	–	Madany and Khalil (2017)
Wheat	Drought	−0.6 MPa	35 days	Rooting medium, seed priming and foliar spray	0.5, 1 mM	35 days for rooting medium, 10 h for seed priming, twice for foliar spray	Malik and Ashraf (2012)
Cotton (*Gossypium barbadense* L.)	Drought	50%, 100% field capacity	45 days	Foliar spray	1 mM	45 days	Mekki et al. (2015)

Canola (*Brassica napus* L.)	Drought	60%, full field capacity	28 days	Foliar spray	50, 100, 150 mg/L	14 days	Shafiq et al. (2014)
Cotton	Heat	44–49 °C	5 days	Foliar spray	20, 40, 60 mg/L	5 days	Kamal et al. (2017)
Mungbean (*Phaseolus aureus* Roxb.)	Heat	35/25, 40/30, 45/35 °C	10 days	Hydroponics	50 µM	10 days	Kumar et al. (2011)
Rice (*Oryza sativa* L.)	Heat	30 °C night temperature, 11 h daily	45 days	Foliar spray	5 mM	45 days	Shah et al. (2011)
Brassica spp.	Osmotic	15% PEG	48 h	Hydroponics	1 mM	48 h	Alam et al. (2014)
Caralluma tuberculate	Osmotic	50, 100, 200 g/L PEG	15 days	Hydroponics	100, 200 µM	15 days	Rehman et al. (2017)
Maize	Osmotic	−0.3 MPa	12 h	Hydroponics	0.1 mM	12 h	Terzi et al. (2015)
Tall fescue (*Festuca arundinacea* Schreb.)	Osmotic	−0.25, −0.5 MPa	10 days	Hydroponics	5 mM	10 h	Xu et al. (2015)
Rice	Ozone	0.1, 0.3 cm^3/m^3	3 days	Hydroponics	5, 10 mM	5 days	Kobayakawa and Imai (2017)
Wheat	Salinity	15 dS/cm	50 days	Seed priming	50, 100, 200 ppm	12 h	Afzal et al. (2006)
Barley	Salinity	100, 200 mM	From third leaf stage to 49 days	Seed priming	1 mM	12 h	Agami (2014)

(continued)

Table 1 (continued)

Species	Stress type	Stress parameter	Stress duration	AsA application type	AsA concentration	AsA application time	References
Wheat	Salinity	40, 80, 120, 160 mM	30 days	Seed priming	0.6 mM	6 h	Al-Hakimi and Hamada (2001)
Wheat	Salinity	150 mM	58 days	Hydroponics	50, 150 mg/L	50 days	Athar et al. (2008)
Wheat	Salinity	150 mM	35 days	Rooting medium, seed priming and foliar spray	100 mg/L	28 days, 6 h for seed priming	Athar et al. (2009)
Durum wheat (*Triticum durum* Desf.)	Salinity	150 mM	14 days	Irrigation	0.7 mM	14 days	Azzedine et al. (2011)
Chick pea (*Cicer arietinum* L.)	Salinity	20, 40 mM	56 days	Irrigation	4 mM	56 days	Beltagi (2008)
Maize	Salinity	12 dS/m	35 days	Hydroponics	0.5, 1 mM	35 days	Billah et al. (2017)
Barley	Salinity	300 mM	8 days	Hydroponics	5 mM	8 days	Chen et al. (1999)
Common bean (*Phaseolus vulgaris* L.)	Salinity	100, 400 mM	48 h	Hydroponics	25, 50, 100 mM	21 days	Dolatabadian and Jouneghani (2009)
Canola	Salinity	200 mM	6 days	Foliar spray	25 mM	6 days	Dolatabadian et al. (2008)
Sugarcane (*Saccharum* spp.)	Salinity	100 mM	70 days	Irrigation and foliar spray	0.1, 0.5, 1 mM	70 days	Ejaz et al. (2012)
Flax (*Linum usitatissimum* L.)	Salinity	2000, 4000, 6000 mg/L	60 days	Foliar spray	400 mg/L	Twice	El-Bassiouny and Sadak (2015)

Maize	Salinity	50, 100, 200 mM	40 days	Seed priming and foliar spray	100 ppm	12 h for seed priming; four times for foliar spray	Hassanein et al. (2009)
Six haplophytes	Salinity	10, 20, 30, 40 dS/m	20 days	Seed priming	20, 40, 60 mM	3 h	Khan et al. (2006a, b)
Wheat	Salinity	150 mM	21 days	Foliar spray	50, 100 mg/L	21 days	Khan et al. (2006a, b)
Citrus (*Citrus aurantium* L.)	Salinity	100 mM	30 days	Irrigation	0.5 mM	30 days	Kostopoulou et al. (2015)
Pumpkin (*Cucurbita pepo* L.)	Salinity	10 dS/m	28 days	Seed priming	15, 30 mg/L	12 h	Rafique et al. (2011)
C. tuberculata	Salinity	100, 200, 300 mM	15 days	Rooting medium	100, 200 μM	15 days	Rehman et al. (2014)
Flax	Salinity	3.08, 6.16, 9.23 dS/m	30 days	Foliar spray	1.13, 2.27 mM	Twice	Sadak and Dawood (2014)
Potato (*Solanum tuberosum* L.)	Salinity	20, 40, 60, 80, 100, 120, 140 mM	60 days	Rooting medium	0.5 mM	72 h	Sajid and Aftab (2009)
Tomato (*Lycopersicon esculentum* Mill.)	Salinity	300 mM	9 h	Hydroponics	0.5 mM	24 h	Shalata and Neumann (2001)
Chinese kale (*Brassica alboglabra* L.)	Salinity	50, 100 mM	14 days	Hydroponics	5, 10 mM	13 days	Tayebi-Meigooni et al. (2014)
Broad bean (*Vicia faba* L.)	Salinity and osmotic	43, 86, 128, 171, 214, 257 mM NaCl; 14, 28, 41, 55, 69, 82 mM mannitol	21 days	Hydroponics	4 mM	24 h	Younis et al. (2010)

the most common mechanism for the detoxification of ROS under abiotic stress (Gressel and Galun 1994). Ascorbic acid is involved in antioxidant defense directly by serving as a substrate of antioxidant enzymes to reduce H_2O_2 or indirectly by affecting other antioxidant pathways.

Abiotic stress imposed upon plants causes excessive production of ROS, while AsA is involved in the reduction of H_2O_2 by directly quenching ROS in a nonenzymatic manner, or through APX enzymatic scavenging in the water-water cycle and ascorbate-glutathione (AsA-GSH) cycle, bringing ROS level to a sub-lethal state (Fig. 1). Hydrogen peroxide and O_2^- content are greatly induced under abiotic stress, and AsA application is able to significantly decrease ROS levels in various plant species (Azzedine et al. 2011; Farouk 2011; Kumar et al. 2011; Alam et al. 2014; Terzi et al. 2015; Billah et al. 2017; Madany and Khalil 2017; Wang et al. 2017). However, most of the studies did not report ROS level decreasing to a normal state following AsA treatment, which indicates that other mechanism(s) must be involved in regulating ROS detoxification in plants under abiotic stress.

In contrast, exogenous application of AsA could bring about various regulation patterns on enzymatic activities of ROS-scavenging enzymes, including positive (Athar et al. 2009; Sajid and Aftab 2009; Younis et al. 2010; Farouk 2011; Ejaz et al. 2012; Ahmad et al. 2013; Agami 2014; Alam et al. 2014; Xu et al. 2015;

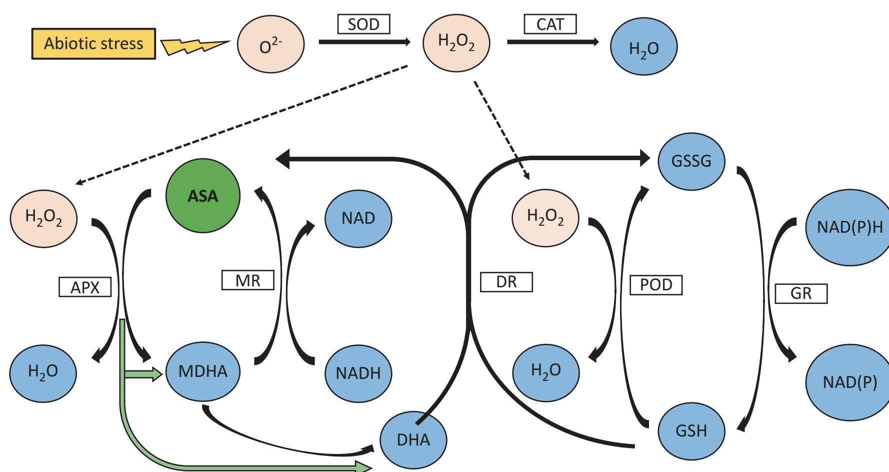

Fig. 1 ROS-scavenging pathway. Green circle shows the position of AsA as the substrate. Yellow color indicates the abiotic stress. Pink and blue circles represent ROS and chemicals involved in the pathway. Green arrows stand for direct ROS quenching. Black arrows stand for enzymatic reactions. Dashed black arrows show the transport of H_2O_2. All boxes are enzymes involved in the pathway. O^{2-} superoxide, H_2O_2 hydrogen peroxide, *AsA* ascorbate, *MDHA* monodehydroascorbate, *DHA* dehydroascorbate, *GSH* glutathione, *GSSG* glutathione disulfide, *NAD* nicotinamide adenine dinucleotide, *NADH* nicotinamide adenine dinucleotide (reduced), *NADP* nicotinamide adenine dinucleotide phosphate, *NADPH* nicotinamide adenine dinucleotide phosphate (reduced), *SOD* superoxide dismutase, *CAT* catalase, *APX* ascorbate peroxidase, *POD* peroxidase, *MR* monodehydroascorbate reductase, *DR* dehydroascorbate reductase, *GR* glutathione reductase

Hafez and Gharib 2016), negative (Dolatabadian et al. 2008; Dolatabadian and Jouneghani 2009; Dolatabadian et al. 2009; Shah et al. 2011; Shafiq et al. 2014), or various regulations (Hassanein et al. 2009; Kumar et al. 2011; Rehman et al. 2014, 2017; El-Bassiouny and Sadak 2015; Tayebi-Meigooni et al. 2014; Billah et al. 2017) for different components in the ROS-scavenging enzyme system, compared with stress conditions alone. One possible explanation for the contradictory findings in these studies is that the ROS-scavenging enzymes perform differently between tolerant and sensitive cultivars. The stress-tolerant cultivars tended to be affected less significantly by abiotic stress conditions, while stress-sensitive cultivars were more subject to oxidative damages, and thus benefited from the application of AsA (Athar et al. 2009; Alam et al. 2014; Shafiq et al. 2014; El-Bassiouny and Sadak 2015; Rehman et al. 2017). Other factors, such as tissue type, abiotic stress type, duration of stress, and AsA concentration, may also have had effects on the discrepancies in these studies, which requires extensive research. Xu et al. (2015) found that the transcript levels of SOD, CAT, APX, GR, DR, and MR in tall fescue were significantly higher after exogenous application of AsA under PEG-induced water stress, compared to water stress alone, while no significant differences in the activities of these enzymes were observed with or without AsA under water stress. It is clear that AsA is involved in antioxidative metabolism at both enzymatic activity and transcriptional levels.

The exogenous application of AsA under abiotic stress promotes other antioxidative mechanisms, such as the glyoxalase system for methylglyoxal (MG) metabolism, and GSH content. Methylglyoxal is a cytotoxic compound recently found in higher plants (Yadav et al. 2005), which is a transition-state intermediate of the triosephosphates (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate) in the glycolysis pathway (Richard 1991). Accumulation of MG inhibits cell proliferation, accelerates protein degradation by modifying arginine, lysine, and cysteine residues, adducts with the guanyl nucleotide in DNA, and inactivates the antioxidant systems (Ray et al. 1994; Martins et al. 2001). The detoxification of MG relies on the glyoxalase system, which utilizes two enzymes, glyoxalase I and glyoxalase II, to convert MG to D-lactate using GSH as a recycling cofactor (Thornalley 1990). Therefore, the maintenance of GSH level and glyoxalase I and II activities is indicative of an efficient glyoxalase system that is vital for plant survival under abiotic stress. An increase in GSH content level brought on by exogenous AsA application was reported under both salt (Hassanein et al. 2009; Billah et al. 2017) and heat stress (Kumar et al. 2011). Both glyoxalase I and glyoxalase II activities were significantly increased in three *Brassica* species after addition of AsA in PEG-induced osmotic stress (Alam et al. 2014). The evidence of AsA as a defensive agent against abiotic stress through such a pathway is believed to attract more attention from researchers with hopes of establishing a novel scenario for manipulating plant abiotic stress tolerance.

3 Cell Membrane Stability

The cell membrane system is one of the first targets for stresses, and it is generally accepted that maintaining its integrity and stability is a major component of plant stress tolerance. The plant cell membrane comprises two components, a lipid bilayer and membrane proteins (Andersen and Koeppe 2007). Under abiotic stress conditions, excessive ROS molecules attack unsaturated fatty acids, which lead to peroxidation of membrane lipids, leakage of cellular contents, rapid desiccation, and cell death (Ahmad et al. 2008). Hydrogen peroxide induces long-lasting oxidative damages on membranes of all the organelles in the cell (Adachi et al. 2009). Malondialdehyde (MDA) is a decomposition product of unsaturated fatty acids in cell membranes, and serves as an index of ROS-induced oxidative damage (Gossett et al. 1994). Electrolyte leakage (EL) from the cell is also applicable to quantify damages to the cell membrane (Bajji et al. 2002). Lower levels of MDA and EL are indicative of improved plant abiotic stress tolerance (Perez-Lopez et al. 2009). Ascorbic acid application scavenges excessive ROS generated from abiotic stress, so that it protects cell membrane stability. As a result, the MDA content or EL was reported to be reduced with AsA application under abiotic stress (Shalata and Neumann 2001; Dolatabadian et al. 2008, 2009; Dolatabadian and Jouneghani 2009; Hassanein et al. 2009; Farouk 2011; Kumar et al. 2011; Shah et al. 2011; Agami 2014; Alam et al. 2014; Tayebi-Meigooni et al. 2014; El-Bassiouny and Sadak 2015; Kostopoulou et al. 2015; Terzi et al. 2015; Billah et al. 2017; Madany and Khalil 2017; Wang et al. 2017). Although the inhibitory effect of exogenous AsA application on plant lipid peroxidation and its promotion of stress tolerance are well established, the underlying mechanisms of this process are not yet clear. Evidence supporting the beneficial effects of AsA application on ROS-induced damages that result from several possible mechanisms, including the oxidation and cross-linking of protein thiols, inhibition of key membrane proteins such as H^+-ATPase (Chen et al. 1999), or changes to the composition and fluidity of membrane lipids, is still lacking.

4 Photosynthesis

Plant photosynthesis is essential for plant growth as it provides carbon compounds and energy that supports various metabolic activities; however, the process also generates ROS in the presence of oxygen, which requires strict control in order to avoid oxidative damage (Foyer and Noctor 2003). Abiotic stress leads to an increase of free radicals in chloroplasts, which interrupt chlorophyll structure and photosynthetic reaction centers (Zhang et al. 2003). Ascorbic acid can counteract the oxidative burst caused by abiotic stress with either direct mitigation of ROS or indirect substrate supply for the water-water cycle and AsA-GSH cycle in ROS-scavenging systems (Gill and Tuteja 2010). Previous research has shown that exogenous

application of AsA enhanced chlorophyll a, chlorophyll b, and/or total chlorophyll contents under various abiotic stress conditions, including salinity (Khan et al. 2006a; Beltagi 2008; Dolatabadian et al. 2008; Athar et al. 2009; Dolatabadian and Jouneghani 2009; Hassanein et al. 2009; Azzedine et al. 2011; Farouk 2011; Agami 2014; Tayebi-Meigooni et al. 2014; Kostopoulou et al. 2015; Billah et al. 2017), drought (Dolatabadian et al. 2009; Malik and Ashraf 2012; Darvishan et al. 2013; Farooq et al. 2013; Sadak and Dawood 2014; Mekki et al. 2015; Madany and Khalil 2017), heat (Kumar et al. 2011), low temperature (Ahmad et al. 2013), and osmotic stress (Alam et al. 2014). The increase in chlorophyll contents in these studies is largely due to the protective role of AsA played in the oxidative stress response. It is also generally known that photochemical efficiency depends upon photosynthetic pigments, such as chlorophyll a and b, which is important in photochemical reactions (Taiz and Zeiger 2006). The improvement of chlorophyll content and structure brought on by AsA application could also increase photochemical efficiency (Dolatabadian and Jouneghani 2009). However, discrepancies between improvement of chlorophyll contents and photochemical efficiency through exogenous AsA application under abiotic stresses also exist, which need further attention and explanation.

5 Ion Homeostasis

The homeostasis of intracellular ions is of fundamental importance for maintaining proper cellular functions. Ion homeostasis is considered to be one of the most important mechanisms for salt stress acclimation in plants (Munns and Tester 2008). Among all types of ions, Na^+/K^+ traffic is especially important when plants are subjected to salinity stress. Under salinity stress (usually caused by accumulation of NaCl), excessive Na^+ in the environment may enter the cell and cause toxic degradation of enzymes (Hasegawa et al. 2000). Meanwhile, the uptake of K^+ is inhibited, and K^+ content decreases under salinity stress, which is a major solute contributing to osmotic pressure and ionic strength (Serrano and Rodriguez-Navarro 2001). Exogenous application of AsA helped maintain plant ion homeostasis, by decreasing Na^+, increasing K^+, and thus decreasing the Na^+/K^+ ratio (Al-Hakimi and Hamada 2001; Athar et al. 2008, 2009; Farouk 2011). The mechanism of AsA-mediated ion homeostasis remains unknown, but it is possibly due to the improvement of cell membrane stability and/or plasma membrane Na^+-H^+ antiporter SOS1 (Zhu 2003). However, a decrease of K^+ content was found in AsA-treated rice under high nighttime temperature stress, compared with non-treated rice (Shah et al. 2011). The effect of AsA application on K^+ content in rice also varied between cultivars and tissue types, further complicating the possible answer to AsA-mediated ion homeostasis.

In addition, other ions, such as magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions, could also play important roles under abiotic stress, which has received little attention. Magnesium ion is responsible for protein synthesis and maintaining chlorophyll

content (Leidi et al. 1991; Flowers and Dalmond 1992). Calcium ion is another important cell signaling molecule involved in a wide range of physiological processes and responses to both biotic and abiotic stresses (Steinhorst and Kudla 2014). Under salinity stress, both Mg^{2+} and Ca^{2+} uptake was reduced, possibly due to the mechanism of antagonistic absorption of Na^{+} and K^{+} on these two cations or to the reductions of transporter activities for Mg^{2+} and Ca^{2+} (Alam 1999; Asik et al. 2009). Exogenous application of AsA ameliorated the reduction of both Mg^{2+} and Ca^{2+} contents under salinity stress (Al-Hakimi and Hamada 2001; Athar et al. 2008, 2009; Farouk 2011), drought stress (Sadak and Dawood 2014), and cadmium stress (Ullah et al. 2016). The relationship between these ions and their potential usage in AsA-mediated regulation are crucial for understanding more about ion homeostasis under abiotic stress.

6 Interactions with Phytohormones and Other Molecules

Plant hormones play essential roles in regulating plant growth and development, as well as in stress tolerance (Peleg and Blumwald 2011). Hormones, such as gibberellic acid (GA) and indole-3-acetic acid (IAA), promote seed germination, cell elongation, and flowering time (Richards et al. 2001; Woodward and Bartel 2005), while abscisic acid (ABA) is known to induce senescence, especially under stress conditions (Tuteja 2007). A few studies have suggested that AsA may affect plant growth and adaptation to abiotic stress through interaction with different hormones. For example, AsA application was found to significantly increase the content of GA and IAA, but decrease the content of abscisic acid (ABA) (Hassanein et al. 2009; Dolatabadian et al. 2009; Terzi et al. 2015). It is possible that the benefits of exogenous AsA application for improving plant abiotic stress tolerance are correlated with the regulation of plant hormones; however, more evidence supporting this idea is required in order to fully understand the mechanisms.

Nitric oxide (NO) is a gaseous signaling molecule first described in mammals, and later found in plants, which plays roles in regulating growth and development, respiratory metabolism, senescence and maturation, and plant responses to biotic and abiotic stresses (Arasimowicz and Floryszak-Wieczorek 2007). A recent study of cadmium (Cd) stress tolerance in wheat seedlings has shown that exogenous AsA application ameliorated Cd stress damage, while addition of cPTIO (NO scavenger) along with AsA in the treatment was able to remove the AsA benefits by prohibiting AsA assimilation and inducing oxidative damage (Wang et al. 2017). The benefits of NO under abiotic stress could be due to its influence on the plant alternative respiration pathway, which produces less ROS than the traditional cytochrome pathway (Xu et al. 2016). However, additional information is still needed to directly validate the interactions between AsA and NO, and their synergistic impact on plant abiotic stress tolerance.

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indolic compound first discovered in animals, and later in plants (Arnao and Hernandez-Ruiz 2006). The

functions of melatonin in plants mainly include defense against oxidative stress, preservation of chlorophyll, and promotion of photosynthesis and root development (Tan et al. 2011). In a study performed by Kostopoulou et al. (2015), the combination of AsA and melatonin applied to salt-stressed citrus seedlings showed enhanced plant salinity tolerance compared to each compound applied individually, which was manifested by improving Cl^- homeostasis, carbohydrate and proline accumulation, antioxidant activity, *myo*-inositol biosynthesis, and transcriptional regulation. Further investigation is required in order to reveal the roles of both AsA and melatonin and the networks in which they are involved relative to plant abiotic stress.

Proline serves as an osmoprotectant, membrane stabilizer, and ROS scavenger (Bandurska 2001; Hartzendorf and Rolletschek 2001; Ashraf and Foolad 2007), and exogenous AsA application on plants could have different regulation patterns under various abiotic stresses, either increasing (Azzedine et al. 2011; Kumar et al. 2011; Ejaz et al. 2012; Farooq et al. 2013; El-Bassiouny and Sadak 2015) or decreasing proline content (Al-Hakimi and Hamada 2001; Dolatabadian et al. 2008, 2009; Darvishan et al. 2013; Agami 2014; Alam et al. 2014; Tayebi-Meigooni et al. 2014; Terzi et al. 2015). Kavi Kishor and Sreenivasulu (2014) proposed that the proline homeostasis existed between the enlargement of proline sink in the reproductive tissue during stress, and providing energy to drive growth in the actively growing tissue during stress. As a result, it is important to evaluate AsA-regulated plant abiotic stress tolerance solely by proline content.

7 Plant Growth and Yield

The main undesirable effects of abiotic stress are the retardation of plant seed germination and growth, and the decrease of nutrient content and yield, which are especially unfavorable for crop species (Wang and Frei 2011). The abiotic stresses imposed upon plants could have different physiological consequences, depending on time and duration. First of all, seed germination may be affected under abiotic stress conditions, especially when seeds are soaked in saline solutions (Khan and Ungar 1984). Seed germination is a complex biological process, starting from the uptake of water by dry seed, and ending with the penetration of radicle out of the seed structure (Bewley 1997). Exogenous application of AsA was able to improve seed germination, by either increasing seed germination rate, or reducing germination time under salinity stress (Afzal et al. 2006; Khan et al. 2006b). The improvement of seed germination under salt stress following AsA application may be ascribed to the increased antioxidant capacity, which would compensate for the increasing ROS levels during carbohydrate reserve depletion and oxidative phosphorylation (Crowe and Crowe 1992; Khan et al. 2006b). Seeds can contain various types of antioxidant components in small amounts, and AsA may not be present among them (Gidrol et al. 1992; AsAda 1997). In contrast, seed germination in *Zinnia elegans* was enhanced by the oxidation of germination inhibitors via ROS (Ogawa and Iwabuchi 2001). This indicates the complexity of the seed germination

process and underscores the importance of continuing research on the benefits of AsA application on seed germination under abiotic stresses.

Plant cell growth undergoes a series of distinct developmental phases, including mitotic cell division and later cell expansion (Verbelen et al. 2006). Plant cell wall expansion is further based on the thermodynamics of hydrogen-bonded networks, which requires expansin (EXP) and xyloglucan endotransglucosylase (XET) to maintain a proper cell wall matrix (Cosgrove 2005). EXPs bind at the interface between cellulose microfibrils and the polysaccharide matrix in the cell wall and induce extension by reversibly disrupting non-covalent bonds in the network (McQueen-Mason and Cosgrove 1995). XET catalyzes the breakdown of xyloglucan chains and the linking of newly generated reducing ends of one chain to the non-reducing ends of another (Schroder et al. 1998). Recently, Xu et al. (2015) reported that the transcript levels of *EXP-A3* and *XET-2* in tall fescue (*Festuca arundinacea*) roots were decreased under PEG-induced water stress, while exogenous application of AsA significantly enhanced their transcript levels, indicating the correlation between increasing transcriptional regulation of *EXP* and *XET* genes and AsA-regulated plant growth under osmotic stress. For cell division, it was also reported that exogenous application of AsA greatly induced a massive transition from the mitotic phase G1 to S in onion root cells, which influenced up to 79% of the cells in the quiescent center (Liso et al. 1988). However, direct evidence regarding AsA-regulated cell division under abiotic stress conditions is still lacking, which is an intriguing but neglected aspect of AsA-mediated abiotic stress tolerance.

The imposition of abiotic stress has also caused reductions of plant biomass and yield, which is of utmost concern, as these types of stress will be increasingly important due to climate change, land degradation, and water quality decline (Witcombe et al. 2008; Tester and Langridge 2010). Exogenous application of AsA has been proven to improve plant performance under abiotic stress, and increase crop yield in various studies. For example, foliar application of AsA improved seed yield of cotton (*Gossypium barbadense*) under both drought (Mekki et al. 2015) and heat stress (Kamal et al. 2017). Reduction of crop yield under drought stress could also be ameliorated by exogenous application of AsA, as seen in maize (*Zea mays*) (Darvishan et al. 2013), flax (*Linum usitatissimum*) (Sadak and Dawood 2014), and wheat (*Triticum aestivum*) (Hafez and Gharib 2016). Exogenous AsA application has also mitigated the reduction of fresh or dry weight of biomass, carbohydrate content, and soluble protein content under abiotic stress (Beltagi 2008; Younis et al. 2010; Rafique et al. 2011; Alam et al. 2014; Rehman et al. 2014, 2017; Ullah et al. 2016; Madany and Khalil 2017). The beneficial effects of AsA application on plant biomass accumulation is of great value for improving abiotic stress tolerance.

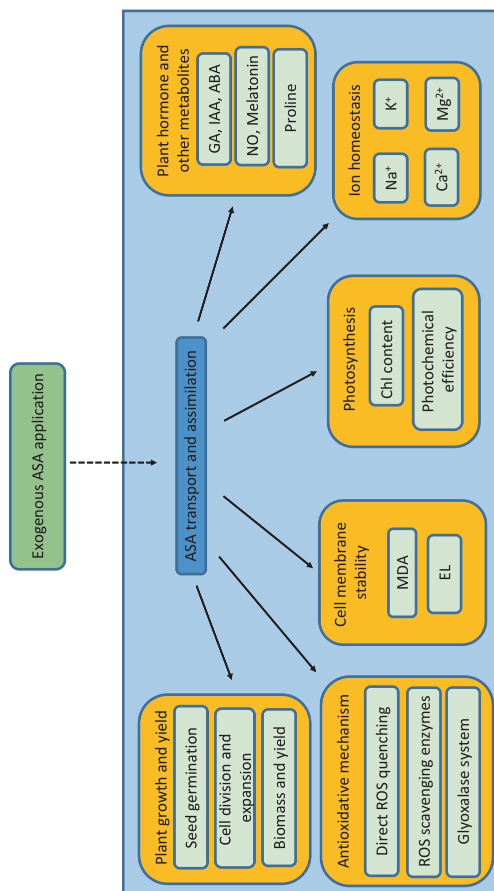

Fig. 2 Proposed mechanisms of exogenous AsA application for improving plant abiotic stress tolerance. *ROS* reactive oxygen species, *MDA* malondialdehyde, *EL* electrolyte leakage, *Chl* chlorophyll, *GA* gibberellic acid, *IAA* indole-3-acetic acid, *NO* nitric oxide

8 Concluding Remarks and Future Research Perspectives

There has been increasing evidence supporting the critical roles of AsA involved in regulating plant developmental, physiological, and biochemical responses to abiotic stress within the last few decades. The key roles that AsA has been shown to play in improving plant abiotic stress include increasing plant antioxidative capacity by direct ROS scavenging and promoting ROS-scavenging enzyme and glyoxalase activities. Several other benefits conferred by AsA application, such as improving plant seed germination, cell division and expansion, biomass and yield, better maintenance of chlorophyll content, photochemical efficiency, cell membrane stability, ion homeostasis (Na^+, K^+, Mg^{2+}, Ca^{2+}), and interactions with plant hormones and other metabolites, may also have either direct or indirect links with its properties as an antioxidant (summarized in Fig. 2). Most previous studies reporting the positive effects of exogenous AsA application on plant growth and stress adaptation have focused on the physiological impact; however, information at the molecular or genomic levels is lacking. Few studies have reported the upregulation of transcript levels of antioxidant enzymes (i.e., SOD, CAT, APX, GR, DR, and MR) and cell elongation (i.e., XET, expansins). Despite the strong efforts of the currently available research, global regulatory patterns for AsA-mediated plant abiotic stress still need to be analyzed further. Therefore, extensive research addressing transcriptomic, proteomic, and metabolomic profiles will provide further insights into a better understanding of improved abiotic stress tolerance brought by exogenous application of AsA.

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The Role of Ascorbic Acid in Plant–Pathogen Interactions

Hatem Boubakri

Abstract Ascorbic acid (AsA) is one of the most abundant antioxidant molecules in plants. AsA provides the first line of defense against damaging reactive oxygen species (ROS), protecting plant cells from many environmental factors that induce oxidative stress, including wounding, ozone, high salinity, and pathogen attacks. AsA interacts with key elements of a complex network orchestrating plant defense mechanisms, thereby influencing the outcome of plant–pathogen interaction. It can act in coordination with glutathione (GSH) and important enzymatic antioxidants in the AsA-GSH cycle to provide the appropriate redox environment regulating diverse defense pathways such as the expression of defense genes through the activation of the NPR1 (Nonexpressor of Pathogenesis-Related protein 1) regulatory transcription factor, the strengthening of cell walls, and the modulation of defense-hormonal signalling networks. On the other hand, AsA was found to act either as an inducer *per se* or as a component of induced resistance (IR) process to pathogens when elicited by other inducers/elicitors such as β-aminobutyric acid (BABA, a non-proteinic amino acid), jasmonic acid (JA) and its methyl ester (methyljasmonate, MEJA), and extracellular polysaccharides (EPSs). This chapter provides a broad picture on the mechanisms by which AsA interacts with key components of a complex network regulating both basal and induced resistance in different pathosystems.

Keywords Ascorbic acid · Biotic stress · Hormonal-signalling · Redox-related defense responses · AsA-GSH cycle · Basal resistance · Induced resistance

1 Introduction

Plants are exposed to attack by an immense array of pathogen microorganisms (viruses, fungal, bacteria, and insect herbivores). To counteract these invaders and protect themselves, they developed sophisticated strategies and complex molecular

H. Boubakri (✉)
Laboratory of Leguminous, Centre of Biotechnology of Borj-Cédria, Hammam-Lif, Tunisia
e-mail: hatem.boubakri@cbbc.rnrt.tn

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mechanisms (Zipfel 2009; Jones and Dangl 2006). Some of these defense-related traits are expressed by a plant constitutively, regardless of a plant's history of attack by pathogens such as the preformed physical barriers in the leaf surface formed by the cuticle (Zipfel 2009). Besides of these pre-existing defense barriers, plants can activate structural and chemical defense mechanisms after pathogen attacks (Jones and Dangl 2006). In incompatible interactions, conserved microbial structures (pathogen-associated molecular patterns, PAMPs) were recognized by plants via transmembrane receptors (pattern recognition receptors, PRRs), triggering intracellular-defense mechanisms (Jones and Dangl 2006). This process, known as PAMP-triggered immunity (PTI), is manifested by extracellular alkalinization, protein phosphorylation, defense gene upregulation, and the generation of reactive oxygen species (ROS). These immune-defense responses are responsible for limiting pathogen spread (Ryals et al. 1996). In the case of compatible interaction, the control of the adapted pathogens is achieved by multiple applications of chemicals known for their negative effect on human health (Boubakri et al. 2016). Thus, the use of disease resistance inducers to protect plants from pathogens has gained important attention by the scientific community on the aim to develop a new alternative strategy to these chemicals in controlling plant diseases. This strategy relies on the manipulation of the natural host-defense repertoire known as "induced resistance" (IR) (Ryals et al. 1996; Walters et al. 2013). IR is characterized by an increased manifestation of plant innate-defense responses against different pathogens triggered by the application of various external factors (Garcia-Brugger et al. 2006; Pieterse et al. 2013). IR implies a complex signalling network involving salicylic acid (SA) defense-signalling pathways (Pieterse et al. 2013) and jasmonic acid (JA)-dependent and ethylene (ET)-dependent signalling pathways (Van Wees et al. 2008), and requires a functional *NPR1* (Nonexpressor of pathogenesis-related protein 1) regulatory gene for the induction of defense genes (Ryals et al. 1996). After pathogen attacks, plants respond by expressing a wide battery of defense-related genes and that in both basal and induced resistance processes. One of these responses is the oxidative burst, a rapid production of ROS. Although they have antimicrobial activity *per se* and can therefore reduce pathogen growth, they also contributed to the damage of crop plants (Lamb and Dixon 1997). However, hydrogen peroxide (H_2O_2) has been shown to play a central role in the expression of disease resistance in different pathosystems. It serves as a substrate for the oxidative cross-linking of cell wall components, and it plays a key role in defense signalling leading to the induction of PR genes mainly through activation of NPR1 transcription factor (Lamb and Dixon 1997). Plants have evolved efficient antioxidant systems to cope with toxicity of ROS (Khan et al. 2012). This includes lipid-soluble membrane-associated antioxidants, such as α-tocopherol and β-carotene, and water-soluble antioxidants, like glutathione (GSH) and ascorbate (AsA) (Khan et al. 2012). A major H_2O_2 detoxifying system in plant cells is the ascorbate-glutathione (AsA-GSH) cycle involving successive oxidations and reductions of AsA and GSH catalyzed by the enzymes constituting the cycle, namely ascorbate peroxidases (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Pignocchi et al. 2003). In the recycling pathways,

which work to maintain the AsA pool size, the oxidized forms of AsA, monodehydroascorbic acid (MDHA) and dehydroascorbic acid (DHA), are reduced back to AsA by MDHAR and DHAR, respectively (Gallie 2013).

Besides its direct antioxidant role, AsA is involved in ROS sensing and defense response signalling. Several studies described that the levels of AsA increased following pathogen attacks (Tsuda et al. 2005; Fujiwara et al. 2013). AsA accumulation promoted oxalic acid (OA) biosynthesis leading to an accumulation of H_2O_2, which is an important defense signalling molecule in plants (Dias et al. 2011). Such increase of H_2O_2 levels influenced the redox state of the cell environment which induced changes in the conformation of the NPR1 transcription factor leading to PR gene expression (Mou et al. 2003; Pavet et al. 2005). Several lines of evidence suggest that the interaction between ROS, AsA and GSH, mediated by the AsA-GSH cycle, and the link between the antioxidative metabolism and redox-related defense responses have a key role in defense signalling mechanisms during both basal and induced resistance in plants. Even though that changes in AsA contents during plant growth and the role of AsA in abiotic stress conditions have been well documented, its function under biotic constraints has not been fully characterized.

2 Key Insights from Studies of Mutations in AsA Synthesis

To gain insights on the role of AsA in plant defense mechanisms, several mutants with altered levels of AsA have been obtained. Seven ascorbate-deficient *vtc* mutants have been identified and represent four different *VTC* loci (Conklin et al. 2000). *VTC1* encodes GDP-mannose pyrophosphorylase (Conklin et al. 1999) and *VTC4* encodes L-galactose 1-P phosphatase (Conklin et al. 2006), which are both enzymes in the proposed GDP-mannose pathway for AsA biosynthesis (Wheeler et al. 1998). *VTC2* encodes GDP-L-galactose phosphorylase, also a biosynthetic enzyme (Dowdle et al. 2007). The identity and function of *VTC3* are unknown. The AsA contents in the *vtc* mutant lines range from 50% of wild-type levels in *vtc2-3*, *vtc3*, and *vtc4* to 25–30% in *vtc1-1*, *vtc1-2*, *vtc2-1*, and *vtc2-2*. The study of some physiological parameters indicated that *vtc1-1* and *vtc2-1* mutant lines have a slower growth rate (Conklin et al. 2000; Pastori et al. 2003; Pavet et al. 2005), but without any effect on photosynthesis rate (Müller-Moulé et al. 2003) in plants grown under moderate light intensity. Additionally, some changes in the intracellular distribution of antioxidant enzymes associated to AsA have been described, but the overall capacity of the antioxidant system is largely unchanged, except for a marked increase in nonspecific peroxidase activity (Conklin et al. 1999). Unperturbed overall leaf antioxidant capacity is indicated further by leaf H_2O_2 contents in the mutant, which are similar to those in the wild type (Veljovic-Jovanovic et al. 2001). Despite this fact, *vtc1* is smaller than the wild-type plant and shows retarded flowering and accelerated senescence (Veljovic-Jovanovic et al. 2001). Because *vtc1* does not accumulate H_2O_2 and the redox states of leaf antioxidants are not changed (Veljovic-Jovanovic et al. 2001), these phenotypic effects are linked to modified amounts of

vitamin C rather than to a general change in cellular redox balance. Thus, this mutant provides an excellent system for studying the effects of physiologically relevant decreases in AsA level.

Transcriptomic analysis on the *vtc1-1 A. thaliana* mutant line compared with the wild type using microarray technology allowed the identification of 171 genes that were differentially expressed in *vtc1-1*; of these, 12.9% were genes involved in defense mechanisms. In particular, PR proteins showed a relatively high level of induction (Pastori et al. 2003). Abscisic acid (ABA) concentration was 60% higher in vtc1-1 than in the wild type, so ABA signalling may provide a link between ascorbate levels and PR protein transcript levels. Other experiments also suggested that AsA-deficient mutants have increased SA, an increased transcript level of genes encoding PR proteins, peroxidase activity, and accumulation of the phytoalexin camalexin (Barth et al. 2004; Pavet et al. 2005). In addition, *vtc1-1* and *vtc2-1* mutant lines both showed increased resistance to infection by virulent pathogens (Barth et al. 2004; Pavet et al. 2005). In fact, an enhanced resistance to *Pseudomonas syringae pv. maculicola* ES4326 and to the oomycete *Hyaloperonospora parasitica pv.* Noco was noted in both *vtc1-1* and *vtc2-1* mutant lines (Pavet et al. 2005; Barth et al. 2004). However, contrarily to the results obtained by Pastori et al. (2003), Barth et al. (2004) reported that PR proteins were not more highly expressed in *vtc1-1* compared to the wild type, but were induced more strongly by pathogen infection, due to higher levels of SA.

Pavet et al. (2005) concluded that AsA deficiency induced PR gene expression through an enhanced GSH accumulation and higher redox levels (more GSH than GSSG). The authors, however, suggested that H_2O_2-signalling and SA-signalling pathways are not implicated in this process (Fig. 1). More recently, Mukherjee et al. (2010) described that the enhanced resistance to *P. syringae* in the AsA-deficient *A. thaliana vtc1-1* mutant correlates with elevated levels of SA, which induced the expression of *PR* genes via the NPR1 pathway. The authors suggested that AsA deficiency causes constitutive priming via a buildup of H_2O_2 that stimulates SA accumulation, leading to an increased disease resistance (Fig. 1). This supports previous findings describing that AsA deficiency led to the expression of PR genes in a SA-dependent manner and the premature senescence positively contributed to this offered resistance (Kus et al. 2002). On the other hand, Botanga et al. (2012) described that the *Arabidopsis vtc1* and *vtc2* mutants were more sensitive to the pathogenic ascomycete *A. brassicicola*. It seems that the deficiency in AsA could either increase or decrease disease resistance levels depending on the pathogen lifestyle.

3 ROS Neutralization by AsA in Response to Pathogen Attacks

One component of plant defense is the generation of ROS, which are mainly produced by NADPH oxidase, amine oxidase, and peroxidases located mainly extracellularly (Kuzniak 2010). The generation of ROS is associated with the induction of

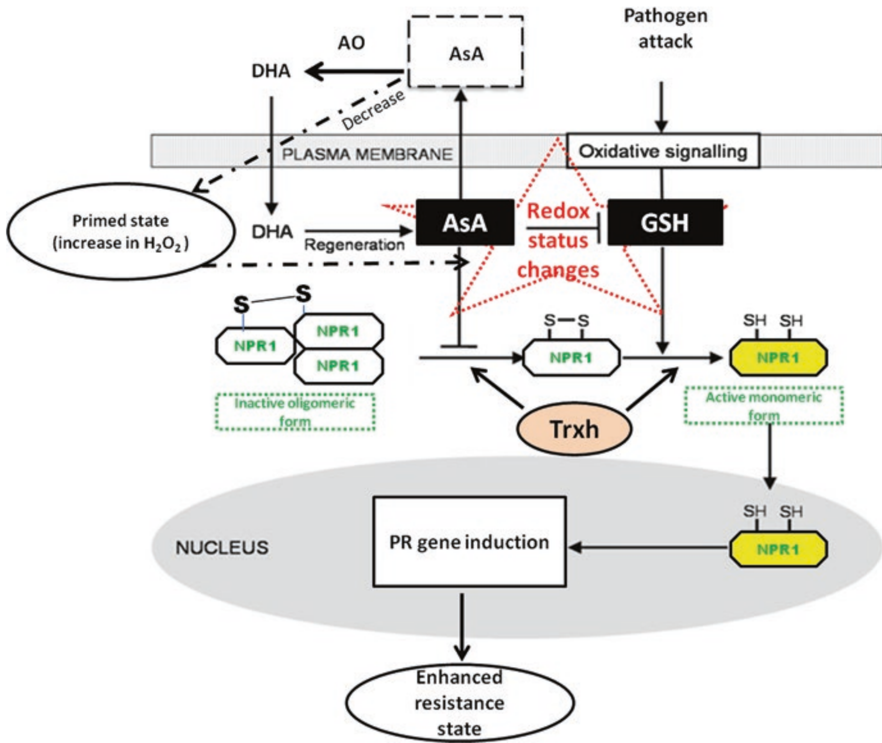

Fig. 1 Key insights from *vtc* mutant *A. thaliana* lines on the role of AsA on defense signalling in response to pathogen attacks. Arabidopsis mutant lines (*vtc*) showed a deficiency in AsA content and increased levels of GSH compared to the wild type (Pastori et al. 2003; Pavet et al. 2005). The apoplastic ascorbate redox state depends on the balance between ascorbate oxidation to DHA by AO and cytosolic regeneration by reduction of DHA by GSH (Pavet et al. 2005; Foyer and Noctor 2005). It was suggested that this increase in GSH content in mutant lines provided an appropriate redox state in the cell environment for activating the NPR1 regulatory transcription (Pastori et al. 2003; Mou et al. 2003). This enables the reduction of cysteine residues in NPR1 by a thioredoxin h-type (Trxh). Thus, NPR1 is reduced from an inactive oligomeric form localized in the cytosol to an active monomeric form through the reduction of intermolecular disulfide bonds (Tada et al. 2008). Monomeric NPR1 is then translocated into the nucleus activating the expression of PR genes, thereby providing an enhanced resistance state to pathogen attacks (Pavet et al. 2005; Tada et al. 2008). It was also suggested that AsA deficiency in *vtc* mutant lines created a priming state characterized by increased levels of H_2O_2 (dashed arrows) which means changes in the redox state of the GSH pool which can easily shift to its oxidized form, GSSG, leading to the activation of NPR1 transcription factor through the SA-signalling pathway (Mukherjee et al. 2010). *AO* ascorbate oxidase, *DHA* dehydroascorbate, *MDHA* monodehydroascorbate, *GSH* glutathione, *GSSG* glutathione disulfide, *AsA* ascorbate, *NPR1* nonexpressor of pathogenesis-related protein 1, *Trxh* thioredoxin h-type, *SA* salicylic acid, H_2O_2 hydrogen peroxide

programmed cell death (PCD), which has been shown to restrict the growth of biotrophic fungal pathogens (Kuzniak 2010). Rapid infection-induced production of ROS in the apoplast could also inhibit pathogen penetration by cell wall lignification, cross-linking of cell wall polymers (Almagro et al. 2009) as well as by the production of antimicrobial phytoalexins (Lamb and Dixon 1997). In addition, ROS have the potential to be directly toxic to pathogens and to induce the expression of defense genes (Desikan et al. 2001). Furthermore, compartmentalization of both ROS production and activation of antioxidants was found to contribute to fine-tuning of ROS levels and their signalling properties (Torres et al. 2006). Although the primary oxidative burst following pathogen recognition occurred in the apoplast, ROS are produced in other organelles with oxidative metabolism components or characterized by rapid electron flux, e.g., chloroplasts, mitochondria, and peroxisomes may also have functions in defense (Kuzniak 2010). Thus, ROS are main players contributing to the inducible defense responses to pathogen attacks; however, their amounts in plant cells are strictly regulated to prevent plant tissue damage by a sophisticated antioxidant system (Desikan et al. 2001; Torres et al. 2006).

In plants, AsA plays an important role on the detoxification of ROS either directly or through the AsA-GSH cycle. The AsA-GSH cycle was found to be functional in different cellular compartments including the apoplast, cytosol, chloroplasts, mitochondria, and peroxisomes (Noctor and Foyer 1998). AsA could be found either in a reduced form (AsA) or in two oxidized forms (MDHA and DHA). A remarkable accumulation of ROS was observed in plant cells after pathogen attack, as well as large amounts of DHA as a result of AsA oxidation. For example, in tomato (*Solanum lycopersicum*) fruits, AsA levels rose ~40% after infection with an attenuated strain of *Cucumber mosaic virus* (Tsuda et al. 2005). In addition, an increase in AsA levels was observed in *Brassica rapa* resistant cultivars after infection with *Turnip mosaic virus (*TuMV) (Fujiwara et al. 2013). However, a susceptible Chinese cabbage cultivar showed a significant decrease in AsA content in the inoculated leaves. These results suggest that AsA accumulation is not induced in the compatible interactions between *B. rapa* and TuMV (Fujiwara et al. 2013). The mechanisms underlying AsA accumulation in response to pathogen infection are still unknown. Although, it has been demonstrated that the AsA level was positively correlated with the extent of viral resistance, negative correlations has also been reported. For example, an AsA deficiency can increase resistance to other pathogens such as *P. syringae* and *P. parasitica* in *vtc* mutant lines of *Arabidopsis* (Barth et al. 2004).

It was suggested that the increase in AsA amounts after pathogen infection shifts the AsA pool towards a more oxidative state (Foyer and Noctor 2005). In this reaction, the reduced form of AsA was oxidized to MDHA. Thereafter, MDHA was either reduced by MDHAR to AsA or, because it is very unstable, reacted to DHA. DHA is reduced by DHAR to AsA. In this reaction, the reduced form of glutathione (GSH) is oxidized to glutathione disulfide (GSSG). GSSG is then reduced by glutathione reductase (GR) to GSH. The electron acceptor NADP is regenerated during the reduction of MDHA and GSSG by the respective enzymes (Noctor and Foyer 1998; Foyer and Noctor 2005). The regeneration of the AsA pool (reduced form) through the different enzymatic recycling pathways (DHAR and MDHAR) is

needed to restore the antioxidative capacity of plant cells. In other terms, the ratio between reduced and oxidized AsA is pivotal for the ability of the plant to fight oxidative stress (Noctor and Foyer 1998; Foyer and Noctor 2005). Additionally, some reports described that both pathogen attacks and treatments with disease resistance inducers can change total AsA contents in plants which makes AsA an important stress marker during biotic stress situations (Khan et al. 2012; Tsuda et al. 2005; Boubakri et al. 2016).

4 Host-Innate Defense Facets Regulated by AsA-Related Redox Status

Important defense facets which are known to be expressed after pathogen attacks could be influenced by AsA-related redox status, including the induction of PR genes after changes in the conformation of the NPR1 regulatory transcription factor (from the S–S form to the SH–SH form), strengthening of the cell wall, and modulation of defense-hormonal signalling pathways.

4.1 NPR1 Transcription Factor Activation

AsA is a main cellular redox buffer and the related redox signalling contributed to the orchestration of plant defense responses (Dias et al. 2011; Khan et al. 2012; Bala and Thukral 2011). In fact, through its interplay with GSH in the AsA-GSH cycle, AsA can modulate the ROS signalling in different cellular compartments and determine its intensity, duration, and localization, i.e., the ROS signature (Mittler et al. 2004), that can determine in turn the type and intensity of the response to a specific pathogen (Kuzniak 2010).

The increase in AsA content following pathogen attacks favored an accumulation of oxalic acid (OA) and, thereby, an accumulation of H_2O_2 because OA is a major contributor in the biosynthesis of H_2O_2. The increase in H_2O_2 amounts influenced the redox status of the GSH pool that can easily shift to its oxidized form, GSSG (Pavet et al. 2005). Knowing that the redox state of GSH is concentration-dependent, changes in the content of GSH even with constants GSH/GSSG ratios would alter the cellular redox state, thereby, activating redox-related defense reactions (Fig. 1). To date, one of the best known examples of how the GSH-related redox signalling pathways may work, inducing alterations in plant genes expression is the activation of NPR1 protein (Mou et al. 2003).

NPR1 is present in the cytosol as an oligomer with subunits aggregated via disulfide bonds (Tada et al. 2008). The TGA1 transcription factor bound to the promoter of SA-responsive gene (TGACG) is not competent to activate defense genes, exemplified as PR1. Mobilization of enzymatic and nonenzymatic antioxidant

mechanisms results in a shift of the cellular redox environment towards reducing conditions. This enables the reduction of cysteine residues in NPR1 and TGA1, by a thioredoxin h-type (Trxh). Thioredoxins are small proteins catalyzing thiol-disulfide interchange in the target proteins. Thus, NPR1 is reduced from an inactive oligomeric complex localized in the cytosol to an active monomeric state through the reduction of intermolecular disulfide bonds (Tada et al. 2008). The reduction of NPR1 preceding defense gene induction requires an increase in GSH content and a concomitant shift in the cellular redox environment towards the reducing conditions (Mou et al. 2003; Fobert and Despres 2005). Monomeric NPR1 is then translocated into the nucleus where it interacts with transcription factors of the TGA class, such as TGA1 and TGA2 (Fig. 1) (Mou et al. 2003; Pieterse and Van Loon 2004).

NPR1 is an intrinsic component of the SA-defense signalling pathway, during both basal and induced resistance in plants. The redox dependence of the NPR1 pathway implied that biotic stimuli that perturb the cellular redox state upregulated defense gene expression via the NPR1 pathway (Mou et al. 2003; Pieterse and Van Loon 2004). Interestingly, the kinetics of the changes in the GSH pool and the GSH/GSSG ratio required for *in vitro* NPR1 reduction were similar to those evoked during IR process (Mou et al. 2003). Collectively, it seems that the AsA and the antioxidative enzymes undergo dynamic changes under biotic stress modulating the redox state of GSH, thereby, providing the appropriate cellular redox environment for the activation of NPR1 transcription factor.

4.2 Cell Wall Strengthening

The products of plant metabolism are separated from pathogen invaders by the cuticle and cell wall. When pathogens attempt to breach these barriers for colonization, plants respond by expressing several wall-associated defense reactions. The most known cell wall-related defense responses include the inhibition of fungal cell wall-degrading enzymes, secretion of fungitoxic peptides and phytoalexins, and cell wall strengthening via a lignification process (Huckelhoven 2007). Cell wall lignification is a complex process occurring exclusively in higher plants; its main function is to strengthen the plant vascular body. This process involved the deposition of phenolic polymers, the so-called lignins, on the extracellular polysaccharidic matrix. These polymers arise from the oxidative coupling of three cinnamyl alcohols in a nonrandom reaction, in which cell wall polysaccharides appear to influence the freedom of cinnamyl alcohol radicals, being a highly orchestrated process (Hagemeier et al. 2001). In basal resistance, lignification makes the cell wall more resistant to mechanical pressure applied during penetration by fungal appressoria (Huckelhoven 2007). Additionally, a lignified cell wall is water resistant and thus less accessible to cell wall-degrading enzymes.

The findings by Takahama and Oniki (1992) indicated that the apoplastic AsA pool had an important role in the lignification process after pathogen perception. These authors reported that AsA in its reduced form negatively regulated the

peroxidase-dependent oxidation of phenolics by the reduction of the oxidized phenolic intermediates. They suggested that the lignification process occurred when the apoplastic AsA pool was largely oxidized. It was shown that in a resistant tomato cultivar the level of reduced AsA in the apoplast decreased significantly after infection with *Botrytis cinerea*, and this response was correlated with high peroxidase activity (Kuzniak 2010). The importance of the cell wall strengthening processes mediated by the apoplastic class III peroxidases for plant resistance to infection has been identified in different pathosystems (Lamb and Dixon 1997; Almagro et al. 2009). Similarly, the ascorbate-deficient (*vtc*) *A. thaliana* mutants with increased resistance to infection by virulent pathogens (Barth et al. 2004; Pavet et al. 2005) exhibited elevated cell wall peroxidase activity (Colvile and Smirnoff 2008). Therefore, AsA was considered as a major redox active compound in the apoplast and it was found to play an important role after pathogen attacks when the invader was perceived firstly by the apoplast. In general, the apoplastic AsA pool accounts between 5 and 10% of the total cellular pool (Noctor et al. 2002; Noctor and Foyer 1998; Veljovic-Jovanovic et al. 2001).

The redox status of the apoplastic AsA pool is regulated by ascorbate oxidase (AO) (Pignocchi et al. 2003; Sanmartin et al. 2003). This enzyme is considered the first step in the AsA degradation pathway in the apoplast (Sanmartin et al. 2003). The presence of AO may also explain why AsA in the apoplast is markedly more oxidized than cytoplasmic AsA (Pignocchi et al. 2003). AO has long been considered to influence cell expansion via the modulation of redox state of the apoplast (Smirnoff and Wheeler 2000), although the exact mechanism is still largely unknown. It has been suggested that the controlled oxidation of apoplastic AsA via AO could have a similar effect on the apoplastic redox state as an oxidative burst (Foyer and Noctor 2005).

On the other hand, several lines of evidence indicated that the concentrations of AsA and GSH and their redox ratios might also have an important role in both apoplastic metabolism and plant defense. Indeed, the apoplastic levels and redox status of AsA and GSH were described to be influenced during both compatible and incompatible interactions of barley with *Botrytis graminis*. Moreover, the activities of several extracellular antioxidative enzymes were increased upon powdery mildew attack (Noctor et al. 2002; Vanacker et al. 1998, 2000). In general, the activities of the antioxidative enzymes in the apoplast are low compared to those recorded in the cytosolic compartment; however, they can significantly affect the cell wall's redox status, thereby influencing the cell wall rigidity, because AsA, H_2O_2, and hydroxyl radicals had an important role in cell expansion (Smirnoff and Wheeler 2000). Clearly, the balance between AsA and H_2O_2 is a determinant factor for the extent of lignification of the cell wall. Indeed, *Arabidopsis* plants over-expressing AO accumulated the oxidized form of AsA in their apoplast and were found to be less responsive to auxin and more susceptible to the virulent *P. syringae* (Huckelhoven 2007). In addition, both an increase in mitogen-activated protein kinase (MAPK) activity and altered gene expression were observed in the lines with oxidized apoplastic AsA. This suggests that the apoplastic redox status could also modulate pathogen responses following a translation into cytoplasmic signalling. In fact,

different reports indicated that direct oxidation of apoplastic AsA can change the outcome of a plant–pathogen interaction via a cytoplasmic response to the apoplastic redox status (Kuzniak 2010). The homeostasis of AsA and GSH in the apoplast is easily perturbed as this compartment lacks a regeneration system to restore the reduced forms. Regarding the low amount of the total AsA and GSH in the apoplast and its low buffering capacity, the specific plasma membrane transport systems, linking the apoplastic AsA and GSH redox couples to their cytoplasmic counterparts, appear to play a key role in maintaining the AsA-GSH cycle-dependent defensive responses of this compartment during the plant–pathogen interaction (Foyer and Noctor 2005; Kuzniak 2010). The regulation of the apoplastic redox status seems to be important at the point of orchestrating key cytoplasmic defense mechanisms by providing optimum levels of ROS. Eventually, ROS are involved in defense gene expression, phytoalexin production, and cell death (Boubakri et al. 2012, 2013a, b).

4.3 Modulation of Defense-Hormonal Signalling Pathways

Plant responses to biotic and abiotic stresses are mediated by various hormones. Hormones function in a complex signalling-network modulating plant defense response to various external factors. Biotic stress signalling in plants involved mainly four hormones which are: SA, JA, Et, and abscisic acid (ABA). SA regulated defense-signalling to many biotrophs, whereas JA and Et are involved in resistance to necrotrophs, although there are many exceptions (Glazebrook 2005). The induction of SA promotes dissociation of NPR1 oligomers into monomers in the cytosol through reduction of disulfide bonds between the NPR1 monomers, which then enter the nucleus (Despres et al. 2003; Mou et al. 2003). Phytohormones were found to act in an antagonistic or synergistic manner, allowing some of them to prevail on others (Suza et al. 2010). In fact, SA, JA, and Et exhibited antagonistic interactions. The activation of the SA-signalling pathway repressed the JA/Et pathway through NPR1 and WRKY70 transcription factors, and the ABA pathway through NPR1 or its downstream elements. The induction of the JA/Et pathway inhibited the expression of certain defense-related genes regulated by the SA-signalling pathway via MAPK4 (Mitogen-Activated Protein Kinase 4) (Glazebrook 2005; Suza et al. 2010).

A unifying feature of the diverse stresses that activate JA signalling is that they all generate ROS accumulation, and JA signaling-deficient mutants exhibited an enhanced susceptibility to oxidative stress (Dombrecht et al. 2007; Suza et al. 2010). In addition, jasmonates are known to induce ROS production when applied exogenously (Zhang and Xing 2008) as well as when produced *de novo* in response to leaf wounding (Suza et al. 2010). Thus, JA likely plays a role in regulating the redox balance of stressed plant tissues. Given that JA and AsA both contribute to plant defenses against oxidative stresses, the potential interaction between these two factors is very possible and merits further investigation. In addition, AsA was reported to play an important role in the biosynthesis of certain plant hormones, including Et

and gibberellic acid (GA), and cell wall glycoproteins and secondary metabolites with antimicrobial properties (Suza et al. 2010). Further, the quantity and redox state of AsA may also influence JA signalling. For example, in tomato, silencing of the terminal enzyme in the Man/Gal pathway resulted in a decrease in the ratio of AsA to DHA and also increased several JA-responsive transcripts such as proteinase inhibitors and arginine decarboxylase (Suza et al. 2010). Pathogen attacks induced changes in the levels of AsA in plants (Sasaki-Sekimoto et al. 2005; Wolucka et al. 2005; Tsuda et al. 2005). For example, Tsuda et al. (2005) reported an accumulation of AsA levels after viral infection in tomato (*Solanum lycopersicum*) fruits. In fact, AsA content rose ~40% after infection with an attenuated strain of *Cucumber mosaic virus* (genus *Cucumovirus*, family *Bromoviridae*, order unassigned). Abscisic acid (ABA) concentration was 60% higher in *vtc1-1* than in the wild type, so ABA signalling may provide a link between AsA levels and PR protein transcript level. Other experiments have shown that AsA-deficient mutants have increased SA content, an increased transcript level of genes encoding PR proteins, peroxidase activity, and accumulation of the phytoalexin camalexin (Pavet et al. 2005).

5 Involvement of AsA in the Establishment of Induced Resistance (IR)

5.1 AsA as a Component of IR Process

Several reports have described the effect of different inducers/elicitors of disease resistance on AsA metabolism in plants. Elicitors are known to induce host-defense responses by mimicking a pathogen attack or other stress, and can be substances of pathogenic origin or compounds released by the plants in response to the action of a pathogen (Boubakri et al. 2013a). Elicitors such as JA and its methyl ester (methyljasmonate, MEJA), SA, BABA (β-aminobutyric acid), and EPSs (extracellular polysaccharides) have been shown to influence AsA metabolism when exogenously applied.

Both JA and MEJA are known to be potent elicitors of innate-defense responses in several plant species (Turner et al. 2002; Almagro et al. 2014). Jasmonates (JAs) application induced a transcriptional reprogramming leading to an enhanced resistance to various pathogens (Almagro et al. 2014; Turner et al. 2002). It was reported that treatment of *N. tabacum* and *A. thaliana* suspension cells with MEJA stimulated the *de novo* biosynthesis of AsA (Wolucka et al. 2005). On the basis of transcript profiling data, it was found that this AsA accumulation in tobacco cells was accompanied with an important induction of *VTC1* gene encoding a GDP-mannose pyrophosphorylase, a key enzyme in AsA biosynthesis pathway (Wolucka et al. 2005). However, it is not yet clear whether induction of AsA by exogenous JAs is a direct JA signalling response that requires all components of JA synthesis and signalling, or is triggered by an effect of JA treatment such as ROS generation, which is caused by but not specific to JA.

SA is known to regulate plant innate-defense responses to several pathogens, mainly the biotrophic fungi, through NPR1 pathway and to induce defense responses and resistance to various pathogens when exogenously applied through eliciting the SAR-related molecular mechanisms (Sudhamony et al. 2009). It was reported that SA treatment induced AsA accumulation in *A. thaliana*; however, the molecular mechanisms underlying such event are still unknown (Suza et al. 2010).

In addition, BABA, a non-proteinic amino acid known to be the most efficient priming agent of SAR in plants, induced *VTC1* expression in *Arabidopsis*, upon infection with *Plectosphaerella cucumerina*, while *APX1* transcription was inhibited (Pastor et al. 2013). This might lead to a more oxidized environment in the cell and allow increased ROS accumulation. Additionally, in BABA-treated plants, the level of mRNA transcripts of *VTC1* gene was enhanced, but not that of *GSH1* gene (Pastor et al.2013). This transcriptomic regulation would lead to a more oxidized state in the cytoplasm by the augmented H_2O_2 accumulation as suggested by the lower levels of GSH/GSSG in BABA-treated plants following *P. cucumerina* inoculation (Pastor et al. 2013). As mentioned above, the cytosolic thiol-disulfide status plays an important role in regulating PR gene expression through NPR1 transcription factor which might affect the outcome of the plant–pathogen interaction (Mou et al. 2003).

The EPSs (extracellular polysaccharides) that modulate the activity of PAL (phenylalanine ammonia-lyase), which is the first enzyme in the phenylpropanoid pathway, also induced AsA accumulation (Mittler 2002). In addition, these phytopathogenic molecules decreased the levels of cytosolic APX which is known to detoxify ROS using AsA as substrate. However, the amount of DHA increased after treatments of cells with the active EPSs. This suggests that an increasing amount of ASC has been used by different metabolic pathways (Mittler 2002). As the activity of APX was decreased by EPSs, the level of ROS increased and thereby an increase in DHA amount. The changes in the redox balance of the ASC/DHA pair and in the activities of the AsA-redox enzymes specifically induced by these phytopathogenic molecules suggest an involvement of these molecules in plant–pathogen interactions. Further, cell death is known as an important event during both incompatible plant–microbe interactions and IR process. It is initiated by the interaction between the *R* gene product and the *Avr* gene product and leads to the restriction of pathogen spreading (Boubakri et al. 2016). The establishment of cell death in EPS-treated cells was preceded by changes in the cytosolic APX activity and a decrease in the total AsA pool (AsA + DHA), as well as a remarkable shift of this redox pairs towards the oxidized form (de Pinto et al. 2002). Overall, these findings suggest an important role for AsA during the establishment of IR by elicitors from different origins.

5.2 AsA as an Inducer of Disease Resistance

Besides its role as a component of IR process, AsA was found to act as an inducer of disease resistance in some pathosystems (Egan et al. 2007; Fujiwara et al. 2013). *Turnip mosaic virus* (TuMV) is known to infect a wide range of hosts including

economically important *Brassica* species (Walsh and Jenner 2002). Recently, it was reported that the exogenous application of DHA and AS derivatives l-(+)-ascorbic acid 2-sulfate disodium salt dehydrate (AS-SO_4) and fat-soluble ascorbyl palmitate (AS-Pal) induced resistance to *TuMV* in turnip (*B. rapa* subsp. *rapa*) plants (Fujiwara et al. 2013). Additionally, AsA was shown to be active not only against viruses but also against other pathogens. For instance, treatment of the rice blast fungus *Magnaporthe oryzae* with AsA decreased the percentage of normal appressorium formation (Egan et al. 2007). Further, the simultaneous application of JA and AsA enhanced the accumulation of sakuranetin, a phytoalexin identified from blast resistance rice cultivars, compared to JA alone (Tamogami et al. 1997). Li et al. (2016) reported that the exogenous application of AsA at 600 μM induced resistance in citrus plants against Huanglongbing (HLB), the most devastating disease worldwide under greenhouse and field conditions. Besides, AsA-IR to HLB was accompanied with enhanced traits of the citrus fruit (yield and quality) under field conditions (Li et al. 2016). On the other hand, AsA might possess direct antifungal activities; for example, Botanga et al. (2012) described that AsA application directly affected the hyphal development of *Alternaria brassicicola*. Overall, these findings indicated that AsA had both direct and indirect effects in plant responses to pathogens.

6 Conclusion

Besides its role in ROS neutralization following pathogen attacks, AsA was found to regulate other important host-defense reactions. In basal resistance, based on transcriptomic and genetic approaches, AsA was found to act in coordination with GSH in the AsA-GSH cycle and with the respective antioxidative enzymes providing the appropriate redox environment for the activation of plant defense responses. The modulation of AsA amounts in different cell compartments after pathogen infection influences the redox state of the cell environment, leading to: the expression of various defense responses such as the induction of PR genes through the NPR1 pathway, cell wall strengthening, and the modulation of defense-hormonal signalling pathways. In addition, AsA was shown to be an important component of IR process when elicited by different inducers. On the other hand, AsA was found to act as an inducer of disease resistance mechanisms in various pathosystems. Nevertheless, and contrarily to its role in abiotic stress which is largely detailed and understood, knowledge on the role of AsA in biotic stress is rudimentary. Thus, further studies on how the AsA works in the AsA-GSH cycle to influence and interact with other important components of the innate defense system based on AsA-related redox changes are of great interest.

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Ascorbate Oxidase in Plant Growth, Development, and Stress Tolerance

Rebecca Stevens, Vincent Truffault, Pierre Baldet, and Hélène Gautier

Abstract Ascorbate oxidase (AO) is a member of the multi-copper oxidase family of enzymes and converts ascorbate to monodehydroascorbate at the same time as reducing oxygen to water. The enzyme is present in the apoplastic space of plant cells and seems to be particularly highly expressed in roots and fruits of the *Cucurbitaceae* family. Cell expansion and division seem to be affected by AO activity and at the whole-plant level resource allocation and yield. The enzyme is suggested to play a role in signaling between the external environment and the cell, and AO gene expression responds to wounding, plant hormones, and stress. The enzyme appears to function in plant growth and development and multiple links have been found with tolerance to abiotic and biotic stress, mostly through the use of transgenic plants. This chapter will discuss the roles of AO in plant growth, development, and stress tolerance that current research has highlighted and will also examine further roles the enzyme could play at a cellular level.

Keywords Monodehydroascorbate · Multi-copper oxidase · Growth · Oxygen sensing · Stress perception

1 Introduction

Ascorbate oxidase (AO) is a member of the multi-copper oxidase family, which includes laccases and ceruloplasmin, its distinguishing feature being its specificity for ascorbate as an electron donor (Messerschmidt 1997). Multi-copper oxidases

R. Stevens (✉)
Genetics and Breeding of Fruit and Vegetable Research Unit, INRA, Avignon, France
e-mail: rebecca.stevens@inra.fr

V. Truffault
Institute of Applied Research on Fruit and Vegetables, CTIFL, Nantes, France

P. Baldet
Fruit Biology and Pathology Research Unit, INRA, Bordeaux, France

H. Gautier
Plants and Cropping Systems in Horticulture Research Unit, INRA, Avignon, France

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are enzymes that have optimized rates for electron transfer, via copper ions, from substrates towards dioxygen and are thus terminal oxidases (forming water or hydrogen peroxide from oxygen), oxygenases, or enzymes involved in superoxide degradation or oxygen transport.

Ascorbate oxidase was first identified as a thermolabile catalyst for the oxidation of hexuronic acid (ascorbic acid) in minced cabbage leaves by Szent Györgyi, a discovery dating back to 1931 (Szent-Györgyi 1931). The activity was labelled hexoxidase and was shown to be specific to the presence of ascorbate and to consume oxygen. Between 1930 and 1970, the biochemical properties of the enzyme gained attention and were the subject of several publications demonstrating that AO activity was present in different (plant) species and tissues, for example Edelman and Hall showed that AO activity developed in washed aerated disks of *Helianthus tuberosus* and that this was not due to protein synthesis (Edelman and Hall 1965). Other investigations revealed that far red light increased AO enzyme activity and synthesis in mustard, *Sinapis alba* L. (Attridge 1974; Hayashi and Morohashi 1993; Newbury and Smith 1981). The body of work over several decades made the enzyme the subject of an annual review in 1975 (Dawson et al. 1975). Much of the early biochemical work on this enzyme is covered in detail in an excellent book chapter in a book entirely dedicated to multi-copper oxidases to which the reader is also referred (Messerschmidt 1997).

The early biochemical interest over the enzyme and its high concentration in, notably, species of the *Cucurbitaceae* family were strong elements favoring the resolution of the crystal structure of the enzyme, which was obtained in the 1970s–1980s (Ladenstein et al. 1979; Messerschmidt et al. 1988, 1989, 1992). The first cDNAs were then cloned from cucumber (Ohkawa et al. 1989) and pumpkin in 1990 (Esaka et al. 1990). Since this period, and this is the major focus of this chapter, efforts have been orientated towards the functional study of the enzyme in higher plants in order to establish its exact function(s).

2 Ascorbate Oxidase Structure

A preliminary crystal structure for AO from green zucchini squash was published in 1979 (Ladenstein et al. 1979). The primary structure of AO was deduced from the cDNA sequence of cucumber (*Cucumis sativus*) in 1989 (Ohkawa et al. 1989) and was found to have high homology with blue copper proteins. A refined crystal structure at 1.9 Å resolution of the enzyme from zucchini was then published in 1992 (Messerschmidt et al. 1992) (Fig. 1). Ascorbate oxidase is a dimeric enzyme of 140,000 $M_{r,}$ which can dissociate into monomers, particularly at neutral to alkaline pH (Avigliano et al. 1983; Di Venere et al. 2011). Each monomer subunit is formed of three distinct domains: it is hypothesized that the enzyme evolved from a type B two-domain protein by duplication of one of the domains: analysis of genome sequences has concluded this is probably the case (Messerschmidt et al. 1993; Nakamura et al. 2003). Each monomeric subunit contains four copper ions, three of which form a trinuclear center near the interface between domains. The trinuclear

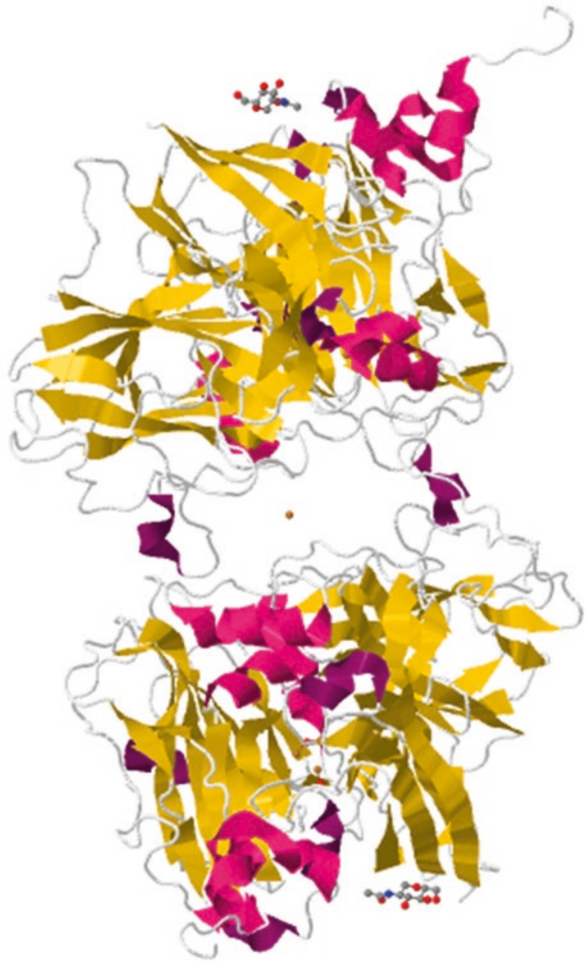

Fig. 1 Refined crystal structure of ascorbate oxidase at 1.9 Å resolution: Protein Data Bank code 1aoz. http://www.rcsb.org/pdb/explore/jmol.do?structureId=1AOZ&bionumber=1

copper center binds oxygen and facilitates electron transfer from the substrate to oxygen first from the type I copper to the type 3 copper (II) pair, the rate of transfer being increased in the presence of oxygen (Farver and Pecht 1992; Farver et al. 1994). More recent work has shown that ascorbate binds in a pocket near the type I copper and its binding is stabilized by several hydrogen bond interactions with protein residues, one of which is a histidine (Santagostini et al. 2004).

The copper present in the protein appears to affect protein stability but not conformation (Savini et al. 1990) and maybe also synthesis (Esaka et al. 1988). Certain studies have shown that copper free AO is more sensitive to photo-induced damage (Maccarrone et al. 1993) or that increases in AO protein are seen by adding copper (Esaka et al. 1988). There is also some evidence that the enzyme is activated by its substrate, ascorbate, in a way that is consistent with the hypothesis of generation of extra active sites (Gerwin et al. 1974).

3 Enzyme Mechanism and Substrate Specificity

The solving of the crystal structure of AO has also allowed the catalytic mechanism to be elucidated (Messerschmidt et al. 1992): the enzyme catalyzes the four electron reduction of oxygen by the one electron oxidation of the substrate (ascorbate) via the type 1 copper. The ascorbate is converted to monodehydroascorbate which then dismutates to dehydroascorbate in solution (Yamazaki and Piette 1961). The reaction therefore does not produce reactive oxygen species or hydrogen peroxide as side-products. One water molecule is released fast and the second more slowly (Messerschmidt et al. 1992). The intramolecular transfer required is suggested to be the rate-determining step but the activation barriers to this transfer are low (Farver and Pecht 1992). Other authors suggested that this intramolecular electron transfer was improved in the presence of oxygen (Farver et al. 1994).

There are indications that reaction with other substrates, i.e., electron donors, is possible: for example, AO has been shown to have catechol oxidase activity (Marchesini et al. 1977) and could oxidize 2,6- and 2,5-dichlorohydroquinone and hydroxyhydroquinone (Dayan and Dawson 1976). Several phenolic compounds could also act as competitive inhibitors binding at or near the site of ascorbate binding (Gaspard et al. 1997).

4 Posttranscriptional Modifications of Ascorbate Oxidase

Ascorbate oxidase from zucchini (*Cucurbita pepo*) has been shown to possess an N-glycan moiety described to be a core-pentasaccharide with a xylose (Altmann 1998; D'Andrea et al. 1988). Ascorbate oxidase is therefore a glycoprotein, glycan moieties may have regulatory activity on the catalytic role of the enzyme, loss of sugars—deglycation (or nonenzymatic deglycosylation)—seemed to improve catalytic efficiency, maybe because of improved exposure of the catalytic sites upon exoglycosidase treatment (D'Andrea et al. 1989). However, enzymatic deglycosylation of purified AO from *Cucurbita pepo medullosa* did not lead to loss of activity compared to the non-glycosylated form of the enzyme (D'Andrea et al. 1993).

5 Ascorbate Oxidase Is Present in Higher and Lower Plants and Fungi

Ascorbate oxidase was first discovered in cabbage (Szent-Györgyi 1931). The enzyme is highly active in *Cucurbitaceae* species which explains why the enzyme has been purified and crystallized from plants such as cucumber and zucchini (see section above). Activity has also been detected in the cytosol, cell wall, and chloroplasts of the filamentous chlorophyte *Chaetomorpha linum*, a green macroalga (Caputo et al. 2010). The enzyme is also present in several fungi such as *Pleurotus*

ostreatus, where purification of a cytosolic AO has shown it to be a hemoprotein similar to b-cytochrome (Kim et al. 1996), and *Acremonium* sp. HI-25 (Hirose et al. 1994). The latter study has shown the fungal ascorbate oxidase to contain four copper atoms and to have equivalent catalytic activity (Km and kcat) towards ascorbic acid as the monomeric cucumber ascorbate oxidase.

The availability of genomic data has allowed the cataloging of genes from numerous plant species. Recent results have revealed three to seven AO genes in *Oryza sativa*, Arabidopsis, *Glycine max*, *Zea mays*, and *Sorghum bicolor,* which were differentially regulated by stress and throughout development (Batth et al. 2017). This size gene family seemed to be typical of other species as a small gene family exists in melon where two of the four identified genes showed detectable expression (Diallinas et al. 1997). A single gene was detected in pepper which was verified by Southern blot (Garcia-Pineda et al. 2004) and two genes have been detected and mapped in tomato (Stevens et al. 2007; Zou et al. 2006).

Putative AO sequences have also been found in lower plants including the lycophyte *Selaginella moellendorffii*, the moss *Physcomitrella patens,* and the green algae *Chlorella* and *Chlamydomonas* (De Tullio et al. 2013). A phylogenetic tree representing a number of AO sequences from higher and lower plants is presented in Fig. 2.

Interestingly, no AO activity was detectable in the parasitic plant *Cuscuta reflexa* Roxb, despite its ability to synthesize ascorbate from the L-galactono-1,4-lactone precursor (Tommasi et al. 1990), indicating that this enzyme might not be essential, or at least its functions can be fulfilled in alternative ways. This parasitic non-photosynthetic plant also had only very low monodehydroascorbate reductase activity and high levels of dehydroascorbate versus ascorbate compared to most photosynthetic plants. Genomic analyses are useful, when available, in confirming the results of previous biochemical observations on the presence/absence of AO activity in different organisms.

6 Tissue Specificity and Cellular Compartmentation

Ascorbate oxidase activity is very high in cucumber and squash mesocarp and also roots and stems of pepper (Garcia-Pineda et al. 2004). In general, transcript levels tend to be high in young fruits and roots, particularly the quiescent center and differentiating vascular tissues (Ioannidi et al. 2009; Liso et al. 2004). In melon, two genes have been identified as well-expressed, one in petals and ovules and the second more highly in roots, stems, leaves, shoot apices, and ovules (Diallinas et al. 1997; Sanmartin et al. 2007). Seeds have apparently no AO activity (Arrigoni et al. 1992).

In zucchini squash (*Cucurbita pepo* L.): AO protein and mRNA followed a similar pattern in that they were high 4–6 days after anthesis corresponding to the phase of expansion in fruit, and also in the growing region of young leaves (Lin and Varner 1991). High activity was found in the epidermis of developing fruit and the lowest activity in seeds.

Fig. 2 Phylogenetic tree of plant ascorbate oxidases taken from de Tullio (2013). Tree based on amino acid sequences for representative AO sequences. The sequences were aligned using Muscle (http://www.ebi.ac.uk/Tools/msa/muscle/) and the alignment tails were trimmed. The phylogenetic tree was constructed with the maximum likelihood method, using the MEGA software, Version 5.0.30. Bootstrap analyses were conducted on the basis of 1000 re-samplings of the sequence alignment. A fungal AO sequence has been used as an outgroup (Permission received from Taylor & Francis 16/08/2017)

A number of studies have confirmed the apoplastic location of the AO protein, either by use of immunological techniques, where it has been detected in cell walls and vacuoles, or by biochemical ones (Liso et al. 2004; Nanasato et al. 2005). In transgenic tobacco under-expressing AO, the apoplastic redox state was more reduced than in wild-type plants, at least in young fully expanded leaves (Pignocchi et al. 2003) and inversely overexpression of cucumber AO in tobacco reduced the redox state of the apoplast to 3%. Outside the plant field, there is evidence for AO activity localized in the endoplasmic reticulum of rat liver cells (Szarka et al. 2002).

7 Ascorbate Oxidase Gene Regulation: Clues to Function?

A number of publications have looked at the induction of AO transcripts under different conditions; fewer have studied the correlations with enzyme activity. Ascorbate oxidase gene expression was induced by light (Nanasato et al. 2005; Pignocchi et al. 2003), auxin (Esaka et al. 1992; Pignocchi et al. 2003), and sometimes by other hormones such as jasmonic acid (upregulation (Sanmartin et al. 2007)) but downregulated by salicylic acid (Pignocchi et al. 2003). The response to hormones is complex and probably species-dependent (Suza et al. 2010). The gene was also induced under situations of oxidative stress such as heat (Sanmartin et al. 2007). For wounding, the situation is not always clear as the gene has been shown to be induced (Sanmartin et al. 2007) and repressed by wounding (Diallinas et al. 1997) or induced transiently (Garcia-Pineda et al. 2004). Ascorbate oxidase is also induced during nodulation (Balestrini et al. 2012) and has been identified as responding to *Verticillium dahliae* in sunflower (Guo et al. 2017). It is noteworthy that the genes for different isoforms are induced, or repressed, in different ways in response to the same stress (Batth et al. 2017). Interestingly, a novel zinc-finger DNA binding protein has been identified that binds to a region of the AO gene promoter in pumpkin (Kisu et al. 1998).

One of the few studies that looks at both mRNA and protein activity was published in 1992: the authors showed that in pumpkin AO activity increased during seedling growth, in expanding fruit and following addition of auxin and copper. The mRNA tended to parallel changes in activity, except for the addition of copper which did not affect mRNA accumulation but stimulated enzyme activity. Ascorbate oxidase activity has also been shown to be very high in squash fruit mesocarp and roots more than other tissues and transiently induced by ascorbate (after 1 h) (Garcia-Pineda et al. 2004). Ascorbate oxidase activity has been shown to be proportional to light intensity in *Cucurbita pepo* and increased with additional oxygen in darkness (De Tullio et al. 2007).

8 Ascorbate Oxidase and Auxin

A number of links have been made between AO and auxin metabolism. Auxin (indole-3-acetic acid, IAA) is the major hormone driving plant growth and morphogenesis. Auxin availability within plant cells depends on its (a) biosynthesis, (b) conjugation, (c) degradation, and (d) transport. (a) Biosynthesis of auxin occurs through two major routes (Ljung et al. 2001): one includes tryptophan-dependent pathways, whereas the other one is independent of the amino acid tryptophan (Woodward and Bartel 2005). Biosynthesis has been reported in leaves, cotyledons, and roots. Young leaves show the highest biosynthetic capacity, whereas no biosynthesis has been observed in fruit. (b) Auxin can be found either in an active (free auxin) or inactive form (represents the vast majority of auxin reported in plants)

within the cell. To permanently inactivate auxin, or for storage purposes, conjugation to both high and low molecular-weight compounds occurs, including conjugation with carbohydrates (glucose, myo-inositol), amino acids, peptides, and proteins (Korasick et al. 2013; Ludwig-Muller 2011). Conjugation of auxin is considered to be the first step towards the irreversible catabolism of auxin, the first precursor is presumably oxIAA, through oxidative decarboxylation of auxin (Woodward and Bartel 2005). (c) Degradation of auxin leads to the release of the auxin transcription factors (ARFs) which are involved in the induction of auxin-responsive genes (Calderon-Villalobos et al. 2010) and consequently to elevation of auxin content in cells through de novo synthesis or release of auxin from conjugated sugars to maintain the cellular growth rate. (d) Transport of auxin is complex and highly regulated (Woodward and Bartel 2005). Transport of auxin from biosynthesis sites (mainly young leaves) to distant sites (fruits for example) is clearly required for normal development (Peer et al. 2011).

The redox state of the thioredoxin and glutathione pools is involved in the regulation of auxin metabolism (mainly signaling, transport, and homeostasis) in plants (Bashandy et al. 2011). Glutathione and ascorbate are of course closely related through the ascorbate-glutathione cycle (Foyer and Noctor 2011). Due to its relatively high abundance in plant cells, the ascorbate redox status influences the redox status of the cell, and is a major contributor to the apoplastic redox status due to the absence of glutathione and NAD(P)H in this compartment (Foyer and Noctor 2005). The equilibrium between oxidative species and antioxidants is a strong signal regulating antioxidant metabolism but also auxin metabolism (Bartoli et al. 2013; Tognetti et al. 2012).

Thus, many direct links between AO and auxin metabolism have been found. The AO promoter contains an auxin-responsive element, as reported in pumpkin (Kisu et al. 1997) where a cis-acting region responsible for auxin regulation has been highlighted. Promoter analysis in *Gossypium hirsutum* (cotton) showed that part of the AO promoter is active in leaf epidermal hairs and roots and is responsive to auxin (Xin et al. 2016). In parallel, AO gene expression has been shown to be induced by auxin treatment (Newcomb 1951). Ascorbate oxidase transcripts increased in the presence of auxin as mentioned in zucchini squash (Lin and Varner 1991), pumpkin (Esaka et al. 1992), tobacco (Pignocchi et al. 2003), and cotton (Xin et al. 2016). Auxin-responsive gene expression also increased in AO RNAi lines in tomato (Garchery et al. 2013) showing a feedback control may exist.

Increased AO activity is correlated with the oxidative decarboxylation of auxin (i.e., auxin catabolism), suggesting a role for AO in the regulation of auxin (Kerk et al. 2000; Ostin et al. 1998), and thus highlighting a regulatory loop between AO and auxin, probably via the auxin-responsive element. It has been shown that an auxin-binding protein (ABP1) located in the apoplast was crucial for auxin-mediated responses, and that auxin insensitivity occurred in the presence of an excess of oxidized ascorbate in the apoplast (Pignocchi and Foyer 2003) or after enhancement of AO activity in transgenic plants (Pignocchi et al. 2003, 2006; Xin et al. 2016). The suppression of auxin responsiveness leads to a decreased cellular growth rate. As firstly reported by Newcomb (1951), and

widely highlighted afterwards (Dumville and Fry 2003; Esaka et al. 1992; Ioannidi et al. 2009; Joo et al. 2001; Kato and Esaka 1999; Lin and Varner 1991; Muller et al. 2009; Sanmartin et al. 2003), high AO activity is often found in actively growing tissues, underlying possible links between auxin metabolism AO activity, cell division, and cell expansion.

9 Cell Division and Cell Expansion

Plant growth is dependent on cell division and cell expansion. A large number of studies have mentioned that ascorbate metabolism is closely linked to these processes. Firstly, ascorbate stimulates mitosis via the G1 to S phase transition (de Pinto et al. 1999; Liso et al. 1984, 1988), unlike dehydroascorbate which is an inhibitor of the G1 phase (de Pinto et al. 1999; Potters et al. 2004). Ascorbate has been shown to stimulate proliferation of competent cells but cannot induce competence in arrested cells (Citterio et al. 1994). The ascorbate/dehydroascorbate ratio could be a signal controlling the transition from cell division to cell expansion and AO could therefore be involved in this transition.

Plant cells expand in three steps (Cosgrove 1993): firstly by an influx of water across the plasma membrane through osmotic uptake related to the gradient in water potential. Consequently, the turgor pressure rises due to the rigidity of the cell wall (second step). Finally, biochemical wall loosening occurs, allowing the cell to expand in response to turgor pressure. The cell wall surrounding plant cells is physically strong and needs to be loosened before cell elongation can occur. Auxin plays a role in increasing the cell wall extensibility (Masuda 1990). A major contribution to the extensibility of the cell wall is ensured by the production of reactive oxygen species in the apoplast: the hydroxyl radical is able to cleave polysaccharides in the cell wall (Sharova and Medvedev 2017). Interestingly, auxin promotes the generation of hydroxyl radicals in the apoplast as mentioned in maize and in association with growth by elongation (Schopfer et al. 2001). Ascorbate is also considered to act as a pro-oxidant in the apoplast (Fry 1998): it has been proposed that nonenzymatic reductions of O_2 into H_2O_2, and Cu^{2+} into Cu^+, occur in the presence of ascorbate and its oxidative and degradation products (notably oxalate) in the apoplast. In a second step, H_2O_2 and Cu^+ react to form hydroxyl radicals, which are proved to induce scission of plant cell wall polysaccharides (Airianah et al. 2016; Fry 1998). Therefore, cell wall extensibility also depends on the ascorbate concentration in the apoplast and its redox status.

More generally, it has been shown that plants with altered ascorbate metabolism, i.e., ascorbate biosynthesis and/or recycling, showed no development beyond the seedling stage (Dowdle et al. 2007) or had altered growth (Garchery et al. 2013; Truffault et al. 2016), highlighting the key role of ascorbate, and its redox state, in plant growth and development processes (Gallie 2013). However, we also note that an Arabidopsis AO mutant with 20% of wild-type activity has no obvious growth phenotype under normal conditions (Yamamoto et al. 2005).

Ascorbate oxidase has been identified as a transmembrane protein (Li et al. 2016). Its product, the monodehydroascorbate radical, resulting from the loss of a single electron, is correlated with stimulation of cell expansion (González-Reyes et al. 1995; Hidalgo et al. 1989) and monodehydroascorbate is involved in cell wall loosening (Schopfer et al. 2002) and vacuole enlargement (Cordoba and Gonzalez-Reyes 1994). Cytochrome b561, localized in cell walls, has also been shown to have MDHAR activity and can reduce the MDHA radical (Asard et al. 2013; Horemans et al. 1994, 2000). This reduction leads to transport of electrons across the cell wall and thus to membrane depolarization (Cordoba and Gonzalez-Reyes 1994; Kato and Esaka 1999, 2000). The activation of membrane proton pumps allowed cell wall expansion through ion uptake which increases osmotic pressure (Kato and Esaka 2000).

Ascorbate oxidation is the first step in the catabolism of ascorbate. Dehydroascorbic acid is formed by disproportionation of monodehydroascorbate and is correlated with inhibition of cell expansion processes in onion (González-Reyes et al. 1995). Overexpression of the cotton AO gene (regulated by auxin-mediated signals) in tobacco cells led to significant accumulation of dehydroascorbate and H_2O_2 in the apoplast, resulting in apoplast oxidation (Li et al. 2017). The authors also revealed enhancement of cell growth which may be linked to increasing Ca^{2+} channel activity in the plasma membrane following apoplast oxidation, with the proviso that only one transgenic line was used (Li et al. 2017).

Dehydroascorbate can be reduced into ascorbate by dehydroascorbic acid reductase (DHAR), or further enzymatically oxidized or nonenzymatically hydrolyzed in irreversible degradation processes previously described (Green and Fry 2005). Oxidation of dehydroascorbate leads to oxalate, threonic acid, and oxalyl-L-threonates (Parsons et al. 2011) which are proposed to be end-products of ascorbate degradation in tomato (Truffault et al. 2017), and spinach (Dewhirst et al. 2017). The role of some of these compounds in cell growth is of particular interest, notably for oxalate. Oxalate could remove calcium ions from the cell wall by formation of calcium oxalate crystals, thus reducing cross-linking between cell wall polysaccharides and increasing cell wall loosening (Nakata 2012). Ascorbate degradation is supposed to be the primary source of oxalate required for the formation of calcium oxalate crystals in many species (Franceschi and Nakata 2005; Loewus 1999), with the exception of rice (Yu et al. 2010) and probably a few other species. Also, in some species (mainly monocots), oxalate oxidase, which induces H_2O_2 accumulation, is linked to cell growth, and interestingly oxalate oxidase is activated by auxin-mediated acidification of the apoplast (Loewus 1999; Pignocchi and Foyer 2003).

When oxidation is minimized, dehydroascorbate degradation occurs via hydrolysis into 2,3 diketogulonate (DKG). DKG can be rearranged to form lactones identified as carboxypentonates, which can themselves be de-lactonized but are otherwise stable in vivo (Parsons et al. 2011). DKG can itself also be oxidized (Parsons and Fry 2012). Thus, DKG generates several by-products, notably H_2O_2 and consequently hydroxyl radicals in the apoplast (Green and Fry 2005). Recently, Karkonen et al. (2017) proposed that some by-products of DKG could delay cell wall cross-linking which leads to cell wall loosening.

The biochemical era and cloning of AO cDNAs was followed by use of transgenic technology to study this enzyme. In many cases, adjustment of AO activity has revealed functions in different plant developmental and physiological processes and of course in response to stress. The results from these studies are summarized in Table 1 and in the subsections below.

10 Plant Physiological Changes: Stomata, Flowering Time, Senescence

Several investigators observed changes in stomatal conductance when AO levels were manipulated (Fotopoulos et al. 2008; Garchery et al. 2013). Stomatal closure is promoted by hydrogen peroxide which supposedly has a signaling role in this process: in dehydroascorbate reductase overexpressing plants, stomatal opening is increased as the increased scavenging by recycled ascorbate decreases hydrogen peroxide levels leading to stomatal opening (Chen and Gallie 2004). In the same way, stomatal opening is negatively correlated to AO activity and was reduced in overexpressing tobacco lines (Table 1) (Fotopoulos et al. 2008) and increased in RNAi tomato lines (Table 1) (Garchery et al. 2013). Similar phenotypic "inversions" have been seen for flowering time where delayed flowering has been observed in an Arabidopsis mutant and tobacco antisense lines (Yamamoto et al. 2005), whereas overexpression of AO in tomato causes earlier flowering (R Stevens unpublished results). Ascorbate oxidase appears to play a role in limiting the progression of dark-induced senescence: after 6 days of dark, tobacco plants overexpressing cucumber AO showed lower dark-induced senescence, as defined by chlorophyll content, compared to controls. Whereas under normal conditions the transgenic plants had higher levels of hydrogen peroxide, the delayed dark-induced senescence correlated with lower hydrogen peroxide and an induction of antioxidant enzymes (Table 1) (Fotopoulos and Kanellis 2013). Other cases where repression of AO activity improved stress tolerance are included in the following section on stress.

11 Tolerance to Abiotic and Biotic Stresses

Many studies have evoked the roles of AO in environmental perception or signaling of stress responses. These hypotheses are largely based on studies subjecting transgenic plants to different types of stress and analyzing their physiological, genetic, and metabolic responses. These responses have been shown in many cases to be multiple and the pleiotropic nature of the AO phenotypes is evidence for the enzyme's probable role as a hub for numerous cellular processes linked to the environment. In general, as shown in Table 1, silencing of AO leads to improved tolerance to stress or sub-optimal environmental conditions: for example, Garchery et al.

Table 1 Summary of transgenic plant-based experiments to analyze the role of AO in plant physiology

Species	Modification of expression	Stress tested	Major observations	Reference
Nicotiana tabacum	Overexpression (pumpkin AO) and antisense tobacco	High light	Higher photosynthesis in antisense lines. Lower threonate in antisense lines, higher in sense lines	Karpinska et al. (2017)
Arabidopsis and tobacco	Overexpression in *Arabidopsis thaliana*, silencing in tobacco	Viral infection	No effect from overexpression. Silenced tobacco showed decreased CMV accumulation 5 days post inoculation	Kumari et al. (2016)
Nicotiana tabacum	Overexpression	Dark-induced senescence	AO overexpressing lines showed decreased loss of chlorophyll and cell necrosis and increased GR and APX activity following 6-day dark treatment of leaf discs	Fotopoulos and Kanellis (2013)
Cherry tomato (*Solanum lycopersicum*)	RNAi	Reduced water, leaf removal	Improved yield of RNAi lines under drought, leaf removal. RNAi lines had increased stomatal conductance and increased sugars. Increased size and growth rate of young fruits	Garchery et al. (2013)
Tomato	RNAi	Drought	Improved tolerance to drought	Zhang et al. (2011)
Populus tremula × *Populus alba*; Salicaceae	Overexpression		AO levels were unlikely to perturb herbivores such as *Lymantria dispar* L. (caterpillars) or *Melanoplus sanguinipes* (grasshoppers)	Barbehenn et al. (2008)
Nicotiana tabacum	Overexpression		Partial stomatal closure led to decreased stomatal conductance in sense lines. Plants had more hydrogen peroxide and more ABA	Fotopoulos et al. (2008)
Nicotiana tabacum	Overexpression	*Botrytis cinerea*, methyl viologen, methylene blue and hydrogen peroxide	Overexpressing plants showed increased susceptibility to *Botrytis* and agents provoking oxidative stress.	Fotopoulos et al. (2006)
Nicotiana tabacum	Overexpression (pumpkin AO) and antisense (tobacco)	Pathogen—*Pseudomonas syringae*	Sense lines had decreased auxin sensitivity Sense lines were more sensitive to *Pseudomonas syringae* infection	Pignocchi et al. (2006)

Tobacco and Arabidopsis	Overexpression and antisense tobacco. Arabidopsis T-DNA mutant	Salt	Antisense plants showed a delay in flowering under normal conditions. Antisense plants had higher germination, photosynthetic activity, growth and seed yields under salinity Arabidopsis mutant had delayed flowering and improved seed yield under salt stress	Yamamoto et al. (2005)
Nicotiana tabacum	Overexpression (pumpkin AO) and antisense (tobacco)		Increased growth rate (increase in shoot fresh weight per week) of AO sense plants and highly oxidized apoplastic ascorbate pool	Pignocchi et al. (2003)
Nicotiana tabacum	Overexpression of cucumber AO	Ozone	Increased foliar injury in transgenic overexpressing lines following ozone treatment Increased oxidation of ascorbate pool, decreased oxidation of glutathione pool	Sanmartin et al. (2003)
Nicotiana tabacum L. cv. Bright yellow no. 2	Overexpression (pumpkin AO)		Increased protoplast elongation in presence of external hormones	Kato and Esaka (2000)

(2013) showed that tomato AO RNAi lines had improved yield under unfavorable conditions where control plants suffered yield losses. Antisense tobacco plants showed higher germination rates, photosynthesis levels, and seed yields under salinity in tobacco (Table 1) (Yamamoto et al. 2005). Underexpression of this enzyme also led to increased tolerance to salt stress in Arabidopsis and tobacco (Table 1) (Yamamoto et al. 2005). In some cases, decreased AO levels led to decreased virus accumulation (Kumari et al. 2016) and inversely, overexpressing plants were more sensitive to infection by pathogens such as *Botrytis cinerea* and *Pseudomonas syringae* (Fotopoulos et al. 2006; Pignocchi et al. 2006). In tobacco, overexpression of cucumber AO increased sensitivity to ozone injury (Sanmartin et al. 2003). These observations have often led authors to question the utility of such an enzyme, but it is logical that an enzyme relaying signals from the environment to allow the cell, and ultimately the plant, to adapt must also relay information to slow or adjust metabolism under what is interpreted as being stressful conditions.

Other hypotheses which are proposed to explain the observation that reduced AO activity often improves stress tolerance include a protective effect of the increased redox state of the apoplast of antisense plants against the rise in hydrogen peroxide levels following stress, the reverse being true in the overexpressing plants. The overexpression of AO and the increased sensitivity to stress have been correlated with a general suppression of the plant's antioxidant metabolism (in particular the ascorbic acid recycling genes) under stress (Fotopoulos et al. 2006).

Ascorbate oxidase was identified in a yeast two-hybrid screen as interacting with the movement protein of cucumber mosaic virus. Silencing of AO reduced viral spread 5 days after inoculation compared to control plants and transcripts tended to be upregulated in infected plants. Ascorbate oxidase is suggested to help targeting of movement proteins during the initial infection stages by virus (Kumari et al. 2016).

Some studies have suggested that AO could act as a defense protein against herbivores by oxidizing the ascorbate that insects require (Felton and Summers 1993). However, Barbehenn et al. (2008) concluded that elevated levels of AO in poplar are unlikely to defend against herbivores such as *L. dispar* (caterpillar) or *M. sanguinipes* (grasshopper).

12 Photosynthesis, Metabolism, and Resource Allocation

In Arabidopsis, underexpression of AO led to increased seed yield under stress conditions compared to wild type (Yamamoto et al. 2005). In tomato, reduced AO activity improved fruit yield under unfavorable environmental conditions including low water and leaf removal (Garchery et al. 2013). Interestingly, the opposite was found in tomato plants silenced for monodehydroascorbate reductase activity (Truffault et al. 2016). In the former tomato AO RNAi lines, many biochemical and

physiological changes were observed: fruit apoplastic sucrose levels decreased in the RNAi lines which could have contributed to improve assimilate transfer to fruit. The variations in expression of genes such as *fw2.2* (Frary et al. 2000; Nesbitt and Tanksley 2001) and UDPgalactose-epimerase (Dormann and Benning 1998; Seifert et al. 2002) support hypotheses concerning altered resource allocation in AO RNAi fruit. In an independent study in tobacco, antisense AO lines with a more highly reduced apoplastic ascorbate redox state showed increased photosynthesis (CO_2 assimilation) compared to AO sense lines and wild-type plants (Karpinska et al. 2017). It is interesting to note that photosynthesis was decreased in the tomato MDHAR silenced lines cited above. However, in the tobacco antisense AO lines, few transcripts were dependent on AO activity but tobacco overexpressing pumpkin AO had more PSII polypeptide transcripts and antisense plants increased expression of aromatic amino acid synthesis genes and decreased expression of proteinase inhibitor genes under high light compared to low light.

13 Ascorbate Oxidase: Roles in Oxygen Sensing and Cofactor Control

Some interesting theories on the role of AO in oxygen sensing have been put forward by the group of De Tullio based on a series of independent observations. Ascorbate oxidase activity was upregulated in the light compared to the dark and upregulated in dark conditions when oxygen levels were increased (De Tullio et al. 2007). The authors suggested that AO was regulated by light-induced oxygen production and indeed its activity was proportional to oxygen availability. If this is the case, AO could be a signal for sensing external oxygen concentrations. Indeed, this makes sense if we consider that AO is there as a regulator of oxygen concentrations, rather than the ascorbate-centered view that the enzyme is removing a potentially useful antioxidant. The enzyme could avoid risks of hyperoxia or serve to create an oxygen gradient between the apoplast and internal compartments of the plant cell (chloroplast or mitochondria).

These theories are borne out by a study in *Lotus japonicas* where an AO gene is induced in two symbiotic interactions: firstly after rhizobia inoculation and secondly in mycorrhizal roots infected with arbuscular mycorrhizal fungi. The enzyme was also immunolocalized at the host-symbiont interface and induced during nodule development and could be involved in creation of an oxygen diffusion barrier for efficient nitrogen fixation (Balestrini et al. 2012), or as a control on oxygen status necessary for cell differentiation.

In a recent review the same authors postulated that AO could have a role in regulation of peptidyl-prolyl-4-hydroxylases (PHDs), which require both oxygen and ascorbate as co-substrates, and are enzymes that hydroxylate, among other proteins, the transcription factor HIF-1α in mammals (Badawi and Shi 2017; De Tullio et al. 2013).

This hydroxylation targets the protein for degradation, the non-hydroxylated form being available for migration to the nucleus. Ascorbate oxidase could therefore modulate transcription by controlling the availability of two co-substrates for PHDs and thus participate in signaling pathways via this posttranslational modification. PHDs have been identified in plants and are involved in processes such as root hair expansion (Velasquez et al. 2015) and often induced by hypoxia or anoxia (Vlad et al. 2007).

In a similar vein, as ascorbate is necessary as a cofactor for 1-aminocyclopro pane-1-carboxylate oxidase (Mirica and Klinman 2008), an enzyme necessary for ethylene synthesis during fruit ripening, it is hypothesized that repression of AO activity during fruit ripening would not only liberate ascorbate for the activity of ACC-oxidase but also increase oxygen availability for respiration (De Tullio et al. 2004). Similarly, repression of AO following wounding might increase oxygen and ascorbate levels for wound respiration and for activity of peptidyl-prolyl-4-hydroxylases, necessary for posttranslational modification of proline in hydroxyproline-containing-protein synthesis in wound repair activity (De Tullio et al. 2004). These appealing hypotheses of course require further testing in the future.

14 Conclusions, Future Directions

At the end of this chapter, we would like to highlight certain points that we have found worthy of note while writing the chapter that could serve as a basis for future research.

Firstly, the amount of data freely available in the form of genome sequences or in expression databases allows questions concerning the presence—or absence—of AO (like) gene sequences in different organisms to be answered. Expression data can also be extremely helpful in determining in which organs and under which conditions the gene is expressed and its co-expression partners: co-expression analysis can also give clues to novel functions for the enzyme.

Secondly, the correlation between AO activity and growth rate is intriguing—is the high activity of species from the *Cucurbitaceae* family meaningful or merely an anecdotal correlation? What are the precise links with the cell wall and growth? Has this enzyme been selected for in the domestication of fruits with rapid growth rate and large final size? Are the links purely hormonal-driven (for example by the interaction with auxin) and what is the role of the monodehydroascorbate radical? The subject is also intriguing because despite a number of studies (often on cellular systems) showing a relationship between AO and cell enlargement or growth, contradictory literature exists (lack of phenotype of Arabidopsis T-DNA mutant line under normal conditions for example).

Thirdly, if it is clear that AO participates in stress responses, apoplastic redox state and possibly several developmental processes including establishment of yield

maybe via a link with major growth hormones—what is the link with oxygen sensing developed above and can this explain the high expression of the enzyme often found in root tissue? In the case that AO does have a role in oxygen sensing, can we make plants capable of better sensing environmental variations in oxygen levels—or reactive oxygen species—and could this have applications in fields such as fruit quality research? Indeed, understanding the regulation, stability, and catalytic activity of enzymes such as AO has already been shown to be necessary for more applied research: an example is the postharvest regulation of ascorbate content of fruit and vegetables, during food preparation and processing which is necessary to maintain antioxidant capacity and health value (Leong and Oey 2014).

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AsA/DHA Redox Pair Influencing Plant Growth and Stress Tolerance

Javier Alberto Miret and Maren Müller

Abstract In a continuously changing environment plants are exposed to adverse stress conditions, such as sunlight, drying, cold, salinity, pollution, or heavy metals, which influence plant growth and result in the generation of reactive oxygen species (ROS). These small and highly reactive molecules have important cell signalling information concerning the change in the environmental and developmental conditions when maintained at proper cellular concentrations. However, during stress conditions, ROS levels in cells can greatly increase and cause oxidative stress by modifying other reactive species, proteins, or lipids. Therefore, appropriate regulation of ROS has a significant impact on plant development, growth, and survival. Ascorbic acid (AsA) as a major antioxidant in plant cells and its oxidized form dehydroascorbate (DHA) play a key role in redox state-based signalling mechanisms by detoxification of ROS and its products, as well as transmission of redox signals. Furthermore, DHA by itself also presents unique functions: cell cycle progression sensing and regulation, modulation of metal stress responses, and DHA adducts seem to be involved in oxidative stress-mediated cellular toxicity. It has become clear that the changes in the pool and ratio of the AsA/DHA redox pair by both growth and environmental cues modulate gene expression and protein levels resulting in increased stress tolerance. In the recent years, this important redox couple (AsA/DHA) has been of increasing interest to better understand the mechanisms of adaptive plant responses and stress tolerance towards abiotic and biotic stress. In this chapter, an overview of the literature is briefly presented in terms of the role of AsA/DHA redox pair in plant growth, and abiotic and biotic stress tolerance.

Keywords Ascorbic acid · Dehydroascorbate · Redox state · Plant growth · Abiotic stress · Biotic stress · Reactive oxygen species

J. A. Miret · M. Müller (✉)
Department of Evolutionary Biology, Ecology and Environmental Sciences, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain
e-mail: maren.muller@ub.edu

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1 Introduction

The introduction of molecular oxygen to our early reducing atmosphere led to O_2-evolving photosynthetic active organisms in the evolution of aerobic life and, as a consequence, to the formation of reactive oxygen species (ROS). Initially, ROS were recognized as toxic by-products generated constantly by aerobic metabolism. However, recently it has become apparent that ROS also play important signalling roles, controlling processes such as growth, development, and especially responses to abiotic and biotic stresses. The major members of the ROS family include free radicals like superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and non-radicals like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Mittler 2002). During the life cycle of plant cells, ROS are formed under normal and stressful conditions in the different cellular compartments. The major generators are chloroplasts, mitochondria, and peroxisomes; but other compartments also produce relevant amounts of ROS: plasma membrane, endoplasmic reticulum, and apoplast (Jubany-Marí et al. 2009; Roychoudhury and Basu 2012). In the presence of light, chloroplasts and peroxisomes are the major sources of ROS production during reactions that participate in the mechanism of photosynthesis and photorespiration, respectively. Meanwhile, mitochondria are the leading producer of ROS during respiration under dark conditions (Choudhury et al. 2013; Mittler et al. 2004).

Under stress-free conditions, ROS, especially $O_2^{\bullet-}$ and H_2O_2, are constantly generated at rather low basal level acting as second messengers and key regulators of growth and plant development. These molecules, although being toxic to the cell, are unable to cause cellular damage as they are scavenged and tightly controlled by a complex antioxidant machinery (Foyer and Noctor 2005a; Mittler et al. 2004). However, under different kinds of environmental stresses, such as high light, high or low temperatures, salinity, drought, nutrient deficiency, and pathogen attack, the cellular homeostasis is disrupted; and this imbalance between ROS production and their detoxification causes oxidative stress (Mittler 2002; Chalapathi and Reddy 2008). ROS are oxidizing agents that are able to subtract electrons from essential organic molecules and thus disturb the cellular function of proteins, nucleic acids, lipids, and sugars, which may lead to cell damage and ultimately cell death. Therefore, the survival of stressed plants, as sessile organisms, depends on adaptation and avoidance strategies like change in growth conditions, severity and duration of stress conditions, and the capacity to quickly adapt a battery of antioxidant strategies (Miller et al. 2010; Foyer and Shigeoka 2011).

The antioxidant defense machinery includes: (1) enzymatic molecules like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) and (2) nonenzymatic antioxidants like AsA, reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids, and the osmolyte proline (Nobuhiro and Mittler 2006). The latter are low molecular weight antioxidants that facilitate the cell to be detoxified during extreme environmental stress conditions, and also to keep ROS at the optimum level allowing

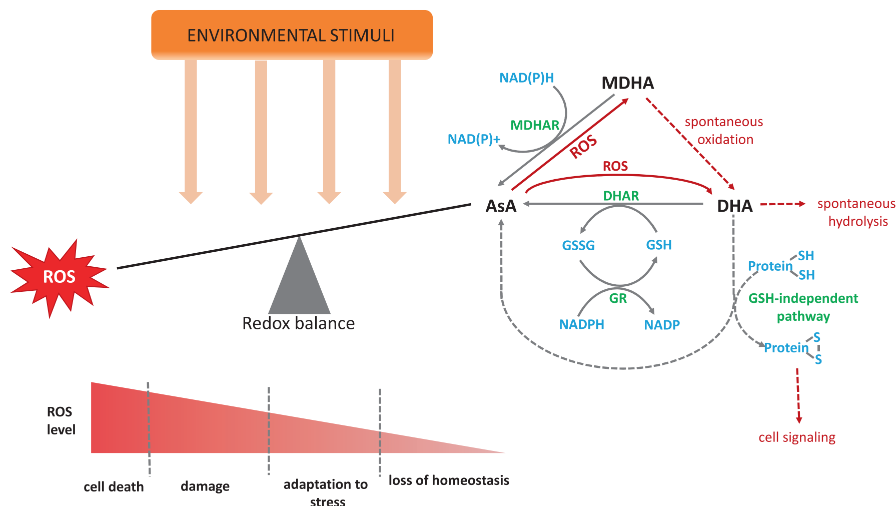

Fig. 1 Schematic representation of the oxidoreduction function of AsA/DHA redox pair during ROS detoxification. Optimum level of ROS keeps cell in homoeostasis condition for normal growth and development of plants. In contrast, different kinds of environmental stimuli trigger enhanced ROS formation, which disrupt cellular homoeostasis and may lead to cell damage or cell death. The survival of stress plants depends in part of quickly ROS detoxification by AsA/DHA redox pair. The AsA recycling involves DHA reduction, which includes (**a**) the AsA-GSH cycle and (**b**) a GSH-independent pathway has been proposed. *AsA* ascorbic acid, *DHA* dehydroascorbate, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *MDHA* monodehydroascorbate, *MDHAR* monodehydroascorbate reductase, *ROS* reactive oxygen species

redox-sensitive signal transduction and balance information from environment and developmental stimuli (Foyer and Noctor 2005b).

AsA is the primary water-soluble antioxidant and has been detected in a wide range of cellular compartments like cytosol, chloroplast, vacuoles, mitochondria, and apoplast. The molecule is considered to be as one of the most powerful antioxidants in plant cells by a virtue of its ultra-crucial ability to function as a donor of electrons by delocalizing electrons around a 5-carbon ring. Due to its oxidoreduction potential, AsA interacts with hydroxyl radicals, singlet oxygen, superoxide, and also with glutathione and tocopherol radicals (Noctor and Foyer 1998). AsA can directly scavenge and neutralize ROS, but it can also repair oxidized molecules such as α-tocopherol or can serve as an enzyme cofactor, for example, for violaxanthin de-epoxidase (VDE) (Blokhina et al. 2003; Müller-Moulé et al. 2002). The regeneration system for AsA involves two key enzymes known as dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) (Fig. 1). The primary oxidation product is the one-electron oxidized unstable radical monodehydroascorbate (MDHA). Two MDHA molecules can react each other to form one molecule of AsA or dehydroascorbate (DHA), which is the two-electron oxidized form of AsA. MDHAR uses NAD(P)H as an electron donor to reduce MDHA

before spontaneous oxidation to DHA. In the chloroplast and mitochondrion, the MDHA radical can also be reduced by ferredoxin and complex II, which is more effective than reduction by NAD(P)H-dependent MDHAR (Asada 1999; Szarka 2013). DHAR catalyzes the reduction of DHA to AsA using glutathione (GSH) as a hydrogen donor (ascorbate-glutathione cycle or Halliwell–Asada cycle). AsA recycling by DHAR, therefore, serves to recycle DHA into AsA before it is lost from the AsA pool. DHA, which predominates in the apoplastic space, must reenter the cell for reduction to AsA because the apoplast contains little GSH and DHAR amounts. In the absence of enough recycling activity, however, DHA is hydrolyzed to irreversible form 2,3-diketogulonic acid (Chen et al. 2003; Potters et al. 2002; Pignocchi and Foyer 2003; Munné-Bosch et al. 2013). Moreover, DHA seems to be able to directly interact not only with GSH, but also using an active GSH-independent pathway for DHA reduction (Fig. 1) (Potters et al. 2004; Fotopoulos et al. 2008).

In higher plants, the AsA/DHA redox pair functions as a reliable sensor that perceives and coordinates their action depending on the cellular redox state. Besides their action as reductant and reacting with and scavenging many types of ROS, AsA/DHA also influence many enzyme activities. Redox-sensitive proteins play crucial role in the ROS signal transduction, because they can undergo directly or indirectly reversible oxidation/reduction, which activate or deactivate them depending upon the cellular redox state. Whereas redox-sensitive metabolic enzymes can modulate directly appropriate cellular responses, redox-sensitive signalling proteins conduct their function via downstream signalling components including kinases, phosphatases, and transcription factors. Moreover, disulfide-thiol conversion is likely important in this redox signal transduction (Shao et al. 2008). It has been hypothesized that DHA could have a key role in signalling due to its peculiar reactivity with specific proteins, such as thiol-containing proteins (Potters et al. 2004; Fotopoulos et al. 2008). However, possible DHA-regulated target proteins are still to be identified in plants. In animal cells, for instance, protein-disulfide isomerase (PDI), a major protein of the ER lumen, is known to have DHA reductase activity. This enzyme accepts protein thiols as the source of reducing equivalents during protein thiol oxidation by DHA, while simultaneously AsA is formed (Bángegyi et al. 2003; Nardai et al. 2001).

Several studies conducted in a number of plant species under environmental stress conditions lead to the assumption that high AsA/DHA ratios accompanied by increasing AsA levels or decreasing DHA levels could be a key element coordinating efficient ROS protection (Szalai et al. 2009). Developmental and environmental stressors can rapidly challenge the AsA pool; however, changes in AsA biosynthetic capacity can take hours or longer and are tightly regulated by light and respiration electrons (Bartoli 2006). Although AsA and its redox state have a fundamental role in the plant defense system, they have also effects on many physiological processes including growth regulation, differentiation, and the metabolism of plants.

In this chapter, we present an overview of the literature that reveals aspects of the role of AsA/DHA redox pair in plant growth and development as well as tolerance responses to abiotic and biotic stresses.

2 The Role of AsA/DHA Redox Pair in Plant Growth and Development

The cellular redox state has emerged as an important determinant for plant growth and development due to the fact that the activity of genes and enzymes has specific redox requirements. Small changes of subcellular distribution of ROS, antioxidants, and redox state in different cell compartments can induce gene expression, defense signalling, and cell death (Kocsy et al. 2013). AsA and its redox state are the most important redox buffer for the detoxification of ROS in the apoplast and vacuoles. The cytosol acts as a hub for the AsA recycling as it reduces the oxidized forms produced in the cytosol or imported from other cell compartments. As AsA/DHA redox pair is also involved in protein synthesis and modification, it can be concluded that they act as oxidative stress sensors playing a key role in the fine-tuning of plant growth and development (Zechmann in press).

2.1 AsA and its Redox State Regulate Cell Cycle

Cell proliferation, the basis of plant growth, is under redox control. Low concentrations of ROS or shorter exposure of ROS seems to promote cell division in contrast to their excess or longer exposure, which can result to cell death. Concurrently, AsA and its redox state seem to play a role in the control of the cell cycle. An increase of AsA and MDHA promote cell division; whereas mutants of tobacco BY-2 cell lines, with 30% less AsA, showed a reduced cell division rate and growth (De Pinto et al. 1999; Kato and Esaka 1999). Moreover, increased level and activity of AsA/DHA redox pairs and ascorbate oxidase (AO) expression suggest that the oxidation of AsA seems to be crucial during cell elongation (Kato and Esaka 1999).

Within the quiescent center of onion root cells, AsA promotes cell division by inducing G1 to S progression (Liso et al. 1984), whereas exogenous DHA treatment reduced the mitotic activity of onion root meristems (De Cabo et al. 1993). In addition, exogenous addition of the plant hormone auxin in quiescent cells induced AO expression and activity, suggesting that high auxin levels induce AO expression, which seems to reduce AsA contents and maintain quiescent cells in G1 state of roots (Kerk and Feldmann 1995). In tobacco BY-2 cell cultures, the addition of DHA during G1 phase, but not during G2 phase, showed inhibitory effects on cell progression. Interestingly, exogenous DHA treatment only increased AsA levels, but not internal DHA concentrations, suggesting a rapid reduction of DHA to AsA. Increased AsA levels are associated with faster cell proliferation rates and not with inhibitory effects. Therefore the effect of DHA treatment may be, in part, by the depletion of GSH, as the latter is a cofactor during the reduction of DHA. Moreover, depletion of GSH seems to inhibit cell cycle progression (Potters et al. 2000, 2004). The reduction of DHA has also been proposed by a GSH-independent pathway and depletion of these reductants, such as thiol-containing

proteins, could also be involved in the inhibition of cell division by DHA treatment (Potters et al. 2004). Supporting this hypothesis is the observation that although AsA levels increased by exogenous L-galactono-1,4-lactone, the precursor to AsA, thiol-containing proteins were not oxidized (Paciolla et al. 2001). Given the effects of AsA and its redox state on the cell cycle, AsA promotes cell division whereas DHA seems to inhibit cell division. A hypothesis explained the impact of AsA and its redox state in stimulation of cell wall expansion and cellular growth (Smirnoff 1996). The model is based on the ability of AsA to give up electrons while the oxidized forms can accept electrons. Cytoplasmic AsA is oxidized by cytochrome b and electrons are transferred to MDHA in the apoplast. The transfer of electrons stimulates H^+-ATPase activity and leads to cell wall loosing stimulating cell growth. Moreover, AsA inhibits peroxidative cross-linking of cell wall polysaccharides and lignin polymerization by scavenging hydrogen peroxide radicals. Meanwhile, DHA may prevent cross-link to matrix polysaccharides and may react with amino acid side chain of cell wall glycoproteins. The AsA/DHA redox pair is also involved in protein synthesis and modification, which could contribute to the observed stimulatory effect of AsA on cell growth (Zechmann in press). The cell cycle progression could be controlled by an oxidative stress checkpoint pathway that responses to one or more redox-sensing systems, as has been proposed (Reichheld et al. 1999).

2.2 AsA and its Redox State Regulate Tissue and Organ Level

AsA and its redox state regulate not only at the cellular, but also at tissue and organ level growth and development. For example, seed development is characterized by dramatic changes of the AsA/DHA redox pair. First, levels of reduced AsA are high during early embryo development, followed by a decrease in the AsA redox state during cell elongation by the way that DHA levels exceed AsA levels (Tommasi et al. 2001). Orthodox seeds are characterized to withstand storage by a desiccation period at the end of the seed development, whereas recalcitrant seeds are desiccation sensitive seeds and are characterized by a short storage life. In orthodox seeds, when start to dry out at the end of seed development, AsA levels are completely oxidized in the embryo (Tommasi et al. 2001; Arrigoni et al. 1992); however, during germination DHA can be rapidly reduced to generate AsA (Tommasi et al. 2001). In contrast, recalcitrant seeds are able to germinate directly after seed abscission and remain metabolically very active. Therefore these seeds accumulate high levels of AsA/DHA redox pairs (Tommasi et al. 1999).

During embryo development, it has been observed that increasing endogenous AsA levels induce monozygotic twinning and polycotyly due to increasing DHAR expression in tobacco (Chen and Gallie 2012). Normally, early embryo development includes the transverse division of a zygote into an apical and a basal cell. However, the effect of AsA on monozygotic twinning generates two genetically identical zygotes, each of which develops into an independent embryo of equal size. The monozygotic twinning by AsA is limited to the first 2 days after pollination,

whereas polycotyly is induced when AsA levels increased just prior to cotyledon initiation. The frequency of polycotyly increases during cell division throughout the specification of cotyledon-forming.

Due to the fact that AsA/DHA redox pair is involved in cell division and elongation, their levels are correlated with leaf growth. However, AsA synthesis declines with the decrease in leaf function as a part of senescence and cell death process (Borraccino et al. 1994; Chen and Gallie 2006). Growth reduction could also be observed in tobacco plants with repressed DHAR expression resulting in lower recycling rate of AsA and its redox state (Chen and Gallie 2006). The *vtc1* mutants of *Arabidopsis*, deficient in AsA biosynthesis, showed significant growth reduction compared to wild-type plants (Veljovic-Jovanovic et al. 2001).

During flower development, characteristic changes in ROS levels suggest that various ROS may have specific functions during flowering and AsA/DHA redox pairs are mainly involved in modulating the required redox homoeostasis (Zafra et al. 2010). Analysis of AsA-deficient *Arabidopsis* mutants *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* growing under short and long day length conditions has proposed that AsA may affect flowering time. Circadian clock and photoperiodic pathways genes were significantly higher in the *vtc* mutants, which exhibited an early flowering phenotype compared to wild type. Moreover, genetic analysis demonstrated that periodic and autonomous pathway mutants were epistatic to the *vtc1-1* mutant (Kotchoni et al. 2009). This conclusion could be supported by the study in *Oncidium* orchid, where a decreased AsA redox state acts as a signal to initiate flowering (Chin et al. 2016).

3 The Role of AsA/DHA Redox Pair Under Abiotic and Biotic Stress Responses

A plant subjected to stress will suffer a series of strains, the intensity of each strain will be directly proportional to the magnitude and duration of the stressor and inversely proportional to the strain tolerance of the plant (Blum 2016; Levitt 1972). Each plant might present constitutive resistance. Besides, the strain itself and/or physiological effects caused by the strain may represent a signal perceived by the plant to promote an adaptive resistance (Blum 2016). For many different stresses, there are mechanisms of avoidance, tolerance, and perception that rely in the physicochemical properties of the redox pair AsA/DHA and its central role in plants metabolism and signalling together with the glutathione redox pair (Gest et al. 2013; Noctor 2006). Generally, plants with low AsA biosynthesis or AsA recycling capacity are more sensitive to environmental stressors. Besides, increases in DHAR activity that represent changes in AsA redox state but not pool size cause enhanced tolerance to many stress: low temperature, salinity, toxic metals, methyl viologen (generating $O_2^{\bullet-}$), or H_2O_2 (Gallie 2013; Kwon et al. 2003). In the next sections, tolerance responses to different stresses in which ascorbate and its redox state play a role will be presented and summarized in Table 1.

Table 1 Summary of the discussed environmental stresses and the corresponding strains generated in the plant

Stress	Strain	Avoidance/adaptation mechanisms	Molecular mechanisms	References
High light	Photo-oxidative stress	Excess energy dissipation. ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts, xanthophylls cycle, ROS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Demmig-Adams et al. (2012), Kozuleva et al. (2016), Mano et al. (2004), Murata et al. (2012), Tóth et al. (2011)
High/low temperature	Metabolism disruption. Photo-oxidative stress	Excess energy dissipation. ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; xanthophylls cycle; ROS, RNS and oxidative damage scavenge, antioxidants regeneration; ascorbate synthesis modulation	Asada (2006), Demmig-Adams et al. (2012), Mano et al. (2004), Bita and Gerats (2013), Lázaro et al. (2013), Martí et al. (2011), Míguez et al. (2015), Szarka et al. (2004, 2013), Tóth et al. (2011)
	Lipid peroxidation	Oxidative damage detoxification	Oxidative damage scavenge, antioxidants regeneration (notably alpha-tocopherol)	Munné-Bosch(2005)
Water shortage	Osmotic strain	Stomatal regulation	Apoplastic oxidative burst modulation, redox signalling	Chen and Gallie (2004), Fotopoulos et al. (2008)
	Photo-oxidative stress	Excess energy dissipation. ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts; xanthophylls cycle; ROS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Demmig-Adams et al. (2012), Mano et al. (2004), Murata et al. (2012), Pastore et al. (2006), Schroeder et al. (2001), Tóth et al. (2011)
Water excess	Hypoxia/anoxia	Excess energy dissipation. ROS and oxidative damage detoxification. Respiratory metabolism modulation	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; xanthophylls cycle; ROS, RNS and oxidative damage scavenge, antioxidants regeneration; ascorbate synthesis modulation; DHA phloem transport	Asada (2006), Demmig-Adams et al. (2012), Franceschi (2002), Herschbach et al. (2010), Mano et al. (2004), Murata et al. (2012), Tóth et al. (2011)
Salinity	Osmotic strain	Stomatal regulation	Apoplastic oxidative burst modulation, redox signalling	Chen and Gallie (2004), Fotopoulos et al. (2008)
		Osmotic adjustment	Free amino acids modulation	Gulyás et al. (2017), Sharma and Dietz (2006), Vranova et al. (2011)

	Ion toxicity	ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; xanthophylls cycle; ROS, RNS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Demmig-Adams et al. (2012), Mano et al. (2004), Murata et al. (2012), Tóth et al. (2011)
Metal toxicity	Metabolism disruption. Oxidative stress and photo-oxidative stress	Excess energy dissipation. ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; xanthophylls cycle; ROS, RNS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Buettner and Jurkiewicz (1996), Demmig-Adams et al. (2012), Mano et al. (2004), Murata et al. (2012), Tóth et al. (2011)
		Chelation	Free amino acids modulation	Gulyás et al. (2017), Sharma and Dietz (2006), Vranova et al. (2011)
Environmental pollutants	Oxidative stress	Stomatal regulation	Oxidative burst modulation, redox signalling	Vainonen and Kangasjärvi (2015), Yoshida (2005)
		ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; ROS, RNS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Burkey et al. (2003), Demmig-Adams et al. (2012), Mano et al. (2004), Murata et al. (2012), Plöchl et al. (2000), Tóth et al. (2011)

Next to each strain, associated avoidance/adaptation mechanisms are summarized in which the ascorbate pool or it redox state plays a role in their known molecular mechanisms.

3.1 AsA Redox State in the Chloroplast Under High Light Stress

Sudden or long exposure to high light causes stress to the chloroplast functionality (Asada 2006). The main detrimental consequence of high irradiance is oxidative stress caused when absorbed light exceeds photosynthetic capacity, namely photo-oxidative stress. But other environmental stressors can generate photo-oxidative stress in the chloroplast without increased incident energy but impairing photosynthetic capacity and the linked metabolism (see Table 1).

Under excess light, two major ROS generating events occur in chloroplasts. An excess of excited chlorophyll (due to high irradiance and/or a disturbed photosynthetic metabolism) converts molecular oxygen (O_2) into singlet oxygen ($^1O_2$) (Krieger-Liszkay 2005). Downstream the electron transport chain, excess of reduced phylloquinone, ferredoxin and Fe-S clusters in the PSI reaction center complex relative to limiting electron and reducing power acceptors can use molecular oxygen as an alternative electron acceptor to generate superoxide anion (O_2^-), a highly reactive species that rapidly reacts to generate oxidative damage and/or other ROS (Asada 2006). Under optimal conditions, these ROS generating reactions occur at low rates because light energy transferred to excited chlorophylls is efficiently used to power PSI and PSII and ferredoxin readily reduces NADP+ in PSI as final electron acceptor. ROS will be formed at increased rates only under excess light because of (1) a limitation in the capacity of the plant to consume NADPH in chloroplasts due to environmental stressors (e.g., drought induced stomatal closure limiting Calvin cycle NADPH consumption, anoxia, temperature stress), (2) high irradiance (e.g., midday conditions in a sunny day), and/or (3) environmental stressors disrupt the chloroplast electron transport chain itself and/or its repair mechanisms (e.g., high temperature denaturing electron transport chain proteins or toxic metal ions disrupting the electron transport). Thus, several environmental stressors such as drought, salinity, nutrient deficiency, high and low temperature, or environmental pollutants lead to increased ROS production in chloroplasts (Demidchik 2015; Kozuleva et al. 2016; Murata et al. 2012). Excessive absorbed energy is dissipated by a number of mechanisms to limit photodamage: LHCII state transitions, xanthophyll cycle, photorespiration, ROS production and detoxification mechanisms (such as the water–water cycle) (Demmig-Adams et al. 2012; Li et al. 2009). Notably, the AsA pool and its redox state play an essential role in most of these mechanisms.

AsA is necessary for the xanthophyll cycle. In this mechanism of dissipation of excessive excitation energy in the PSI, the enzyme violaxanthin de-epoxidase uses excess energy to convert the carotenoid violaxanthin to zeaxanthin, requiring a readily available pool of reduced ascorbate as cosubstrate (Eskling et al. 1997; Yamamoto et al. 1962). Meanwhile, photorespiration reactions dissipate excess reducing power and energy directly by using ATP, NAD(P)H, and reduced ferredoxin (Peterhansel et al. 2010; Wingler et al. 2000). At the same time, the required ribulose-1,5-bisphosphate regeneration generates H_2O_2 in the peroxisome, possibly involved in signal transduction and redox homeostasis adjustment (Scheibe and

Dietz 2012; Takahashi et al. 2007; Voss et al. 2013). AsA contributes to H_2O_2 detoxification in the peroxisome as cosubstrate of APX (Karyotou and Donaldson 2005). Thus, the maintenance of AsA redox state and pool in the peroxisome is essential for the detoxification and fine-tuning of the redox homeostasis feedback signal generated by photorespiration metabolites.

The above described mechanisms dependent of the AsA pool and its redox state reduce the probability of ROS production. Once ROS generation rises, the availability of reduced ascorbate and readily available recycling mechanisms can maintain minimal ROS steady levels through the water–water cycle (Asada 1999, 2006). This cycle consists in the photoreduction of molecular oxygen to superoxide in PSI by electrons generated in PSII from water, while half of the electrons are used to regenerate ascorbate. The generated superoxide can generate oxidative damage to nearby macromolecules, but is readily transformed in H_2O_2 by superoxide dismutases. The regenerated AsA provides reduced equivalents to detoxify H_2O_2 to water by ascorbate peroxidase (APX), and detoxify products from oxidative damage (Asada 1999, 2006). This constitutes a nonproductive cycle that provides ROS scavenging and protection from photoinhibition, in which readily available reduced AsA is essential. In addition, AsA can nonenzymatically detoxify ROS, such as $^1O_2$ (Chou and Khan 1983) and $OH^•$ (Buettner and Jurkiewicz 1996), as well as recycle tocopheroxyl radicals to tocopherol, generated by the scavenging of lipid peroxyl radicals caused by oxidative damage on lipids (Munné-Bosch 2005). In addition, different APX isoforms reduce H_2O_2 using AsA as reducing power in other plant cell compartments (Pandey et al. 2017). Thus, a readily reduced AsA pool and recycling mechanisms are essential to sustain these detoxification mechanisms for the plant survival.

When the photosynthetic electron transport chain is impaired, reduced and oxidized AsA forms can act as an alternative donor and acceptor of electrons in order to avoid photo-oxidative damage. If the primary electron donor system is impaired, AsA can be a direct electron donor to PSI and PSII generating MDHA in the lumen (Mano et al. 1997, 2004). Hence, AsA enables cyclic electron transport in anoxic conditions, high temperature, or when the electron transport chain reaction centers are impaired under severe nutritional deficits (Tóth et al. 2009, 2013). Meanwhile, when the electron acceptor $NADP^+$ becomes limiting, photoreduction of MDHA to AsA by ferredoxin can maintain electron transport (Forti and Ehrenheim 1993; Miyake and Asada 1994) protecting the photosynthetic machinery by slowing down photo-oxidative damage (Tóth et al. 2011). In addition, it has recently been described that light acclimation processes and the resulting photosynthesis rate are influenced not only by chloroplastic AsA redox state, but also by the apoplastic redox state (Karpinska et al. in press), largely regulated by AO activity (discussed in latter sections).

Accumulated DHA can conjugate with peptides and proteins cysteine thiol groups to generate DHA-peptide or DHA-protein adducts (Kay et al. 2013). These modifications might be generalized while at the same time specific to certain peptides and proteins (i.e., glutathione, glutaredoxin) in oxidative stress-mediated cellular toxicity (Flandrin et al. 2015; Regulus et al. 2010). The relevance of this

modification mechanism for oxidative stress perception and response in vivo in plants and other organisms remains to be explored.

As discussed elsewhere, AsA has plenty of functions as cofactor or cosubstrate of many enzymatic reactions (Arrigoni and De Tullio 2002). But DHA also plays a role as an oxidant in the protein-disulfide isomerase-catalyzed protein disulfide formation, essential for oxidative protein folding in the endoplasmatic reticulum (Bánhegyi et al. 2003; Csala et al. 1999; Nardai et al. 2001), an essential step of the secretory protein pathway. The established disulfides can act as thiol switches for regulatory processes like short-term adaptation to normal daily environmental changes such as dark–light cycle, but also to sense oxidative challenges during severe environmental stresses (Onda 2013).

The photosynthetic electron transport can be strained by an imbalance between incident energy and electron flux capacity caused by many environmental stressors, potentially generating ROS and photo-oxidative damage. The size of the ascorbate pool and its redox state are essential in a number of energy dissipation mechanisms associated to photo-oxidative stress, preventing the over-reduction of the photosynthetic electron chain and ultimately avoiding photo-oxidative damage. All these energy dissipation mechanisms require a readily available pool of reduced AsA in different compartments and AsA recycling mechanisms. The potential regulatory role in stress signalling and responses of DHA adducts and DHA in oxidative protein folding remain to be examined.

3.2 AsA Redox State in the Mitochondria Under Temperature Stress

Temperature stress is one of the main environmental limitations affecting plant production. Extreme temperatures affect plants by three strains: lowering enzymatic activity—by thermodynamic limitations or loss of conformation in low or high temperature, respectively—; loss of membrane function, by changes in membrane fluidity and lipid peroxidation promotion; and ice formation and mechanical damage (Bita and Gerats 2013; Thomashow 1999).

High or low temperature exposure results in direct disruptions in proteins and membranes due to temperature itself, and indirectly because of the subsequent increased ROS generation. Therefore chloroplast and mitochondrial metabolism can be severely disturbed, being energy dissipation and ROS detoxification mechanisms essential in the response to temperature stress (Iba 2002; Larkindale and Knight 2002; Míguez et al. 2015). In previous sections, it has been already discussed how the disturbance of the chloroplastic electron chain by excessive absorbed energy is determined by ascorbate content due to its redox state and its double role as an alternative electron donor and acceptor, as well as its involvement in ROS detoxification mechanisms. These mechanisms are also relevant in temperature stress adaptation, where enzymes and membranes functionality can be compromised (Bita and Gerats

2013). Thus, the ascorbate pool and redox state are essential for minimizing the damage to photosynthetic apparatus caused by temperatures stress.

Although the mitochondrial ROS production is considerably less than in illuminated chloroplasts or in peroxisomes, in most conditions, but especially in the dark or in non-green tissues, mitochondria are major generators of ROS (Lázaro et al. 2013; Noctor and Foyer 2016). The DHA transporter located in the inner mitochondrial membrane is an important member of the ascorbate regeneration machinery (Szarka et al. 2004). Similarly to the mechanisms described to prevent excessive energy in the chloroplastic electron chain, DHA can be an alternative acceptor in the mitochondrial complex II while ascorbic acid can act as an electron donor to complex IV (Szarka et al. 2007, 2013). This electron route through the AsA/DHA pair may provide an alternative route in case of complex III damage by taking up electrons at complex II (by the reduction of DHA to ascorbate) and providing electrons through a bypass to complex IV (by the oxidation of ascorbate to DHA) (Szarka et al. 2013). Plant mitochondria have also emerged as an important site for reactive nitrogen species (RNS), and these reactive species, like ROS, can generate oxidative damage but also present signalling roles generating reversible changes in the redox state of target molecules. In addition, RNS are involved in S-glutathionylation and S-nitrosylation protein modifications (Lázaro et al. 2013). These functions are important in response to stress conditions and are actively modulated by antioxidant mechanisms like the ascorbate-glutathione cycle (Martí et al. 2011).

It should be reminded that the last step of AsA biosynthesis is dependent on respiratory electrons, and therefore the respiratory electron flow (and thus the respiratory rate) has a regulatory role in ascorbate biosynthesis (Millar et al. 2003). In mitochondria, both AsA synthesis and recycling depend on respiratory electrons, thus perturbations of mitochondrial metabolism will cause respiration-dependent changes of AsA metabolism that would regulate retrograde signalling as a common signal from both mitochondria and chloroplasts (Szarka et al. 2007; Talla et al. 2011). An example of such an inter-organelle communication is the AsA produced in the mitochondria and then transported into the apoplast. Thus, AsA levels and redox state have been shown to modulate photosynthesis through mitochondrial metabolism in order to protect photosynthesis against photoinhibition (Lázaro et al. 2013; Talla et al. 2011), especially under heat, salinity, and drought stress (Lázaro et al. 2013; Pastore et al. 2006).

Extreme temperatures can impose severe disturbances to electron transport chains in chloroplast and mitochondria, requiring excess energy dissipation mechanisms, as well as ROS and oxidative damage detoxification mechanisms where ascorbate pool and redox state play an essential role. As AsA is synthesized and recycled in mitochondria with electrons from the respiratory electron chain and is sensible to its electron flux, there is a possible role for ascorbate in coordinating the rates of respiration, the tricarboxylic acid cycle, and photosynthesis to environmental and developmental stressors.

3.3 Drought and Salt Stress

The stomatal closure caused by drought and subsequent limitation of CO_2 entry reduces NADPH consumption for assimilation, and thus the available $NADP^+$. When this final electron acceptor is limited, the chloroplastic electron chain can accumulate excess energy, dissipated by the above described mechanisms that require a readily available ascorbate pool and redox state (see Table 1 and Sect. 3.1). Nevertheless, these are not the only tolerance mechanisms in which AsA pool and redox state are essentials.

In the apoplast, ROS are not only damaging reactive species, but also signalling molecules, like H_2O_2 in guard cells which controls gas exchange. Absorbed light energy excess, for example during peak sunlight when irradiance can exceed photosystem's photosynthetic capacity, can increase H_2O_2 production, triggering stomatal closure (Schroeder et al. 2001). During the day, AsA is consumed for H_2O_2 reduction and the rate-limiting DHAR activity causes an increase of DHA contents. Thus, the signalling role of H_2O_2 in stomatal conductance is modulated by both its rate of productions and removal, the latter determined by the AsA pools, its redox state, and DHAR recycling activity. The manipulation of DHAR activity, either overexpressed or suppressed, changes the efficiency of AsA recycling and thus H_2O_2 scavenging. DHAR overexpression increases AsA/DHA redox state towards a more efficient H_2O_2 scavenging, maintaining H_2O_2 at levels that do not trigger stomatal closure, therefore presenting increased transpiration and water loss under both normal and water stress conditions (Chen et al. 2003; Chen and Gallie 2004). Meanwhile, the suppression of DHAR expression causes an accumulation of H_2O_2, inducing more stomatal closure under the same water conditions (Chen and Gallie 2004). Actually, exogenous DHA promotes rapid stomatal closure, while AO overexpressing plants present impaired control of stomatal aperture, suggesting that not only the redox state but also the DHA levels and its modulation are regulators of stomatal dynamics, a key trait of tolerance to drought (Fotopoulos et al. 2008).

The size and redox state of the AsA pool influences plant metabolism and its plasticity for stress tolerance responses not only through AsA antioxidant functions. For example, influencing free amino acid levels. The manipulation of levels of AsA, DHA and the AsA redox state by the combination of mutants and chemical treatments with AsA, reduced glutathione and the synthetic reductant dithiothreitol (DTT), showed their influence on total free amino acid levels, and individual proteinogenic and non-proteinogenic amino acids levels (Gulyás et al. 2017). The actual redox state of AsA (and GSH) regulates free amino acids levels because this regulation was not observed by the synthetic reductant DTT (Gulyás et al. 2017). Free amino acids are essential in response against stresses, specially salt, heavy metal, and drought stress where free amino acids and derived molecules contribute to osmotic adjustment, metal binding, antioxidant defense, and signalling (Sharma and Dietz 2006; Vranova et al. 2011).

The AsA redox state in different compartments can actively modulate tolerance responses like stomatal closure and free amino acids contents, contributing to limiting transpiration, and promoting osmotic adjustments and metal chelation.

3.4 Flooding

Generation of reactive oxygen species (ROS) is characteristic for hypoxia and especially for reoxygenation. Consequences of hypoxia-induced oxidative stress depend on the tolerance to anoxia, membrane properties, endogenous antioxidant content, and on the ability to induce the response in the antioxidant systems. The switch to anaerobic metabolism and the preservation of the redox status are necessary for survival. Overexpressing experiments leading to increased antioxidants production do not always result in the enhancement of the antioxidant defense shield, and hence increased antioxidant capacity does not always correlate positively with the degree of protection (Herschbach et al. 2010). Plants tolerant to anaerobic conditions present significant increase in the reduced forms of ascorbate and ascorbate recycling enzymes, but if the stress endures recycling enzymes and AsA/DHA ratio decrease. DHA is the transported form of ascorbate through the phloem, generally from photosynthetic tissues to autotrophic organs and tissues with limited oxygen availability like root tips (Franceschi 2002; Herschbach et al. 2010). The interruption of this transport changes ascorbate contents and redox state across the entire root system (Herschbach et al. 2010). This transport might be important to act as a signal of redox imbalance due to stress between different tissues/organs, as shoot to root signal to coordinate growth and response to environmental stressors.

3.5 Metal Stress

Heavy metals generate a disturbance of the cellular redox balance and impair metabolism, leading to a rise of ROS generation. AsA pool and redox state play essential roles in avoidance (chelation) and tolerance mechanisms (e.g., ROS detoxification).

As discussed above, DHA content modulates free amino acids contents, notably many with chelating functionality. For example, hydroxyl radicals ($^{\bullet}OH$) generated by trace levels of transition metals by the metal-catalyzed Haber–Weiss reaction (producing $^{\bullet}OH$ from H_2O_2 and O_2^-) can nonenzymatically be detoxified by AsA (Buettner and Jurkiewicz 1996). Previously presented enzymatic and nonenzymatic ROS scavenging mechanisms that require a readily available pool of reduced AsA are also relevant in metal stress. Moreover, trace levels of transition metals can catalyze the oxidation of AsA (Buettner and Jurkiewicz 1996). Similarly, it has been observed that under high concentrations of iron together with high rates of AsA recycling leading to a sustained reduced AsA redox state, confer sensitivity to

exposed plants. This result suggests the existence of pro-oxidant activity of reduced AsA in the presence of high concentrations of iron *in planta* (Wu et al. 2017).

3.6 AsA Redox State in the Apoplast: Biotic Stress and Environmental Oxidative Pollutants

Ozone and other oxidative pollutants (notably nitrogen and sulfur oxides) rapidly degrade into hydroxyl when entering a plant through guard cells, rapidly spreading the oxidative strain to other cells (Sandermann et al. 1998). Damage extension is limited by avoidance, closing stomata to reduce their entrance into leaves; or by tolerance, detoxifying ROS that get into the plant (Vainonen and Kangasjärvi 2015). Apoplastic AsA acts directly as a first line of defense against environmental oxidants such as ozone, SO_2, and NO_2 (Plöchl et al. 2000), being ozone tolerance correlated to the levels of apoplastic AsA (Burkey et al. 2003). As described previously, AsA recycling capacity and apoplastic AsA redox state and DHA levels regulate the responsiveness of guard cells to ROS accumulation. Thus, apoplastic DHA levels and AsA redox state modulate stomatal closure as a pollutants avoidance mechanism (Yoshida 2005).

There is significant overlap between the signalling pathways and response factors activated by pathogens and those induced by ozone or reduced ascorbate contents (Bostock et al. 2014; Sandermann et al. 1998). Probably, common signalling pathways are triggered by increased apoplastic oxidative stress, as promoted by both environmental oxidants and the pathogen-induced oxidative burst during the hypersensitive response. AsA levels, AsA redox state, and AsA recycling capacity are strongly associated with resistance to pathogenic virus (Fujiwara et al. 2016), bacteria, and fungi (Botanga et al. 2012).

The AsA pool and metabolism in the apoplast regulates environmental perception and its signalling transduction. Environmental oxidative pollutants and pathogen attack oxidize the apoplast promoting a phenomenon named oxidative burst. As the major antioxidant buffer in the apoplastic compartment AsA modulates this signalling and it is associated to developmental and tolerance responses (Horemans et al. 2000; Pignocchi and Foyer 2003). Actually, AsA levels and especially AsA redox state are actively modulated by the apoplastic AO. A number of AO isoforms are differentially expressed in different organs, being actively regulated by both developmental cues and in response to stress conditions (Batth et al. 2017; Ioannidi et al. 2009). The deregulation of AsA redox state via AO overexpression enhances sensitivity to oxidative stress-promoting agents like ozone with an associated suppression of ascorbate recycling genes expression (Fotopoulos et al. 2006; Sanmartin et al. 2003). However, increased AO activity also increased sensitivity to fungal infection, although without showing suppression of AsA recycling genes expression (Fotopoulos et al. 2006). AsA redox state and its modulation through AO are essential for adequate responses to pathogens, becoming a target of virulence factors.

AO function can be actively disrupted by pathogens like the cucumber mosaic virus. In early infection, the movement protein of cucumber mosaic virus associates with apoplastic AO disrupting the formation of functional AO dimers, thus enhancing the spread of virus to nearby cells and reducing the redox defense of the plant during initial stages of infection (Kumari et al. 2016). Open stomata are the main access point of many pathogens, thus the promoted oxidative bursts lead to stomata closure, generating an avoidance response (Bostock et al. 2014). Stomatal closure dynamics are also modulated by AO activity (Fotopoulos et al. 2008).

Oxidized forms of AsA are essential for trans-plasma membrane electron transfer, through the family cytochromes b561 dual function as MDHAR and as Fe^{3+}-reductases (Asard et al. 2013; Griesen et al. 2004; Picco et al. 2015). It is also present in the vacuoles membrane where it may participate in storing excessive iron, but its biological functions are better described in the plasma membrane as essential for recycling AsA and for iron uptake. In addition, they act as buffering oxidant pollutants and modulating the signalling associated to the oxidative burst caused by pathogens.

Apoplastic AsA pool and redox state is actively modulated by apoplastic AO and plasmalemma cytochromes b561, and this modulation is essential for proper abiotic and biotic stress avoidance and tolerance responses. Mechanisms regulated by the AsA/DHA redox pair in the apoplast include signalling modulation (oxidative burst), stomatal closure, antioxidant buffer and environmental oxidants detoxification.

4 Conclusions and Perspectives

Ascorbate is the most abundant hydrosoluble antioxidant in plants, with multiple antioxidant and non-antioxidant functions. It is not only the major redox buffer with regulatory roles in many compartments, but it is also a cosubstrate of essential enzymatic reactions. The active modulation of ascorbate redox state in specific compartments is essential to successfully regulate development and growth while integrating environmental stimuli to modulate stress responses.

The modification of AsA/DHA relative contents by exogenous applications and molecular techniques has revealed many developmental and growth processes regulated by the AsA pool and redox state. However, our explanation of the underlying mechanisms still lacks specific targets or receptors of these disturbances. A highly active focus of ascorbate research is the regulation of the transport of the specific AsA, DHA and its redox power between compartments and between tissues, and its relevance in developmental and stress responses. The maintenance of specific AsA/DHA redox state in different compartments is essential to successfully regulate development and growth and to modulate avoidance and tolerance stress responses. Ascorbate is synthesized in the mitochondria but required for all compartments for

its detoxifying and regulatory roles, as well as a cosubstrate of enzymatic reactions and to coordinate plant energy metabolism. In addition to the plasma membrane DHA transporter (Horemans et al. 1997), other regulatory elements of the relationship of ascorbate contents and its redox state between compartments have only recently been described, and the study of their functional regulation is an active field. A recent study characterized the chloroplast transporter AtPHT4;4 while providing indications of a number of other possible plastidial ascorbate transporters (Miyaji et al. 2015). Yet many putative transporters remain to be identified and characterized, necessary to unravel the dynamics between different compartments. In addition, the recently described cytochrome b561 capacity to use ascorbate reduction power across compartments (Picco et al. 2015) raises many questions about the relationship between ascorbate pools and redox states in different compartments, its regulation, and its potential regulation role.

Despite plant cell energetic metabolism is essential for adaptation to environmental and developmental stressors; mitochondrial metabolism under stress is still largely unknown but there are hint that AsA and its redox state participate in its adaptation and coordination with other organelles. Both AsA and DHA participate in energy dissipation mechanisms in the electron transport chains of mitochondria and chloroplasts, while modulating ROS and RNS detoxification and related redox sensing. Therefore, the AsA pool and redox state may have a signalling role coordinating the chloroplast/cytosol/mitochondrion cooperation, especially under stress conditions, aimed at modulating cell redox homeostasis across all intra- and extracellular compartments.

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The Role of Plant High-Throughput Phenotyping in the Characterization of the Response of High Ascorbate Plants to Abiotic Stresses

Jessica P. Yactayo-Chang, Lucia M. Acosta-Gamboa, Nirman Nepal, and Argelia Lorence

Abstract L-Ascorbic acid (AsA, ascorbate or vitamin C) is the most abundant water-soluble antioxidant found in plants. Ascorbate is synthesized via four pathways involving D-mannose/L-galactose, D-galacturonate, L-gulose, and *myo*-inositol as main precursors. In addition to protecting plant tissues from damage caused by reactive oxygen species produced through normal oxygenic metabolism or those generated from biotic and abiotic stresses, ascorbate is also an enzyme cofactor and a modulator of cell division, cell expansion, flowering time, and gene regulation. Plants that are deficient in ascorbate are affected in multiple ways including alterations in cell division, cell expansion, seed germination, growth, floral induction, and photosynthesis. Additionally, elevated ascorbate content in plants leads to an increase in their nutritive value, lengthening of their shelf life, enhancement on their growth rate and biomass accumulation, and to an increased tolerance to multiple abiotic stresses including salt, cold, heat, and water deficit. Increasing the productivity of crops is imperative to satisfy the growing demand for food, feed, and fuels in the world, and biotechnology can lead to the development of plants with higher yields capable of thriving under adverse conditions. To this end, phenotype screening and characterization of a large number of plants experimentally obtained is time consuming and requires a significant amount of resources, skills, and expertise. In this chapter, we will present an overview of how the use of high-throughput phenotyping or phenomics is revolutionizing the way plant phenotypes are characterized and will illustrate the power of digital phenotyping in the characterization of plants overexpressing enzymes in the inositol pathway to ascorbate.

J. P. Yactayo-Chang · L. M. Acosta-Gamboa · N. Nepal
Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

A. Lorence (✉)
Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

Department of Chemistry and Physics, Arkansas State University, Jonesboro, AR, USA
e-mail: alorence@astate.edu

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1 Introduction

L-Ascorbic acid (ascorbate, AsA) is the most abundant water-soluble antioxidant found in plants. Ascorbate content varies widely among species but in general AsA concentration in plants is in the mM range (Noctor and Foyer 1998). Ascorbate has a wide variety of physiological roles. It functions as an enzyme cofactor, as a modulator of the cell cycle, and as a scavenger protecting tissues against damage caused by reactive oxygen species (ROS) produced from a multitude of environmental stresses including water deficit, soil salinity, cold, and heat, among others. Alteration of the redox balance resulting in accumulation of ROS (e.g., singlet oxygen, superoxide, and hydrogen peroxide) is a feature common to multiple environmental stresses. When the intensity of those stresses exceeds the antioxidant and repair capacity of cells, ROS can accumulate and cause oxidative damage to all macromolecules, promoting apoptosis and senescence. On the other hand, there is growing evidence of the important role of low levels or ROS as signaling molecules in pathways that stimulate adaptive hormonal and metabolic responses. Therefore whether ROS induce oxidative damage or trigger acclimation depends on the rate and site of production and is controlled by the antioxidant system, of which ascorbate and glutathione are central components (Foyer et al. 1994; Foyer and Noctor 2011).

A better understanding about how ascorbate is made in plants and the factors that regulate its content is of chief importance because this molecule provides the dietary source of vitamin C to humans, primates, and other animals. Mutations in the terminal enzyme of the animal vitamin C pathway (L-gulono-1,4-lactone oxidase) are what render its synthesis impossible for humans and make this an essential vitamin that we must acquire through diet (Linster and Van Schaftingen 2007). Deficiency of ascorbic acid is often associated with human health problems such as anemia, infections, bleeding gums, scurvy, poor wound healing, capillary hemorrhage, muscle degradation, atherosclerotic plaques, and neurotic disturbances (Chambial et al. 2013). Vitamin C deficiency is a common issue for malnourished people in developing countries, and also for various subpopulation groups in developed countries including those who are incarcerated, smokers, widows, and people who suffer various eating and nutrient absorption disorders (Beal et al. 2017).

Vitamin C is involved in essential physiological processes that impact the agronomical value of crops and its physiology. The growth and the world population present unique challenges to agriculture. We need to find ways to make crops more productive with less inputs. One of the potential solutions to this great challenge is the development of improved crops with enhanced nutritional value, higher tolerance to environmental insults, and superior yields.

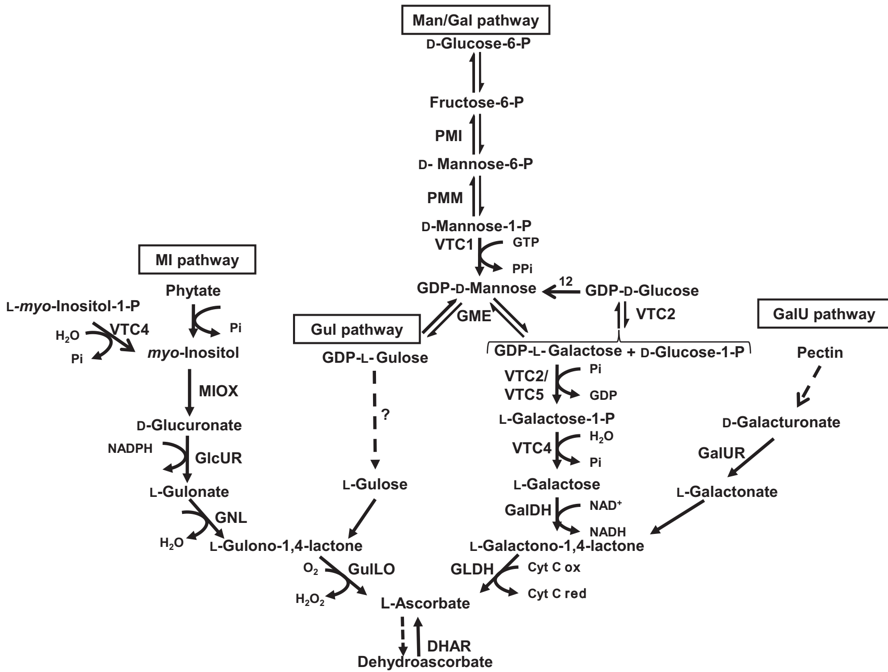


Fig. 1 The ascorbate metabolic network. There is a single biosynthetic pathway for L-ascorbic acid (ascorbate, vitamin C) in some animals, while four distinct routes lead to its formation in plants: MI (*myo*-inositol) pathway, Gul (L-gulose) pathway, Man/Gal (D-mannose/L-galactose) pathway, and GalU (D-galacturonic acid) pathway. The enzymes involved in the four different routes are: MI pathway: *myo*-inositol oxygenase (MIOX); glucuronate reductase (GlcUR); gluconolactonase (GNL); and L-gulono-1,4-lactone oxidase (GuILO). Man/Gal pathway: phosphomannose isomerase (PMI); phosphomannose mutase (PMM); GDP-mannose pyrophosphorylase (VTC1); GDP-3′5′-epimerase (GME); L-galactose guanyltransferase (VTC2); L-galactose-1-phosphate phosphatase (VTC4); L-galactose dehydrogenase (GalDH); L-galactono-1,4-lactone dehydrogenase (GLDH). GalU pathway: D-galacturonate reductase (GalUR). The enzymes in the recycling pathway are monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Adapted from Suza et al. 2010

There are five routes for the production of AsA, one in animals and four in plants (Fig. 1). The biosynthetic pathway for AsA in animals was elucidated in the early 1950s (Ishikawa et al. 2006). On the other hand, plants possess four possible ways to make this key molecule. These are the D-mannose/L-galactose (Wheeler et al. 1998), L-gulose (Wolucka and Van Montagu 2003), D-galacturonate (Agius et al. 2003), and *myo*-inositol (Lorence et al. 2004) routes (Fig. 1). The best characterized of these pathways is the D-mannose/L-galactose route. The degree of which the so called "alternative routes" is operational in each plant species and the tissue and temporal specificity of these alternative routes is currently unclear.

In this chapter, we present an overview of the successful strategies to metabolically engineer elevated AsA content in model plants and crops and summarize the

studies in which the stress tolerance of those high ascorbate plants has been analyzed. We also discuss how the use of high-throughput phenotyping or phenomics is revolutionizing the way plant phenotypes are characterized and illustrate the power of digital phenotyping in the development of model plants with elevated AsA and enhanced ability to withstand stresses.

2 Successful Metabolic Engineering Strategies to Enhance Ascorbate Content in Plants

During the last 20 years, we have witnessed an unprecedented progress in the understanding of the multiple pathways leading to AsA formation in plants. Table 1 summarizes the successful strategies that have been reported in the engineering of elevated AsA content in plants.

All genes in the D-mannose/L-galactose pathway have been cloned and characterized, and several of those have been used to increase AsA content in more than one species. Overexpression of those genes has led to a 1.2-fold to 6-fold AsA increase.

Phosphomannose mutase (PMM) is one of the key enzymes in the regulation of the initial steps of the AsA synthesis via the D-mannose/L-galactose pathway (Fig. 1). Badejo et al. (2009) developed transgenic tobacco (*Nicotiana tabacum*) overexpressing PMM from acerola (*Malpighia glabra*), leading to a 2-fold AsA increase compared to controls.

Overexpression of kiwifruit GDP-L-galactose phosphorylase increased AsA content 6-fold in tomato fruits, while overexpression of this gene in potato increased AsA content in the tubers (Bulley et al. 2012). This gene has also been expressed in Arabidopsis (Zhou et al. 2012) and rice (Zhang et al. 2015).

The importance of GDP-galactose guanylyl transferase (GGT) as a rate-limiting step in the production of AsA production was confirmed with the overexpression of the *GGT* gene in Arabidopsis resulting in a 2.9-fold increase in AsA content (Zhou et al. 2012).

The overexpression of tomato GME leads to an increase of 1.2-fold in the AsA content of leaves and a boost of 1.6-fold in fruits (Zhang et al. 2011). This gene has also been successfully expressed in Arabidopsis leaves (Zhou et al. 2012; Huang et al. 2014) and alfalfa shoots (Ma et al. 2014).

GDP-D-Mannose pyrophosphorylase has been widely studied in a number of plants including tobacco (Badejo et al. 2008), tomato (Cronje et al. 2012), and Arabidopsis (Zhou et al. 2012; Sawake et al. 2015). The AsA content of transgenic tobacco plants expressing acerola GMP was 2-fold higher compared to wild type (Badejo et al. 2008). Overexpression of L-galactose dehydrogenase (GalDH) in Arabidopsis resulted in a modest increase (1.2-fold) in AsA content (Zhou et al. 2012).

Table 1 Metabolic engineering strategies to increase ascorbate in plants

Pathways regulatory factors	Gene transformed	Gene source	Species transformed	Tissue examined	Max fold increase	Reference
D-Mannose/L-Galactose pathway	Phosphomannose mutase	Acerola (*Malpighia glabra*)	Tobacco (*Nicotiana tabacum*)	Leaves	2.0 fold	Badejo et al. (2009)
	GDP-L-galactose phosphorylase	Kiwifruit (*Actinidia chinensis*) Potato (*Solanum tuberosum*) Mouse ear cress (*Arabidopsis thaliana*)	Tomato (*Solanum lycopersicum* cv. UC82B) Potato (*Solanum tuberosum* cv. Ranger Russet) Strawberry (*Fragaria X ananassa* cv. Camrosa)	Leaves/fruits Tubers Leaves/fruits	2.0 fold/6.0 fold 4.0 fold 2.0 fold/2.0 fold	Bulley et al. (2012)
	GDP-L-galactose phosphorylase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.5 fold	Zhou et al. (2012)
	GDP-L-galactose phosphorylase	Mouse ear cress (*Arabidopsis thaliana*)	Rice (*Oryza sativa* cv. Wuyujing 3)	Leaves	2.6 fold	Zhang et al. (2015)
	GDP-galactose guanylyl transferase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	2.9 fold	Zhou et al. (2012)
	GDP-mannose-3′5′-epimerase	Tomato (*Solanum lycopersicum*)	Tomato (*Solanum lycopersicum* cv. Ailsa Craig)	Leaves Fruits	1.2 fold 1.6 fold	Zhang et al. (2011)
	GDP-mannose-3′5′-epimerase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.4 fold	Zhou et al. (2012)
	GDP-mannose-3′5′-epimerase	Chestnut rose (*Rosa roxburghii*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	2.0 fold	Huang et al. (2014)
	GDP-mannose-3′5′-epimerase	Alfalfa (*Medicago sativa*)	Mouse ear cress (*Arabidopsis thaliana*)	Shoots	1.8 fold	Ma et al. (2014)
	GDP-D-mannose pyrophosphorylase	Acerola (*Malpighia glabra*)	Tobacco (*Nicotiana tabacum*)	Leaves	2.0 fold	Badejo et al. (2008)

(continued)

Table 1 (continued)

Pathways regulatory factors	Gene transformed	Gene source	Species transformed	Tissue examined	Max fold increase	Reference
D-Mannose/L-Galactose pathway	GDP-D-mannose pyrophosphorylase	Yeast (*Saccharomyces cerevisiae*)	Tomato (*Solanum lycopersicum* cv. Money Maker)	Leaves Fruits	1.7 fold 1.5 fold	Cronje et al. (2012)
	GDP-D-mannose pyrophosphorylase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.3 fold	Zhou et al. (2012)
	GDP-D-mannose pyrophosphorylase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.2 fold	Sawake et al. (2015)
	L-galactose dehydrogenase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.2 fold	Zhou et al. (2012)
	L-galactono-1,4-lactone dehydrogenase	No available	Rice (*Oryza sativa* cv. Zhonghua 11)	Leaves	1.5 fold	Liu et al. (2011)
	L-galactono-1,4-lactone dehydrogenase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.8 fold	Zhou et al. (2012)
	L-galactono-1,4-lactone dehydrogenase	Chestnut rose (*Rosa roxburghii*)	Tobacco (*Nicotiana tabacum* cv. Xanthi)	Leaves	2.1 fold	Liu et al. (2013)
	L-galactono-1,4-lactone dehydrogenase	Lettuce (*Lactuca sativa*)	Lettuce (*Lactuca sativa* cv. Iceberg)	Leaves	1.3 fold	Landi et al. (2015)
	L-galactono-1,4-lactone dehydrogenase	Mouse ear cress (*Arabidopsis thaliana*)	Rice (*Oryza sativa* cv. Wuyujing 3)	Leaves	1.5 fold	Zhang et al. (2015)
	GDP-galactose guanylyl transferase + L-galactose-1-phosphate phosphatase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	4.1 fold	Zhou et al. (2012)
	GDP-galactose guanylyl transferase + L-galactono-1,4-lactone dehydrogenase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	3.6 fold	Zhou et al. (2012)

D-Mannose/L-Galactose pathway	GDP-D-mannose pyrophosphorylase + GDP-mannose-3′5′-epimerase	Peach (*Prunus persica*)	Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1)	Young leaves Old leaves	1.0 fold 1.0 fold	Imai et al. (2012)
D-Galacturonate pathway	Galacturonic acid reductase	Strawberry (*Fragaria* x *ananassa*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	3.0 fold	Agius et al. (2003)
	Galacturonic acid reductase	Strawberry (*Fragaria* x *ananassa*)	Potato (*Solanum tuberosum* cv. Taedong Valley)	Tubers	2.0 fold	Hemavathi et al. (2009)
	Galacturonic acid reductase	Strawberry (*Fragaria* x *ananassa*)	Tomato (*Solanum lycopersicum* cv. Money Marker)	Hairy roots	2.5 fold	Wevar Oller et al. (2009)
	Galacturonic acid reductase	Strawberry (*Fragaria* x *ananassa*)	Tomato (*Solanum lycopersicum* cv. Money Marker)	Leaves Fruits	1.3 fold 1.4 fold	Amaya et al. (2014)
	Galacturonic acid reductase	Strawberry (*Fragaria* x *ananassa*)	Tomato (*Solanum lycopersicum* cv. Ailsa Craig)	Leaves Fruits	1.6 fold 2.0 fold	Cai et al. (2014)
	Galacturonic acid reductase	Strawberry (*Fragaria* x *ananassa*)	Tomato (*Solanum lycopersicum*, cherry tomato)	Fruits	2.5 fold	Lim et al. (2016)
Myo-inositol pathway	Phytase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	2.0 fold	Zhang et al. (2008)
	Myo-inositol oxygenase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	3.0 fold	Lorence et al. (2004)
	Myo-inositol oxygenase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.7 fold	Tóth et al. (2011)
	Myo-inositol oxygenase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Fruits	1.4 fold	Cronje et al. (2012)

(continued)

Table 1 (continued)

Pathways regulatory factors	Gene transformed	Gene source	Species transformed	Tissue examined	Max fold increase	Reference
Myo-inositol pathway	*Myo*-inositol oxygenase	Mouse ear cress (*Arabidopsis thaliana*)	Tomato (*Solanum lycopersicum* cv. MicroTom)	Fruits	3.0 fold	Kulkarni (2012)
	Myo-inositol oxygenase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.2 fold	Yactayo-Chang (2011)
	Myo-inositol oxygenase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.6 fold	Lisko et al. (2013)
	Glucuronic acid reductase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	2.0 fold	Yactayo-Chang (2011)
	Gluconolactonase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	2.6 fold	Yactayo-Chang (2016)
	L-Gulono-1,4-lactone oxidase	Rat (*Rattus norvegicus*)	Tobacco (*Nicotiana tabacum* cv. Xanthi) Lettuce (*Lactuca sativa)*	Leaves Leaves	7.0 fold 7.0 fold	Jain and Nessler (2000)
	L-Gulono-1,4-lactone oxidase	Rat (*Rattus norvegicus*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	3.0 fold	Radzio et al. (2003)
	L-Gulono-1,4-lactone oxidase	Rat (*Rattus norvegicus*)	Potato (*Solanum tuberosum* cv. Taedong Valley)	Tubers	1.4 fold	Hemavathi et al. (2010)
	L-Gulono-1,4-lactone oxidase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.8 fold	Lisko et al. (2013)
	Arabino-1,4-lactono oxidase	Yeast (*Saccharomyces cerevisiae*)	Tomato (*Solanum lycopersicum* cv. Money Marker)	Leaves Fruits	1.5 fold 1.3 fold	Cronje et al. (2012)
	Arabino-1,4-lactono oxidase	Yeast (*Saccharomyces cerevisiae*)	Stylo (*Stylosanthes guianensis*, Aublet, Swartz)	Leaves	3.1 fold	Bao et al. (2016)

Recycling pathway	Dehydroascorbate reductase	Wheat (*Triticum aestivum*)	Tobacco (*Nicotiana tabacum* cv. Xanthi) Maize (*Zea mays*)	Leaves Kernels	2.0 fold 4.0 fold	Chen et al. (2003)
	Dehydroascorbate reductase	Wheat (*Triticum aestivum*) Tobacco (*Nicotiana tabacum*)	Tobacco (*Nicotiana tabacum* cv. Xanthi)	Leaves	2.1 fold	Chen and Gallie (2005)
	Dehydroascorbate reductase	Mouse ear cress (*Arabidopsis thaliana*)	Tobacco (*Nicotiana tabacum* cv. SR-1)	Leaves	2.1 fold	Eltayeb et al. (2006)
	Dehydroascorbate reductase	Sesame (*Sesamum indicum*)	Potato (*Solanum tuberosum* cv. Jowon)	Leaves Tuber	1.5 fold 1.6 fold	Goo et al. (2008)
	Dehydroascorbate reductase	Rice (*Oryza sativa*)	Maize (*Zea mays* cv. M37W)	Endosperm (corn)	6.0 fold	Naqvi et al. (2009)
	Dehydroascorbate reductase	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	4.3 fold	Wang et al. (2010)
	Dehydroascorbate reductase	Mouse ear cress (*Arabidopsis thaliana*)	Potato (*Solanum tuberosum* cv. Atlantic)	Leaves	2.8 fold	Eltayeb et al. (2011)
	Dehydroascorbate reductase	Tomato (*Solanum lycopersicum*)	Tomato (*Solanum lycopersicum*)	Leaves Fruits	1.6 fold	Haroldsen et al. (2011)
	Dehydroascorbate reductase	Potato (*Solanum tuberosum*)	Tomato (*Solanum lycopersicum* cv. Sy12f)	Leaves Fruits	1.9 fold 1.4 fold	Li et al. (2012)
	Dehydroascorbate reductase	Chestnut rose (*Rosa roxburghii*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	3.0 fold	Huang et al. (2014)

(continued)

Table 1 (continued)

Pathways regulatory factors	Gene transformed	Gene source	Species transformed	Tissue examined	Max fold increase	Reference
Recycling pathway	Dehydroascorbate reductase	Kiwifruit (*Actinidia chinensis*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.3 fold	Liu et al. (2015)
	Monodehydroascorbate reductase	Mouse ear cress (*Arabidopsis thaliana*)	Tobacco (*Nicotiana tabacum* cv. SR-1)	Leaves	2.2 fold	Eltayeb et al. (2007)
	Monodehydroascorbate reductase	Tomato (*Solanum lycopersicum*)	Tomato (*Lycopersicon esculentum* cv. Zhongshu)	Leaves	1.2 fold	Li et al. (2010)
	Monodehydroascorbate reductase	Tomato (*Solanum lycopersicum*)	Tomato (*Solanum lycopersicum*)	Leaves	1.3 fold	Gest et al. (2013)
Master regulators	Ascorbic acid mannose pathway regulator 1	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	3.0 fold	Zhang et al. (2009)
	KNOTTED-like homeobox gene	Norway spruce (*Picea abies*)	Norway spruce (*Picea abies* cv. Karst)	Embryogenic cells	1.7 fold	Belmonte and Stasolla (2009)
	Ethylene response factor	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.7 fold	Zhang et al. (2012)
	Photomorphogenic factor COP9 signalosome subunit 5B	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Seedlings	1.4 fold	Wang et al. (2013)
	Pyrophosphorylase-like protein	Mouse ear cress (*Arabidopsis thaliana*)	Mouse ear cress (*Arabidopsis thaliana*)	Leaves	1.4 fold	Sawake et al. (2015)
	Dof-type transcription factor	Tomato (*Solanum lycopersicum*)	Tomato (*Solanum lycopersicum* cv. Ailsa Craig)	Fruits	1.3 fold	Cai et al. (2016)
Other pathways	Malate dehydrogenase	Tomato (*Solanum lycopersicum*)	Tomato (*Solanum lycopersicum* cv. Money Marker)	Leaves	5.7 fold	Nunes-Nesi et al. (2005)
	At4g26850 (Uncharacterized gene)	Kiwifruit (*Actinidia chinensis*)	Tobacco (*Nicotiana benthamiana*)	Leaves	3.0 fold	Laing et al. (2007)

L-Galactono-1,4-lactone dehydrogenase (GLDH) catalyzes the last step in the D-mannose/L-galactose pathway to AsA (Fig. 1). Liu et al. (2011) showed that suppression of *GLDH* expression resulted in a loss of chlorophyll, lower ribulose-1,5-bisphosphate carboxylase (RUBISCO) protein content, and a lower rate of CO_2 assimilation. As a consequence, a slower rate of plant growth and lower seed set were observed. However, overexpression of this enzyme maintained high levels of chlorophyll, RUBISCO protein, and a higher rate of net photosynthesis, resulting in higher seed set. Also increased *GLDH* expression correlated with the reduced lipid peroxidation, indicating that this enzyme functions protecting the plant against ROS (Liu et al. 2011, 2013). GLDH has been overexpressed in multiple plants including rice (Liu et al. 2011; Zhang et al. 2015), Arabidopsis (Zhou et al. 2012), tobacco (Liu et al. 2013), and lettuce (Landi et al. 2015) leading to 1.3-fold to 2.1-fold AsA content compared to controls.

Engineering increased AsA content using a combination of genes has been more successful than using a single enzyme. Zhou et al. (2012) overexpressed the combination of GGT + GPP and GGT + GLDH separately in Arabidopsis. Their results indicate that the GGT + GPP transgenic lines performed best leading to a 4.1-fold AsA content.

Ripening strawberry has been reported to synthesize AsA from D-galacturonic acid and its methyl ester (Mapson and Isherwood 1956; Loewus and Kelly 1961). In 2003, Agius et al., discovery of a D-galacturonic acid reductase (*GalUR*) gene from strawberry, open the possibility to engineer AsA content using this new gene. GalUR is the only enzyme identified in this pathway, which has been shown to be cytosolic (Agius et al. 2003). GalUR overexpression has led to a 2- to 3-fold AsA increase in Arabidopsis (Agius et al. 2003). In plant storage organs, possible sources of AsA include in situ synthesis and long-distance transport of AsA synthesized in other tissues. Hemavathi et al. (2009) developed transgenic potatoes via overexpression of GalUR. These resulting potato tubers had two times more AsA than controls. Transgenic tomato hairy roots overexpressing GalUR had 2.5-fold AsA (Wevar Oller et al. 2009) while overexpression of GalUR in fruits led to 1.4- to 2.5-fold AsA increases (Amaya et al. 2014; Cai et al. 2014; Lim et al. 2016).

Biochemical and molecular data indicate that *myo*-inositol can also be a precursor for the biosynthesis of AsA in Arabidopsis (Lorence et al. 2004; Zhang et al. 2008). This pathway involves four enzymes, starting from the oxidation of *myo*-inositol to D-glucuronic acid and further reduction to L-gulonic acid and to L-gulono-1,4-lactone, and further conversion to AsA. These reactions are catalyzed by *myo*-inositol oxygenase (MIOX), glucuronate reductase (GlcUR), gluconolactonase (GNL), and L-gulono-1,4-lactone oxidase (GuILO, a.k.a. GLOase), respectively. These four enzymes have been already characterized by the Lorence Laboratory (Table 1). *A*rabidopsis lines overexpressing MIOX contained up to 3-fold ascorbate in leaves and presented increased biomass accumulation, growth rate compared to controls (Lorence et al. 2004). These results are similar with the findings by Kulkarni (2012) that showed a 3-fold increase in AsA content after transient *AtMIOX4* expression in tomato fruits. Glucuronate reductase (GlcUR) is the second enzyme in the inositol pathway to AsA, and an isoform from Arabidopsis has been already

characterized (Lorence and Nessler 2007; Yactayo-Chang 2011). The overexpression of GlcUR in Arabidopsis led to a 2-fold ascorbate content in leaves compared to the wild-type controls (Yactayo-Chang 2011). The first two enzymes in the inositol pathway to AsA seem to be cytosolic. The Lorence Laboratory has evidence indicating that some isoforms of the last two enzymes in this pathway reside in the chloroplast (Yactayo-Chang 2016) and the endoplasmic reticulum (ER) (Aboobucker 2014; Aboobucker et al. 2017), respectively. Our group recently characterized a GNL isoform that is localized in the plastids. When overexpressed in Arabidopsis, transgenics GNL over-expressers have a 5-fold AsA content in leaves (Yactayo-Chang 2016; Yactayo-Chang and Lorence 2016). The last enzyme in the inositol pathway to AsA is L-gulono-1,4-lactone oxidase (GulLO). GulLO belongs to the aldonolactone oxidoreductases protein family. GulLO participates in both the L-gulose and inositol pathways to ascorbate. Transgenic tobacco and lettuce plants expressing rat GulLO accumulated up to 7-fold more ascorbate than untransformed plants (Jain and Nessler 2000), while Arabidopsis lines overexpressing the rat isoform were reported to contain a more modest AsA increase (Lisko et al. 2013).

Heick et al. (1972) reported that AsA has been found in a number of yeasts including *Saccharomyces cerevisiae*. The arabino-1,4-lactone oxidase (ALO) enzyme catalyzes the last step in the formation of D-erythroascorbate in yeast. This enzyme uses L-galactono-1,4-lactone as efficiently as D-arabino-1,4-lactone to produce AsA. Overexpression of ALO resulted in elevated ascorbate (1.3-fold) in tomato fruits and stylo leaves (3.1-fold) (Cronje et al. 2012; Bao et al. 2016).

Once oxidized, AsA can also be recycled back to its reduced form. The reduced AsA is ready for its role again. Overexpression of dehydroascorbate reductase (DHAR) and monodehydro ascorbate reductase (MDHAR) has been implemented successfully to engineer plants with elevated AsA. Overexpression of DHAR has led to 1.3-fold to 6-fold AsA increase in various plant species (Table 1). Constitutive expression of MDHAR isoforms has also been successfully implemented to increase AsA content in plants (Table 1).

The existence of four different pathways leading to AsA formation in plants indicates the need of a complex regulatory mechanism for this network. Regulation within and between pathways in the network is largely uncharacterized. Six master regulators for AsA have been described in the literature. The ascorbic acid mannose pathway regulator 1 (AMR1) is one of these regulators. AMR1 negatively regulates the D-Man/L-Gal pathway (Zhang et al. 2009).

Another regulator of this network is the three KNOTTED-like homeobox gene (HBK3). Homeobox genes encode a typical DNA-binding domain of 60 amino acids, known as homeodomain that characterizes a large family of transcription factors. These genes encode transcription factors which regulate important events in plant growth and development. The *HBK3* gene from Norway spruce regulates somatic embryo yield through alterations in glutathione and AsA metabolism, which have been previously implicated in controlling development and maturation both in vivo and in vitro (Belmonte and Stasolla 2009).

The size of the AsA pool in plants is controlled by a combination of its biosynthesis, recycling, and transport and translocation. Some factors that regulate the

AsA pool size by modulating biosynthesis are light, development, and environmental stress conditions. The ethylene response factor (ERF98) is considered a positive regulator of AsA. This regulator enhances tolerance to salt stress through direct activation of AsA synthesis in Arabidopsis (Zhang et al. 2012). Arabidopsis ERF98 transcriptionally activates gene expression of *VTC1* to improve AsA content and tolerance to salt stress.

3 Effect of Ascorbate in the Ability of Plants to Withstand Abiotic Stresses

3.1 Low AsA Plants Are Sensitive to Abiotic Stresses

Ascorbate plays vital role in maintaining the plant health status. Mutation of genes involved in the D-mannose/L-galactose pathway led to the characterization of vitamin C defective (*vtc*) mutants. The *vtc* 1-1, *vtc* 2-1, and *vtc* 3 mutants have 50–75% lower AsA content than wild type. Plants with low AsA are sensitive to heat and light stress compared to control plants (Pavet et al. 2005; Conklin et al. 2013). Interestingly, the *vtc* 1-1 and *vtc* 2-1 lines are resistant to bacterial pathogen, *Pseudomonas syringe* (Tóth et al. 2011). The *vtc* mutant plants are sensitive to osmotic stress and oxidative stress (Cho et al. 2016) as shown in Table 2. This table illustrates that lowering AsA content makes plants more vulnerable to abiotic stresses.

3.2 High AsA Lines Are Tolerant to Abiotic Stresses

Table 3 presents a summary of the studies in which diverse research groups have evaluated the tolerance to stresses of plants with enhanced AsA content. High AsA plants overexpressing genes involved in the D-mannose/L-galactose pathway have been found to be tolerant to salt stress (up to 100 mM NaCl), herbicide stress (up to 75 μM methyl viologen), cold stress (4 °C), and oxidative stress (wounding). Plant biomass, plant height, shoot length, root length, leaf color, survival rate, and seed germination rate are the most common readouts used to assess the tolerance to abiotic stresses in plants (Zhang et al. 2015, Zhang et al. 2011; Landi et al. 2015; Li et al. 2012).

Similarly plants overexpressing D-galacturonate reductase were found to be tolerant to salt stress (up to 600 mM NaCl), herbicide stress (up to 75 μM methyl viologen), cold stress (4 °C), and osmotic stress (up to 300 mM mannitol). Chlorophyll content, root length/biomass and shoot length/biomass, germination response, and malondialdehyde content have been the main readouts used to assess the tolerance to abiotic stresses in these plants (Hemavathi et al. 2009; Cai et al. 2014; Lim et al. 2016).

Table 2 Effect of low ascorbate plants in response to stresses

Pathways regulatory factors	Species transformed	Genes down-regulated	Gene source	Max. fold decrease	Tissue examined	Growth conditions	Stress tested	Treatment	Methodology	Response to stress	Phenotype	Reference
D-Mannose/L-Galactose pathway	*Arabidopsis thaliana*	*vtc3*	*Arabidopsis thaliana*	3.8 fold	Leaves	Growth chamber 23 °C 50% relative humidity 16:8 h photo-period 200 μmol/$m^{-2} s^{-1}$	Light stress	Continuous 150 μmol/$m^2 s^{-1}$ light intensity	Redox status of ascorbate and GalDH activity	Sensitive	Manual	Conklin et al. (2013)
							Heat stress	30 °C for 4 days				
	Arabidopsis thaliana	*vtc1-1*	*Arabidopsis thaliana*	2.9 fold	Leaves	Growth chamber 22 ± 4 °C 70% relative humidity 14:10 h photoperiod 250 μmol/$m^{-2} s^{-1}$	*P. syringae*	Bacterial infiltration at 5 × 10^6 cfu/mL	Bacterial growth Plant cell death	Resistant	Manual	Pavet et al. (2005)
		vtc2-1	*Arabidopsis thaliana*	5.4 fold	Leaves							

D-Mannose/L-Galactose pathway	*Arabidopsis thaliana*	*vtc2-3*	*Arabidopsis thaliana*	1.4 fold	Leaves	Growth chamber 20–24 °C 16:8 h photoperiod 150 μmol/m^{-2} s^{-1}	Heat stress Light stress	40 °C for 15 min 300 μmol/m^2 s^{-1} for 10–60 min	Chlorophyll fluorescence Photosynthesis parameters [photosystem II efficiency, NPQt, chlorophyll fluorescence quenching]	Sensitive	Manual	Tóth et al. (2011)
Regulatory factors	*Arabidopsis thaliana*	*CaM-like protein (CML10)*	*Arabidopsis thaliana*	1.4 fold	Leaves	Growth chamber 50% relative humidity 16:8 h photoperiod	Osmotic stress Oxidative stress	0, 100 or 200 mM mannitol for 3 days 10 mM H_2O_2 for 12 h	Chlorophyll content	Sensitive	Manual	Cho et al. (2016)

Table 3 Summary of studies that have evaluated the tolerance to stress of plants wutg enhanced ascorbate content

Pathways regulatory factors	Species transformed	Gene overexpressed	Max fold increased	Tissue tested	Growth conditions	Stress tested	Treatment	Methodology	Phenotype	Reference
D-Mannose/L-Galactose pathway	*Orzya sativa-Japonica cultivar, Wuyujing 3 (WY3)*	*GDP-mannose phosphorylase, GDP-mannose-3′,5′-epimerase*	2.6 fold	Leaves	Growth chamber 25 °C 75% relative humidity 16:8 h photoperiod 700 μmol m^{-2} s^{-1}	Salt stress	100 mM NaCl	Plant height, fresh biomass, root length, and membrane ion leakage	Manual	Zhang et al. (2015)
	Lycopersicon esculentum	*GDP-Mannose 3′,5′-epimerase*	1.4 fold 1.6 fold	Leaves Fruits	Greenhouse, not mentioned	Methyl viologen stress Cold stress Salt stress	75 μM MV 4 °C for 10 days 100 mM NaCl	Chlorophyll and malondialdehyde content survival rate, seed germination rate	Manual	Zhang et al. (2011)
	Lactuca sativa "Iceberg"	L-*Galactono-1,4-lactone dehydrogenase*	3.0 fold	Leaves	Growth chamber 23 ± 1 °C 16:8 h photoperiod 500 μmol m^{-2} s^{-1}	Oxidative stress	Wounding	Leaf browning	Manual	Landi et al. (2015)
	Nicotiana tabacum "xanthi"	L-*Galactono-1,4-lactone dehydrogenase*	2.1 fold	Leaves	Growth chamber 25 ± 2 °C 14:10 h photoperiod 120 μmol m^{-2} s^{-1}	Salt stress Methyl viologen stress	100 mM NaCl 2 μM MV	Shoot length and biomass, phenotypic changes and chlorophyll content on leaf discs	Manual	Liu et al. (2015)
D-Galacturonate pathway	*Solanum tuberosum*	D-*Galacturonic acid reductase*	2.0 fold	Tubers	Growth chamber 22 ± 2 °C 16:8 h photoperiod 100 μmol m^{-2} s^{-1}	Methyl viologen stress Salt stress Oxidative stress	2, 5 and 10 μM MV 200, 400 and 600 mM NaCl 100, 200 and 300 mM mannitol	Chlorophyll content, root length, and shoot length	Manual	Hemavathi et al. (2009)

	Solanum lycopersicum	D-*Galacturonic acid reductase*	2.0 fold 1.6 fold	Fruits Leaves	Greenhouse 28 °C–24 °C (day/night) 70–80% relative humidity	Methyl viologen stress Cold stress Salt stress	75 µM MV 4 °C for 10 days 200 mM NaCl	Chlorophyll and malondialdehyde Shoot biomass, root biomass, and chlorophyll	Manual	Cai et al. (2014)
	Solanum lycopersicum	*D-galacturonic reductase*	2.5 fold	Leaves	Growth chamber 25 ± 1 °C 100 µmol m^{-2} s^{-1}	Methyl viologen stress Salt stress Osmotic stress	0, 10, 20 µM MV 0, 250, 500 mM NaCl 0, 200, 300 mM mannitol	In vitro germination and response, malondialdehyde content	Manual	Lim et al. (2016)
	Solanum lycopersicum	D-*Galacturonic acid reductase*	2.5 fold	Leaves						
Gulose pathway	*Solanum tuberosum*	L-*Gulono-1,4-lactone oxidase*	1.4 fold	Tubers	Growth chamber Greenhouse 22 ± 2 °C 16:8 h photoperiod 100 µmol m^{-2} s^{-1}	Methyl viologen stress Osmotic stress Salt stress	0, 5, 10 µM MV 0, 200, 300 mM mannitol 0, 400, 600 mM NaCl	Phenotypic changes and chlorophyll content	Manual	Upadhyaya et al. (2010)
	Solanum lycopersicum	L-*Gulono-1,4-lactone oxidase*	1.5 fold	Fruits	Growth chamber 25 ± 1 °C 16:8 h photoperiod 100 µmol m^{-2} s^{-1}	Salt stress plates Salt stress leaf discs Methyl viologen stress Osmotic stress	0, 50 or 100 mM NaCl 0, 250, or 500 mM NaCl 0, 10, or 20 µM MV 0, 200, or 300 mM mannitol	Germination response Malondialdehyde content	Manual	Lim et al. (2012)

(continued)

Table 3 (continued)

Pathways regulatory factors	Species transformed	Gene overexpressed	Max fold increased	Tissue tested	Growth conditions	Stress tested	Treatment	Methodology	Phenotype	Reference
myo-inositol pathway	*Arabidopsis thaliana*	*myo-Inositol oxygenase (MIOX4)*	1.5 fold	Leaves	Growth chamber 20–24 °C 16:8 h photoperiod 150 μmol m^{-2} s^{-1}	Heat stress	40 °C for 15 min	Chlorophyll fluorescence Photosynthesis parameters [photosystem II efficiency, non-photochemical quenching, energy-dependent quenching]	Manual	Tóth et al. (2011)
						Light stress	300 μmol photons m^{-2} s^{-1}			
	Arabidopsis thaliana	*myo -Inositol oxygenase (MIOX4)*	1.5 fold	Leaves	Growth chamber 23 °C 65% relative humidity 16:8 h photoperiod 110–120 μmol m^{-2} s^{-1}	Salt stress Cold stress Heat stress Pyrene stress	0, 50, 100, 150, or 200 mM NaCl 16 °C for 3 weeks 29 °C for 10 days 5–200 ppb pyrene	Plant growth was measured as inflorescence height, rosette diameter, and dry weight, root length	Manual	Lisko et al. (2013)
Recycling pathway	*Lycopersicum esculentum*	*Monodehydro-ascorbate reductase*	1.2 fold	Leaves	Growth chamber 4–40 °C 16:8 h photoperiod 100 μmol m^{-2} s^{-1}	Methyl viologen stress	100 μM MV	Measurement of net photosynthetic rate and chlorophyll a fluorescence H_2O_2 measurement and thiobarbituric acid reactive substance (TBARS) content	Manual	Li et al. (2010)
						Heat stress	40 °C for 24 h			
						Cold stress	4 °C for 24 h			

Recycling pathway	*Nicotiana tabacum L.*	*Monodehydro-ascorbate reductase*	1.8 fold	Leaves	Growth chamber 25 ± 1 °C 16:8 h photoperiod 150 μmol m^{-2} s^{-1}	Salt stress	300 mM NaCl	Plant height and number of leaves per plant	Manual	Eltelib et al. (2012)
	Nicotiana tabacum L.	*Dehydroascorbate reductase*	No change		Growth chamber 25 ± 1 °C 16:8 h photoperiod 25 μmol m^{-2} s^{-1}	Methyl viologen stress	0, 2, 5, 10, μM MV	Cell leakage analysis Plant growth and chlorophyll content	Manual	Kwon et al. (2003)
						Oxidative stress	0, 100, 200, 400, 800 mM H_2O_2			
						Salt stress	0, 50, 100, 150 or 200 mM NaCl			
						Cold stress	15 °C for 50 days			
	Nicotiana tabacum	*Dehydroascorbate reductase*	2.1 fold	Leaves	Greenhouse 25 °C 60–70% relative humidity 800–950 μmol m^{-2} s^{-1} 300 ppm CO_2	Ozone stress	Ozone 200 ppb	Transpiration, CO_2 assimilation, stomatal conductance, and H_2O_2	Manual	Chen and Gallie (2005)
Recycling pathway	*Nicotiana tabacum (SR-1)*	*Dehydroascorbate reductase*	2.1 fold	Leaves	Growth chamber 25 °C 45–55% relative humidity 14:10 h photoperiod	Ozone stress Drought stress Salt stress	0.2 ppm ozone, holding water 10% (w/v) PEG 0.3 M NaCl	Net photosynthesis	Manual	Eltayeb et al. (2006)
	Arabidopsis thaliana	*Dehydroascorbate reductase*	1.2 fold	Leaves	Growth chamber/ Greenhouse 22 °C 16:8 h photoperiod 70 μmol m^{-2} s^{-1}	Salt stress	100 mM NaCl	Seedling growth	Manual	Ushimaru et al. (2006)

(continued)

Table 3 (continued)

Pathways regulatory factors	Species transformed	Gene overexpressed	Max fold increased	Tissue tested	Growth conditions	Stress tested	Treatment	Methodology	Phenotype	Reference
	Arabidopsis thaliana	*Dehydroascorbate reductase*	4.0 fold	Leaves	Growth chamber 22 °C 16:8 h photoperiod 100 μmol m^{-2} s^{-1}	Light stress	1000 μmol m^{-2} s^{-1}	Stomatal aperture	Manual	Wang et al. (2010)
						Heat stress	40 °C for 1 week			
						Oxidative stress	10 μM MV			
	Nicotiana tabacum L.	*Dehydroascorbate reductase*	1.3 fold	Roots	Growth chamber 25 °C 16:8 h photoperiod 200 μmol m^{-2} s^{-1}	Aluminum stress	400 μM $AlCl_3$	Aluminum accumulation, malondialdehyde, oxidative DNA damage (8-OHdG), H_2O_2, AsA, and GSH	Manual	Yin et al. (2010)
Recycling pathway	*Solanum tuberosum L.*	*Dehydroascorbate reductase 1*	2.8 fold	Leaves	Growth chamber 22 °C 60% relative humidity 16:8 h photoperiod	Methyl viologen stress Drought stress	3 μM MV 5% (w/v) PEG	Ion leakage, chlorophyll content, and H_2O_2	Manual	Eltayeb et al. (2011)
						Salt stress	50 mM Nacl			
	Solanum lycopersicum	*Dehydroascorbate reductase*	1.4 fold	Leaves	Growth chamber 26 ± 4 °C 50–60% relative humidity 11:13 h photoperiod	Salt stress	0 or 100 mM NaCl	Germination rate, plant height, plant biomass, and chlorophyll content	Manual	Li et al. (2012)
			1.2 fold	Fruits		Methyl viologen stress	0, 2, 50, or 100 μmol/L MV			
	Solanum lycopersicum	*Dehydroascorbate reductase*	1.5 fold	Leaves	Growth chamber 22 ± 4 °C 50–60% relative humidity 14:10 h photoperiod	Salt stress	50 mM NaCl	Chlorophyll and malondialdehyde	Manual	Qin et al. (2015)
						Cold stress	4 °C for 1 week			

	Arabidopsis thaliana	*Dehydroascorbate reductase*	1.3 fold	Leaves	Growth chamber 22 °C 70% relative humidity 16:8 h photoperiod 50 μmol m^{-2} s^{-1}	Salt stress	150 mM NaCl	Plant biomass and root length	Manual	Liu et al. (2016)
Alternative pathways	*Nicotiana tabacum L.*	D-*Arabinono-1,4-lactone oxidase (ALO yeast)*	1.6 fold	Leaves	Greenhouse 25–30 °C 12:12 h photoperiod 700 μmol m^{-2} s^{-1}	Methyl viologen stress	0.1 mM MV	Root biomass, shoot biomass and aluminum accumulation	Manual	Chen et al. (2015)
						Light stress	1200 μmol m^{-2} s^{-1}			
						Aluminum stress	100 mM $AlCl_3$			
	Nicotiana tabacum	*9-Cis-epoxycarotenoid dioxygenase (SgNCED1)*	3.4 fold	Leaves	Growth chamber 25–30 °C Light intensity 800 μmol m^{-2} s^{-1}	Cold stress	3 °C for 3 days (for tobacco) or 6 °C for 5 days (for stylo)	Relative water content, ion leakage, malondialdehyde, and Fv/Fm ratio	Manual	Bao et al. (2016)
	Stylosanthes guianensis	*And yeast* D-*arabinono-1,4-lactone oxidase (ALO) gene*				Drought stress		Withheld irrigation until WT plants showed serious wilting		
Master regulators	*Arabidopsis thaliana*	*Ethylene response factor (AtERF98)*	1.7 fold	Leaves	Growth chamber 22 ± 4 °C 14:10 h photoperiod	Salt stress	180 mM NaCl, 100 μM ACC (1-amino cycloprop ane-1-carb oxylate) or 100 μM H_2O_2	Chlorophyll and malondialdehyde, accumulation of H_2O_2 Proline, soluble sugar glutathione content and Na+/K^+ analysis	Manual	Zhang et al. (2012)

(continued)

Table 3 (continued)

Pathways regulatory factors	Species transformed	Gene overexpressed	Max fold increased	Tissue tested	Growth conditions	Stress tested	Treatment	Methodology	Phenotype	Reference
Master regulators	*Arabidopsis thaliana*	*Photomorphogenic factor COP9 signalosome subunit 5B (CSN5B)*	2.0 fold	Leaves	Growth chamber 22 °C 70% relative humidity 16:8 h photoperiod 50 μmol m^{-2} s^{-1}	Methyl viologen stress Oxidative stress Salt stress	50 mM MV 100 mM NaCl	Total biomass and root length, malondialdehyde	Manual	Wang et al. (2013)
	Solanum lycopersicum	DoF transcription factor *(SlDof22)*	1.3 fold	Fruits	Greenhouse 24–28 °C 70–80% relative humidity	Salt stress	300 mM NaCl	Root biomass, shoot biomass, and chlorophyll content	Manual	Cai et al. (2016)
	Solanum lycopersicum	HD-Zip I transcription factor *(SlHZ24)*	1.6 fold 1.2 fold	Leaves Fruits	Growth chamber 25 °C 16:8 h photoperiod	Light stress Methyl viologen stress 80 μmol MV	Continuous light at 25 °C for 16 h followed by 8 h continuous dark under 25 °C 80 μmol MV	Chlorophyll and malondialdehyde content	Manual	Hu et al. (2016)

Plants overexpressing L-gulono-1,4-lactone oxidase (GulLO), the enzyme involved in the L-gulose and the inositol pathway of AsA, have elevated AsA. When subjected to abiotic stresses, those plants were tolerant to herbicide stress (up to 20 μM methyl viologen), osmotic stress (up to 300 mM mannitol), and salt stress (up to 600 mM NaCl). Phenotypic changes, chlorophyll content, and malondialdehyde content were the variables used to assess the tolerance to stresses in these plants (Hemavathi et al. 2009; Lim et al. 2016).

Our group and others have characterized the response to stresses of high AsA Arabidopsis lines overexpressing MIOX4, one of the enzymes involved in the *myo*-inositol pathway to AsA. Increased level of AsA in those plants correlates with tolerance to salt stress (up to 200 mM NaCl), cold stress (16 °C for 3 weeks), heat stress (29 °C for 10 days/40 °C for 15 min), environmental pollutant (up to 200 ppb pyrene), and light stress (up to 300 μmol m^{-2} s^{-1}). Photosynthetic efficiency, root growth, and shoot growth are the main readouts used to assess abiotic stress tolerance (Tóth et al. 2011; Lisko et al. 2013).

Arabidopsis plants overexpressing a recently characterized GNL containing 5-fold AsA content were found to display enhanced growth rate, tolerance to light stress, and improved photosynthetic efficiency and seed yield (Yactayo-Chang 2016; Yactayo-Chang and Lorence 2016).

Monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) are the recycling enzymes for AsA in plants. MDHAR and DHAR help to maintain redox status of AsA (Haroldsen et al. 2011). Overexpression of MDHAR from *Lycopersicum* spp. and *Malpighia* spp. resulted in 1.2–1.8-fold elevated AsA level in leaves compared to wild-type plants. Elevated AsA level correlated with tolerance to herbicide stress (up to 100 μM methyl viologen), heat stress (40 °C for 24 h), cold stress (4 °C for 24 h), and salt stress (up to 300 mM NaCl) (Li et al. 2010; Eltelib et al. 2012). Similarly, overexpression of DHAR resulted in 1.2–4.0-fold increase in AsA level in leaves, fruits, and roots compared to controls. High AsA plants were found to be tolerant to ozone stress (up to 0.2 ppm), drought stress (5% polyethylene glycol), herbicide stress (up to 3 μM methyl viologen), aluminum stress (up to 400 μM $AlCl_3$), salt stress (up to 150 mM NaCl), light stress (up to 1000 μmol m^{-2} s^{-1}), and cold stress (4 °C for 1 week). Photosynthetic efficiency, plant height, number of leaves, plant growth, ion leakage, H_2O_2 accumulation, transpiration, CO_2 accumulation, and stomatal conductance were the parameters used to assess the abiotic stress tolerance on these plants (Kwon et al. 2003, Chen and Gallie 2005, Eltayeb et al. 2011, Li et al. 2012, Qin et al. 2015; Liu et al. 2011).

Overexpression of homologues of the plant GulLO enzymes from *Saccharomyces cerevisiae,* D-arabiono-1,4 lactone oxidase and *Stylosanthes guianensis* 9-cis-epoxycarotenoid deoxygenase, resulted in a boost of the AsA content in plants. These transgenics showed tolerance to herbicide stress (up to 0.1 mM methyl viologen), light stress (up to 1200 μmol m^{-2} s^{-1}), aluminum stress (100 mM $AlCl_3$), cold stress (3 and 6 °C), and water limitation stress (Chen et al. 2005; Bao et al. 2016).

On the other hand, overexpression of master regulators of the AsA metabolic network, ethylene response factor 8, SID 22, SIHZ24 and CSN5B from *Arabidopsis thaliana* and *Solanum lycopersicum* resulted in 1.2- to 2.0-fold increase in AsA

content in leaves and fruits compared to controls. Plants with elevated AsA were found to be tolerant to salt stress (300 mM NaCl), light stress, herbicide stress (up to 50 mM methyl viologen), and oxidative stress. Shoot biomass, root biomass, aluminum accumulation, relative water content, ion leakage, chlorophyll content, and malondialdehyde content were the parameters used to assess the abiotic stress tolerance in those studies (Zhang et al. 2012; Wang et al. 2013; Cai et al. 2016; Hu et al. 2016).

In summary, increased AsA level in plants is associated with enhanced abiotic stresses tolerance, whereas decreasing AsA level has led to sensitivity to abiotic stresses. Surprisingly, plants with low AsA level (vtc mutants) have been shown to be resistant to some biotic stresses.

To date, the most common readouts of abiotic stress tolerance have been plant size, plant shape, and color and photosynthesis parameters. In all published studies to date research teams have relied on manual phenotyping to make these assessments. Manual phenotyping is time consuming and sensitive to bias and is limited to the resolution of the naked eye. Based on these drawbacks, it is important to implement the use of more advanced tools to analyze the phenotype of high AsA plants to fully realize the potential of these metabolic engineering strategies.

4 Plant Phenomics, a Modern Approach to Characterize Plant Phenotypes

4.1 Closing the Gap Between Genomics and Phenomics

Understanding crop adaptation to abiotic and biotic stress is very important, especially now in the face of climate change and global population growth. Powered by the advances in gene editing (Barrangou and Doudna 2016) and the study of natural genetic variability (Nunes-Nesi et al. 2016), plant genomics has been growing to select and improve crop adaptation and yield. This is why it is important to understand plant plasticity using phenomics. Plant phenomics is defined as the application and development of different methodologies to capture information related with performance, function, and structure of a large number of plants (Houle et al. 2010; Tardieu et al. 2017). The main purpose of plant phenomics is to understand plant behavior under a vast variety of scenarios and how exactly the genotypic traits are expressed through the plant phenotype.

Scientists that have had the opportunity to work with manual plant phenotyping understand how slow this process can be. Measuring plant area, plant height, seed number, panicle number, fruit color, leaf number, and other traits of interest can be time consuming and affected by human bias. Phenomics is a research area that is moving fast due to the progress in the development of new sensors and imaging techniques for several traits, plant organs, and responses (Furbank and Tester 2011).

However, a major challenge that remains in this area is data handling and processing when analyzing the sensor information and translating that information into knowledge.

4.2 Plant High-Throughput Phenotyping

Being able to gather massive amounts of information based on high resolution images is very convenient. The capability of eliminating destructive measurements increases the capacity of adjusting the experimental design and allows gathering information throughout the life cycle of plants. Plant high-throughput phenotyping technology consists of taking images with different sensors using hundreds of plants per day (Fahlgren et al. 2015). Data acquisition and analysis have become more feasible due to advances in automation for plant phenotyping, specifically robotic and sensor based technology (Rahaman et al. 2015). Currently, this technology has expanded not only at the laboratory (controlled environment) and greenhouse levels but also to the field scale.

The adaptability of this new technology is overwhelming, and it has made it possible to have platforms such as the Field Scanalyzer System at Rothamsted Research, UK and the TERRA-REF (http://terraref.org/) project in Maricopa, AZ, examples of large field crop analytic robots in the world. These robots take images by moving on a surface of 100–200 m^2 using different sensors. This technology can also be used on a smaller scale with options that are less expensive, such as taking images using a digital camera, a tripod, and a light box attached (Chitwood et al. 2014). The data gathered with this technology can then be analyzed using commercial software or open source algorithms. A compilation of software options for plant phenomics can be found in the online database plant-image-analysis.org/ (Lobet et al. 2013).

4.3 Key Sensors in Plant Phenomics

Plant plasticity is very complex. When a genomic trait is adjusted, plants show very different architectures and phenotypic responses depending on the environmental conditions they face. Here is where different sensors come into play to help understand these behaviors. Sensor technology has improved since RGB images were used for the first time to determine plant growth, where top view RGB images were taken and correlated to obtain plant fresh weight (Leister et al. 1999).

The sensors available in the market work at different wavelength ranges of the light spectrum (Fig. 2). Color cameras are restricted to the 400–700 nm range and they have three colored sensors (red, green and blue) that are used to calculate the true color of each pixel (Fahlgren et al. 2015). These sensors use pixel-based maps to analyze several phenotypic characteristics such as biomass, area, diameter,

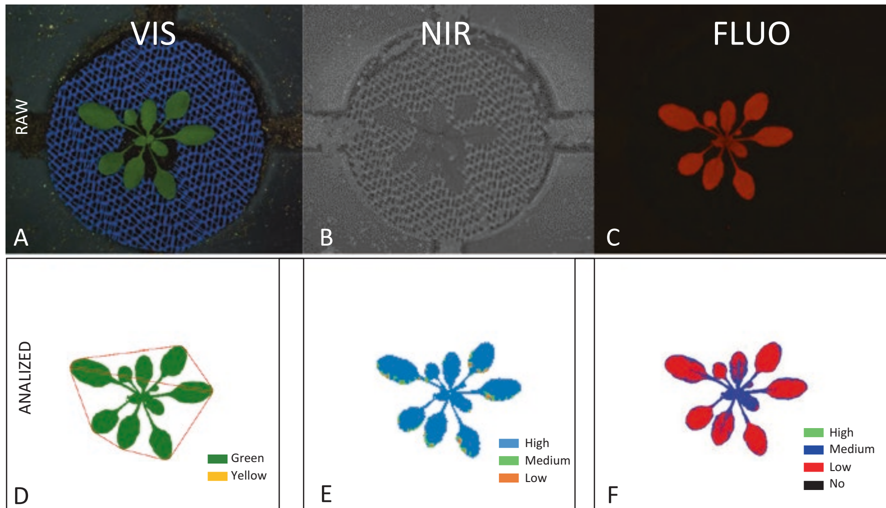


Fig. 2 Representative images acquired with the visible (VIS), near infrared (NIR), and fluorescence (FLUO) sensors. (**a**–**c**) Raw images. (**d**–**f**) Analyzed images where the different parameters were quantified. (**d**) Shows geometrical parameters and color classification computed from VIS images. (**e**) *In planta* water content values computed from NIR images. (**f**) *In planta* chlorophyll fluorescence values computed from FLUO images. Values in E and F have been corrected to be relative to plant area

flowering time, root architecture, plant height, and others. Although these sensors are the most commonly used, there is a disadvantage to them since they cannot distinguish between overlapping leaves or soil background unless some segmentation process is applied to the image (Li et al. 2014; Rahaman et al. 2015).

Due to global warming, scientists have focused their research in finding solutions to reduce the negative effect of abiotic/biotic stresses in plant yield. When a plant is affected by high temperature or soil water is depleted, plants tend to close the stomata to avoid water loss. The physiology of plants is also affected due to the increase in leaf temperature, and responses such as leaf tissue and enzyme damage, lower photosynthetic rate, higher respiration rate, and others occur (Schauberger et al. 2017). For this reason, sensors that detect near-infrared (NIR) and infrared (IR) light are very important for plant high-throughput phenotyping. These sensors use pixel-based maps and are used to determine plant water content (900–1700 nm) and canopy/leaf temperature (700–1000 nm), respectively (Fahlgren et al. 2015).

Plants under stress conditions are affected internally, specifically in their photosynthetic machinery, which will affect crop yield. The use of fluorescence (FLUO) sensors allows for the quantification of chlorophyll molecules in photosystem II by artificial excitation, and this information is used to measure how efficient the plant is during photosynthesis (Jansen et al. 2009; Li et al. 2014). The sensor corresponds to a charge-couple device (CCD) camera with sensitive fluorescent signals. These signals occur when the sample is illuminated with visible or UV

light (Rahaman et al. 2015). The FLUO sensors are pixel-based maps that are used to detect disease, infestation, photosynthetic status, carbon assimilation, quantum yield, non-photochemical quenching, and total chlorophyll content (Kuhlgert et al. 2016).

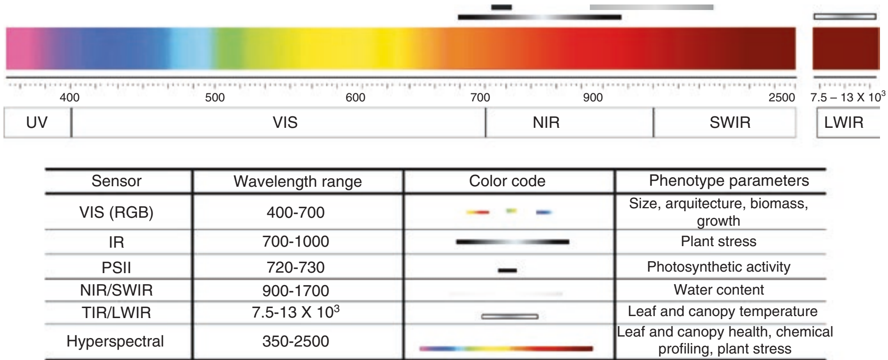
Agronomical traits such as tiller, leaf, and panicle number are key for yield estimation. This is why 3D sensors play an important role in plant phenomics. These type of sensors use depth maps and allow acquiring noncontact and nondestructive measurements. By combining a 3D camera with laser scanning, the amount of point clouds detected enables a valid and accurate description of the plant geometry (Paulus et al. 2013). Some of the phenotype parameters that can be detected with this sensor include shoot structure, leaf angle, canopy health status, leaf growth, coverage density, and panicle health (Li et al. 2014).

The latest sensor developed, and the most expensive one, corresponds to imaging spectroscopy, also called hyperspectral imaging. This technology combines features of RGB imaging based on pixel mapping and spectroscopy based on intensity from different spectral bands. The most important feature about this sensor is that it enables measuring plant chemical traits such as water, nutrients, lipids, sugars, and others (Pandey et al. 2017). This sensor is currently the slowest of all, and the image analysis requires additional computational knowledge due to the data dimensions and complexity.

4.4 Phenomics to Understand Plant Abiotic/Biotic Stress Response

The power of phenomics relies on the fact that it is possible to extract a great deal of information from a single image. Figure 3 presents representative images of Arabidopsis plants that have been phenotyped using RGB, FLUO, and NIR sensors (panels A, B, and C). Once the images were analyzed through a commercial software (panels D, E, and F), the information was plotted and analyzed. Each of the sensors quantifies different phenotype parameters (Fig. 3). For example, from the RGB sensor, data such as plant size, architecture, biomass and growth can be obtained. From the NIR, quantification and localization of water can be obtained. More information can be gathered and analyzed, that will help understand in a more detailed manner plant adaptation to the new environment due to climate change. Subtle changes that are not detectable with the naked eye can be measured and quantified using sensors.

Figure 4 illustrates the use of plant phenomics approaches in the characterization of high AsA Arabidopsis lines. As shown in this figure, AtMIOX4 over-expressers are salt tolerant as indicated by their higher growth rate and biomass accumulation compared to wild-type controls. Although not evident to the naked eye, but captured by the RGB camera AtMIOX4 displayed less chlorosis than wild-type plants exposed to NaCl stress.



Sensor	Wavelength range	Color code	Phenotype parameters
VIS (RGB)	400-700		Size, arquitecture, biomass, growth
IR	700-1000		Plant stress
PSII	720-730		Photosynthetic activity
NIR/SWIR	900-1700		Water content
TIR/LWIR	7.5-13 X 10^3		Leaf and canopy temperature
Hyperspectral	350-2500		Leaf and canopy health, chemical profiling, plant stress

Fig. 3 Comparison of different cameras used in high-throughput plant phenotyping and the parameters that can be measured with each of them. Each sensor captures signals from the visible and infrared spectrum of the light. The VIS (visible range) camera can quantify plant size, shape, and color. The IR (infrared range) camera can quantify plant stress. The PSII (photosystem II efficiency) sensor can quantify plant photosynthetic activity. The NIR/SWIR (near infrared and short-wave infrared) camera can detect plant relative water content. The TIR/LWIR (thermal infrared and long-wave infrared) sensor can detect plant canopy or leaf temperature. The hyperspectral camera can measure plant stress and chemical composition by detecting a wide variety of spectral bands

4.5 Data Analysis and Handling

Plant high-throughput phenotyping studies generate large-scale, multidimensional data sets that require proper analysis (Klukas et al. 2014). One of the main challenges in the field is standardization of the methodologies and data publication. Storage and data collection needs to be consistent and linked with specific phenotypes and genomic information (Krajewski et al. 2015).

Optimization of the experimental design is key to obtain good quality images and, therefore, reproducible data. First, it is necessary to determine the objective of the experiment before acquiring any information. Then, once the objective is determined, it is necessary to select the sensor/sensors to use. Moreover, having consistency in how the images are acquired is imperative. Improving the quality of the images facilitates the analysis. One simple example of this is adding a blue mesh as a soil cover, in order to improve background/object contrast and to reduce evaporation (Junker et al. 2014).

Plant development research has been positively affected by the advance in phenotyping technologies, which help to determine phenotypic traits that change depending on genotype and environment. Further analysis that integrates several sensors and overlaps the data collected is necessary to better understand plant plasticity and adaptation.

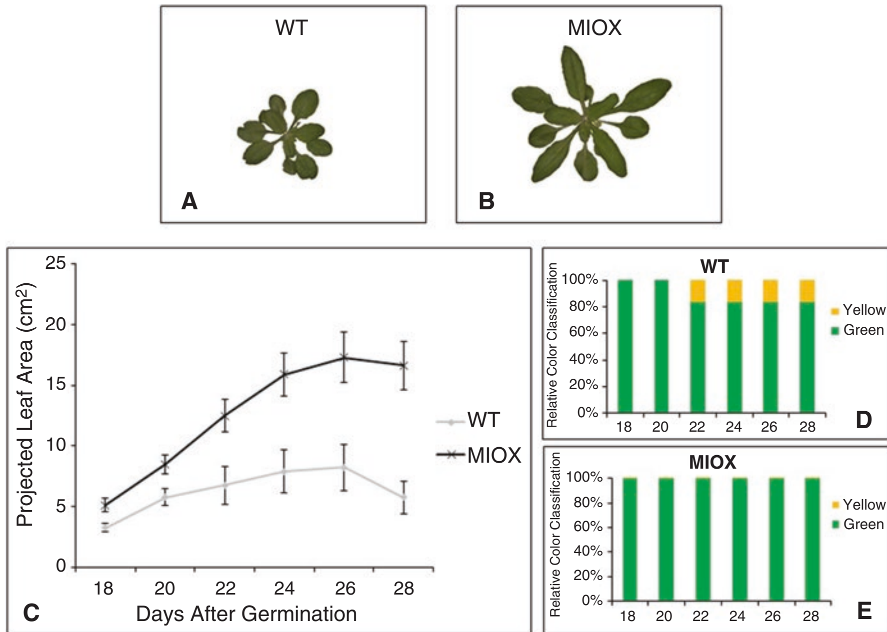


Fig. 4 Characterization of the phenotype of a high ascorbate line compared to controls. Arabidopsis seeds were sterilized, vernalized, and planted on Murashige and Skoog media. After germination vigorous seedlings were transferred to soil and grown in an environment controlled chamber (23 °C, 65% humidity, 16:8 h photoperiod, 200 μmol m^{-2} s^{-1} light intensity). After acclimation to soil, plants were subjected to salt stress (150 mM NaCl) 21 days after germination. (**a**, **b**) Raw RGB images after segmentation. (**c**) Projected leaf area of the Arabidopsis rosette; data are means ± SE, $n = 15$. (**d**, **e**) Color classification of the plants analyzed in (**b**). Values were corrected relative to plant area. This parameter distinguishes yellow color (chlorosis) from green color (healthy tissue). Chlorosis was not apparent by visual inspection, but detected by the RGB camera. *MIOX Myo*-inositol oxygenase over-expresser, *WT* wild-type control

5 Conclusions

During the past two decades, we have significantly advanced our understanding about the different pathways plants use to make AsA, a key molecule involved in the modulation of plant growth and development, plant health, and stress tolerance. Full realization of the potential that this knowledge represents for the development of more nutritious and improved crops must involve the incorporation of novel approaches to engineer crops and to characterize their phenotype. We propose that gene editing (Barrangou and Doudna 2016) and plant phenomics are two approaches that are likely to revolutionize the way high AsA crops are made and characterized before some of them are able to reach widespread adoption.

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Physiological Role of Ascorbic Acid Recycling Enzymes in Plants

Marina Suekawa, Yukichi Fujikawa, and Muneharu Esaka

Abstract Ascorbic acid (AsA) is oxidized to monodehydroascorbate (MDHA), which dissociates to form dehydroascorbate (DHA) instead of detoxifying reactive oxygen species (ROS). MDHA and DHA are directly reduced to AsA by two reductases, monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), respectively. They contribute to maintaining AsA contents and its redox status, which are dependent on the rate of its biosynthesis and recycling. The primary functions of MDHAR and DHAR appear to recycle AsA in the AsA-glutathione (GSH) cycles for diminishing ROS produced during photosynthesis in leaves. In fruits, MDHAR and DHAR might function in a complementary manner to maintain the AsA redox status during fruit development and ripening. Also, MDHAR and DHAR function as part of the AsA-GSH cycles in the different plant cellular compartments, like chloroplasts, mitochondria, and peroxisomes. Taking into account the physiological functions of AsA in plants, MDHAR and DHAR as AsA regenerators are paid much attention in engineering of stress tolerance and nutrient values. Transgenic plants overexpressing MDHAR and DHAR exhibit an increase in AsA contents and enhanced stress tolerance. This chapter focuses on the primary structures and gene expressions of plant MDHAR and DHAR isozymes as well as their contributions to the AsA contents in the leaves and fruits of plants. Also, this chapter provides the information about the roles of MDHAR and DHAR in the chloroplast, the cytosol, the guard cells, and stress tolerance in plants.

Keywords Ascorbic acid-glutathione cycle · Ascorbic acid recycling · Dehydroascorbate reductase · Environmental stress · Monodehydroascorbate reductase · Oxidative stress tolerance

M. Suekawa · Y. Fujikawa · M. Esaka (✉)
Graduate School of Biosphere Sciences, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan
e-mail: mesaka@hiroshima-u.ac.jp

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1 Introduction

Ascorbic acid (AsA) is the major antioxidant of plants, and it detoxifies reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydroxyl radical, and hydrogen peroxide (H_2O_2). When removing H_2O_2, AsA is oxidized to monodehydroascorbate (MDHA) by ascorbate peroxidase and followed by nonenzymatic dissociation to form dehydroascorbate (DHA). In AsA recycling (Noctor and Foyer 1998), after AsA is oxidized to MDHA and DHA, two types of reductases reduce MDHA and DHA respectively back to AsA. The ratio of total AsA (reduced and oxidized AsA) and DHA relates to cellular redox status. Considering the physiological functions of AsA in plants, it is essential to maintain the AsA redox status under condition increasing ROS production, such as light, extreme temperature, metal or salt stress (Davey et al. 2000; Conklin 2001; Anjum et al. 2014). Thus, the AsA redox status, as well as its contents in cells, is a significant indicator of physiological properties to stress tolerance, and it depends on the balance of AsA biosynthesis and recycling. In AsA recycling as described above, two reductases participate in the reduction of oxidized AsA; one of the reductases is monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), which can reduce MDHA to AsA using NAD(P)H as a reductant (Fig. 1; Noctor and Foyer 1998). The other is dehydroascorbate reductase (DHAR; EC 1.8.5.1) for reducing DHA to AsA in a reaction requiring glutathione (GSH) as a reductant. MDHAR and DHAR, as well as AsA biosynthesis enzymes, are essential enzymes to maintain the AsA redox status (Leterrier et al. 2005; Lunde et al. 2006; Tang and Yang 2013; Zhang et al. 2015). As shown in Fig. 1, the AsA recycling is a component of the AsA-GSH cycle. In the AsA-GSH cycle, oxidized glutathione (GSSG), which comes from GSH used by

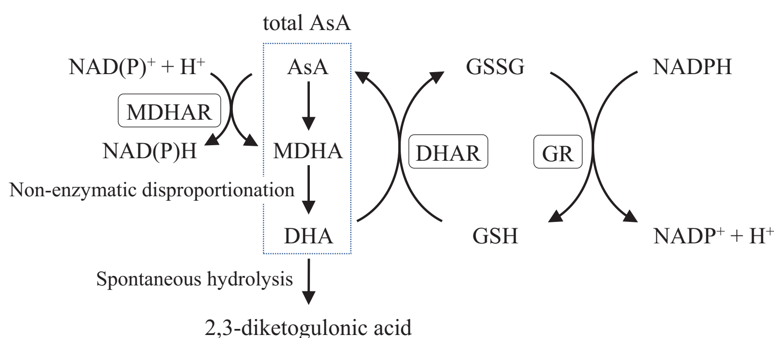

Fig. 1 Ascorbic acid recycling by the ascorbate-glutathione cycle through DHAR and MDHAR. After oxidization of AsA to monodehydroascorbate (MDHA), monodehydroascorbate reductase (MDHAR) converts MDHA to AsA, or MDHA disproportionates nonenzymatically to dehydroascorbate (DHA). Dehydroascorbate reductase (DHAR) reduces DHA to AsA using glutathione (GSH) as the reductant. Oxidized glutathione (GSSG) is reduced by glutathione reductase (GR) to GSH using NADHP as the reductant. DHA will spontaneously hydrolyze to 2,3-diketoguloninc acid unless salvaged by DHAR. Total AsA represents the inclusion of reduced (AsA) and oxidized AsA (MDHA and DHA)

DHAR to recycle AsA from DHA, is reduced by glutathione reductase using NADPH as a reductant. If DHA does not get back to AsA, it spontaneously hydrolyzes to 2,3-diketogulonic acid, which results in irreversible AsA loss. Since irreversible DHA oxidation causes the decrease of AsA contents, the efficiencies of AsA recycling are important in maintaining not only AsA redox status but also AsA contents in cells. In plants, the primary structures of MDHAR and DHAR are determined, and they can be classified into several groups based on their primary structures. In leaves, MDHAR and DHAR mainly participate in scavenging ROS in photosynthesis. The gene expressions of MDHAR and DHAR during fruit development and ripening suggest that MDHAR and DHAR have a complementary relationship in maintaining the AsA redox status. Acerola (*Malpighia glabra*) fruits, containing high AsA contents (Badejo et al. 2009; Eltelib et al. 2011), possess high DHAR activities compared to other plants with relatively low AsA contents (Hossain and Asada 1984; Dipierro and Borraccino 1991; Kato et al. 1997; Shimaoka et al. 2000; Eltayeb et al. 2006). Thus, the AsA recycling appears to contribute high AsA contents in acerola fruits. Under abiotic stress conditions accompanied by ROS accumulation, the gene expressions of MDHAR and DHAR are enhanced in plants, suggesting that they improve the AsA redox status by the AsA recycling from MDHA and DHA. And transgenic plants overexpressing MDHAR and DHAR exhibit enhanced oxidative stress tolerance, even slight increase of AsA contents and AsA redox status (Kwon et al. 2003; Amako et al. 2006; Eltayeb et al. 2006, 2007, 2011; Ushimaru et al. 2006; Li et al. 2010a; Yin et al. 2010; Qin et al. 2011; Eltelib et al. 2012; Chang et al. 2017). On the other hand, biotic stress by pathogen reduced the gene expression of MDHAR in wheat leaves. Moreover, the suppression of MDHAR in wheat leaves improved resistance to pathogen infection (Feng et al. 2014a, b). Under biotic stress, ROS serve as not only the toxic substance but also pathogen resistive element (Wang et al. 2007). Thus, the regulation of MDHAR and DHAR expressions may be of importance for resistance to abiotic and biotic stresses through ROS regulation by the AsA-GSH cycles.

2 The Primary Structures of Ascorbic Acid Recycling Enzymes in Plants

In plants, there are many isozymes of MDHAR and DHAR, and their primary structures have been deposited at public database sites, such as National Center for Biotechnology Information (U.S. National Library of Medicine) and DNA Data Bank of Japan (Japan, National Institute of Genetics). As described in the previous section, MDHAR is the first enzyme to recycle oxidized AsA (MDHA). MDHAR contains the FAD-NAD-binding sites, which play a significant role in its enzyme activity. The alignment of the primary structures of plant MDHAR shows they are classified into four groups (Fig. 2a: Leterrier et al. 2005; Lunde et al. 2006). Each group of the plant MDHAR appears to localize at different cell compartments:

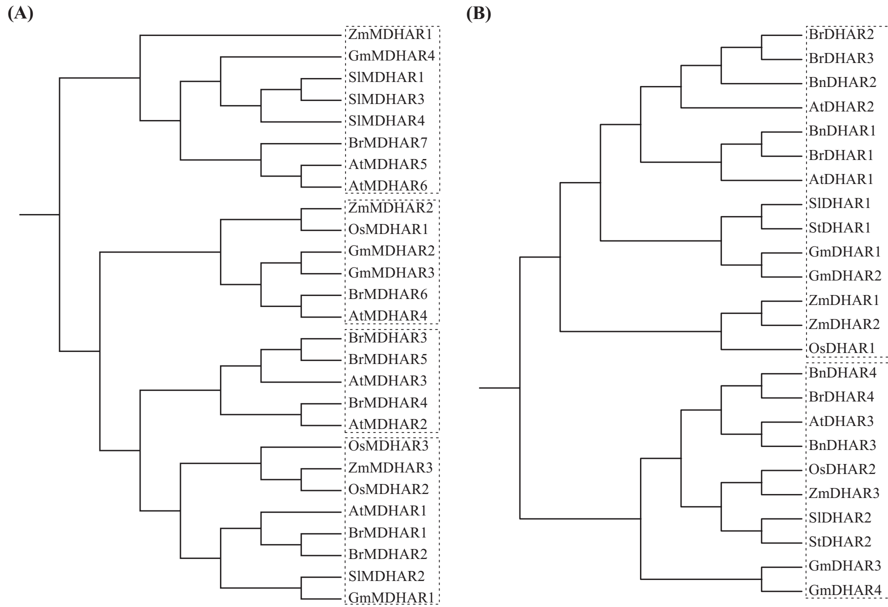

Fig. 2 Phylogenetic tree of plant MDHAR and DHAR. Phylogenetic trees based upon protein sequences of plant MDHAR (**a**) and DHAR (**b**) are presented. The dotted-line box indicated each clade in the phylogenetic trees of plant MDHAR and DHAR. For abbreviations and accession numbers see the following, and accession numbers are given in parentheses. MDHAR; *Arabidopsis thaliana*, AtMDHAR1 (AAM83213), AtMDHAR2 (NP_568125), AtMDHAR3 (NP_566361), AtMDHAR4 (AAM91734), AtMDHAR5 (BAA12349), AtMDHAR6 (NP_564818); *Brassica napus*, BrMDHAR1 (XP_013663253), BrMDHAR2 (XP_013749992), BrMDHAR3 (XP_013681036), BrMDHAR4 (XP_013724800), BrMDHAR5 (XP_013740977), BrMDHAR6 (XP_013646118), BrMDHAR7 (XP_013686116); *Glycine max*, GmMDHAR1 (XP_003557022), GmMDHAR2 (XP_006599107), GmMDHAR3 (XP_003553831), GmMDHAR4 (XP_006584627); *Oryza sativa*, OsMDHAR1 (BAS80528), OsMDHAR2 (BAT06666), OsMDHAR3 (BAT09471); *Solanum lycopersicum*, SlMDHAR1 (NP_001234013), SlMDHAR2 (NP_001318117), SlMDHAR3 (AAZ66138), SlMDHAR4 (ADJ21816). DHAR; *Arabidopsis thaliana*, AtDHAR1 (NP_173387), AtDHAR2 (NP_177662), AtDHAR3 (NP_568336); *Brassica napus*, BnDHAR1 (No. CDY29420), BnDHAR2 (CDY62535), BnDHAR3 (CDY62535), BnDHAR4 (CDX85490); *Brassica rapa*, BrDHAR1 (XP_009149443), BrDHAR2 (XP_009128088), BrDHAR3 (XP_009128089), BrDHAR4 (XP_009126134); *Glycine max*, GmDHAR1 (NP_001236937), GmDHAR2 (KRH36212), GmDHAR3 (KRG97934), GmDHAR4 (KRH30940); *Oryza sativa*, OsDHAR1 (BAS91971), OsDHAR2 (BAS96927); *Solanum lycopersicum*, SlDHAR1 (AAY47048), SlDHAR2 (AAY47049); *Solanum tuberosum*, StDHAR1 (ABX26128), StDHAR2 (ACJ70069). *Zea mays*, ZmDHAR1 (AIQ78396), ZmDHAR2 (AIQ78394), ZmDHAR3 (NP_001151414)

cytosol, peroxisome, chloroplast, and mitochondrion. The MDHAR isozymes which localize in chloroplasts, mitochondria, and peroxisomes have specific extensions at their N-terminus or C-terminus. These extensions act as a transit peptide for mitochondrial or chloroplast targeting (N-terminus) and peroxisomal targeting (C-terminus). In *Arabidopsis*, six MDHAR isozymes (AtMDHAR) were found in the genome (Leterrier et al. 2005; Lisenbee et al. 2005). Two of them, AtMDHAR1

(gene accession number; At3g52880) and AtMDHAR4 (gene accession number; At3g27820) localize in the peroxisome and possess specific C-terminal sequences, which are similar to the type-1 peroxisomal targeting signal (PTS1) and membrane peroxisomal targeting signals, respectively (Lisenbee et al. 2005). Another two which arise from a single gene (gene accession number; At1g639400) at different transcription initiation sites, AtMDHAR5 (gene accession number; D84417) and AtMDHAR6 (gene accession number; NP_564818) with an N-terminal extended region, are targeted to mitochondria and chloroplasts, respectively (Obara et al. 2002). The other two, AtMDHAR2 (At5g03630) and AtMDHAR3 (At3g09940), localize in the cytosol with no sequence characterized by subcellular localizations (Lisenbee et al. 2005). These six isozymes of *Arabidopsis* MDHAR exist in different cell compartments. On the other hand, DHAR is the other enzyme to recycle oxidized AsA (DHA) and prevents DHA from being spontaneously hydrolyzed to 2,3-diketogulonic acid. Plant DHAR contains a highly conserved DHAR peptide motif (CxxS), corresponding to thiol-dependent redox sites in thiol-disulfide oxidoreductase enzymes, and it is involved in redox function (Fomenko and Gladyshev 2002). Unlikely to plant MDHAR, the phylogenetic tree shows the plant DHAR isozymes are classified into two groups (Fig. 2b). In *Arabidopsis*, three DHAR isozymes, AtDHAR1 (gene accession number; At1g19570), AtDHAR2 (gene accession number; At1g75270), and AtDHAR3 (gene accession number; At5g16710), were found in the genome (Yoshida et al. 2006; Noshi et al. 2017). Based on the primary structures of the plant MDHAR isozymes, two (AtDHAR1 and AtDHAR2) of three *Arabidopsis* DHAR are classified into the same group. AtDHAR1 and AtDHAR2 having no targeting signal like sequence, they seemed to localize in the cytosol (Yoshida et al. 2006; Grefen et al. 2010). However, AtDHAR1 was reported to localize in peroxisomes practically (Reumann et al. 2009). AtDHAR3 only possesses a specific N-terminal extension, similar to chloroplast targeting signal, and was found to localize in the chloroplast (Noshi et al. 2016). Zea mays also has four DHAR isozymes (ZmDHAR) in the genome. One DHAR (ZmDHAR2) possesses a similar sequence to chloroplast targeting signal, and another two isozymes (ZmDHAR1 and ZmDHAR3) don't contain any targeting signal. However, the other one (ZmDHAR4) contains signal peptide like sequence and localizes in vacuole (Zhang et al. 2015). In *Pisum sativum*, DHAR were reported to localize in mitochondria as well as peroxisome (Jimenez et al. 1997). Taking into account the multiplicity of plant MDHAR and DHAR isozymes, the MDHAR and DHAR isozymes function differentially at their localization sites in plants.

3 The Role of Ascorbic Acid Recycling Enzymes in Regulating Ascorbic Acid Contents and its Redox State

Leaf tissues abundantly contain AsA, especially in chloroplasts (Foyer et al. 1983; Foyer and Lelandais 1996). Light induces the photosynthesis in chloroplasts. In the photosynthesis process, ROS are unexpectedly generated when electrons from reduced ferredoxin of the photosynthetic electron transport chain at photosystem I

transfer to O_2 instead of to NADP. In chloroplasts, AsA is essential as a substrate for APX in the AsA-GSH cycle, which detoxifies ROS through successive oxidation and reduction with GSH and NADPH. Since MDHAR and DHAR participate in the AsA-GSH cycle, they play important roles in the regulation of cellular redox status to scavenge ROS in photosynthesis. In tobacco (*Nicotiana tabacum*), the levels of enzyme activities and proteins of DHAR were highest in the youngest leaves and declined along with leaf aging (Chen and Gallie 2006). The fluctuation in DHAR activities primarily correlated with the change in the chlorophyll contents and the rate of CO_2 assimilation. The overexpression of DHAR also decreased the levels of lipid peroxidation, whereas the suppression of DHAR expression increased the levels of lipid peroxidation. Moreover, DHAR can affect plant growth development as well as the diminishment of ROS. In rice (*Oryza sativa*), the overexpression of OsDHAR1 exhibited better growth development, phenotypes, and rice yield, including grain yield and biomass (Kim et al. 2013). In potato (*Solanum tuberosum*) possessing two DHAR isozymes (StDHAR1 and StDHAR2), the StDHAR1 was highly expressed in tubers, whereas the expression of StDHAR2 was high in leaves (Qin et al. 2011). Their primary structures suggest StDHAR1 and StDHAR2 localize in cytosol and chloroplast, respectively. The overexpression of StDHAR1 significantly increased AsA contents in leaves and tubers. In contrast, StDHAR2 overexpression resulted in the increase of DHAR activities and AsA contents only in leaves and did not fluctuate them in tubers, suggesting that StDHAR2 might need to be activated by posttranslational processing in chloroplasts. StDHAR1 and StDHAR2 might play important roles in improving the AsA contents at different organs and cell compartments. MDHAR and DHAR expressed not only leaves but also roots. During development of the taproots of carrot and radish, the AsA contents were gradually decreased (Xu et al. 2013; Wang et al. 2015). MDHAR and DHAR may be involved in the fluctuation of AsA contents in root, although their physiological function remains unknown.

Fruit is a primary source of AsA for humans, which cannot synthesize their own AsA due to a lack of L-gluconolactone oxidase. Thus, AsA accumulation during fruit development and ripening has been paid attention to in recent years. The patterns of AsA accumulation during fruit development and ripening depend on plant species. Several studies have reported that the AsA contents and AsA redox status in fruits can be modulated during fruit development and ripening. Acerola (*Malpighia glabra*) is a tropical fruit containing high AsA contents. The AsA content in green fruits is the highest during fruit ripening, and it decreases significantly as ripening progresses (Badejo et al. 2009). In acerola fruits, the high gene expression levels of the AsA biosynthesis enzymes involved in the Smirnoff–Wheeler were observed during fruit ripening, suggesting that the Smirnoff–Wheeler pathway mainly contributes to high AsA contents in acerola fruit (Badejo et al. 2009). In acerola fruits, AsA contents are decreased during fruit ripening, whereas the ratio of reduced AsA to DHA is increased. Concerning AsA recycling, MDHAR activities were greatly higher than DHAR activities in acerola fruits. MDHAR activities are increased gradually and significantly as ripening progressed, although DHAR activities increased at the early and intermediate stages of ripening, and then decreased

dramatically at the later stages (Eltelib et al. 2011). The one of each DHAR and MDHAR isozymes were cloned from acerola, and the gene expression of the DHAR was correlated with its enzyme activities during fruit ripening. The MDHAR mainly expressed at overripe fruits, against high MDHAR activities through fruit ripening. On the other hand, the gene expression of the MDHAR was consistent with enzyme activities in other tissues: root, stem, and young and mature leaves. Furthermore, overexpression of the isolated MDHAR leads to the increase of AsA contents in tobacco plants (Eltelib et al. 2012), suggesting that the MDHAR function mainly in other tissue than fruit. However, acerola fruits possess high MDHAR activities and its activities were increased with fruit ripening. Considering the existence of several MDHAR isozymes in *Arabidopsis*, other MDHAR isozymes may mainly function to maintain the AsA redox status during fruit ripening in acerola. Similar AsA accumulation patterns have been reported in the fruits of blueberry (*Vaccinium corymbosum*) (Liu et al. 2015) and kiwifruit (*Actinidia deliciosa*) (Li et al. 2010b) and the pulps of citrus such as Navel orange (*Citrus sinensis*) and Satsuma mandarin (*Citrus unshiu*) (Lado et al. 2015). In blueberry and kiwifruit (Li et al. 2010b; Liu et al. 2015), the AsA contents were higher in green fruits and decreased as fruit ripening, and the gene expression of MDHAR increased in fruit ripening, while that of DHAR decreased. Also, comparative analysis with AsA accumulation patterns was performed with two blueberry cultivars, “Bluecrop” and “Berkeley.” In the two cultivars, although the AsA accumulation patterns were similar to each other, the decrement of AsA content in “Berkeley” fruit was more significant during fruit ripening than in “Bluecrop” fruit, which was consistent with the gene expressions of MDHAR and DHAR. The results suggest the higher efficiency of AsA recycling was partially responsible for the higher AsA accumulation in “Bluecrop.” In pulps of two citrus fruits, Satsuma Owari Mandarin and Washington Navel orange fruits, the AsA contents were decreased as fruit development and ripening progress, and the AsA contents in oranges were about twofold higher than in mandarins (Lado et al. 2015). The gene expression levels of three citrus MDHAR isozymes (MDHAR1, MDHAR2, MDHAR3) were increased during fruit maturation, suggesting the turnover of AsA in the pulp of oranges and mandarins was enhanced during fruit ripening. In oranges, the high expression levels of citrus MDHAR3 at the early stages of fruit development and citrus MDHAR1 and MDHAR2 during fruit ripening were observed, compared with those in mandarins. Thus, it may be assumed that the coordinated expression of the MDHAR isozymes may contribute to maintaining the AsA recycling in the pulp of fruits accumulating the high AsA contents. In contrast to AsA accumulation patterns of acerola, blueberry, and kiwifruit, the AsA contents of strawberry (*Fragaria* × *ananassa*) (Agius et al. 2003; Cruz-Rus et al. 2011), tomato (Ioannidi et al. 2009), and chestnut rose (*Rosa roxburghii* Tratt) were increased during fruit ripening. In strawberry, the gene expression level of MDHAR was the highest in red fruits and was correlated with the increase in AsA contents during fruit ripening. On the other hand, the gene expression of DHAR was high in green fruits of strawberry. Chestnut rose, one of the high AsA accumulated plants, contained high AsA contents in matured fruits and the AsA redox status was also enhanced during fruit ripening. The gene expression

levels and enzyme activities of DHAR significantly correlated with AsA accumulation during fruit ripening, indicating DHAR contributes to the buildup of high AsA contents in chestnut rose fruits. In fruits, each plant MDHAR and DHAR isozymes contribute to AsA recycling at different phases of the ripening process. The influences of MDHAR and DHAR on AsA accumulation during fruit development and ripening vary among plant species. However, MDHAR and DHAR appear to have a complementary relationship in maintaining the AsA redox status in fruit development and ripening.

4 The Role of Ascorbic Acid Recycling Enzymes in the Chloroplast, the Cytosol, and the Guard Cell

Aerobic metabolism continuously produces ROS at the different plant cellular compartments, like chloroplasts, mitochondria, and peroxisomes. Thus, the AsA redox status is important to scavenge ROS produced under both normal and stress conditions in those cellular compartments (Gallie 2013). As part of the AsA-GSH cycle, MDHAR and DHAR isozymes can localize in these organelles. The chloroplast is a major source of ROS production in plants and produces ROS during the photosynthesis process as described in the previous section. Chloroplasts possess the light harvesting system in thylakoid membranes. The photosystems, photosystem I (PSI) and photosystem II (PSII), form the core of the light harvesting system and are the primary sources of ROS production. In the photosynthesis process, after the PSI reduces ferredoxin (Fd), photoreduced Fd transfers electrons to $NADP^+$, which is used for reduction of CO_2 in the Calvin–Benson cycle. However, especially when $NADP^+$ is limited, excess photoreduced Fd can transfer electrons to O_2 instead of $NADP^+$, which leads to ROS production in the stroma of chloroplasts. Thus, the regulation of the PSII reduction is necessary to maintain electron flow through the PSI and to prevent over-reduction of Fd. H_2O_2 is produced not only under normal conditions but also by oxidative stress. Under water deficit conditions like drought, salinity, and high temperature, intake of CO_2 is restricted because of stomatal closure, and excess light leads to the formation of ROS mainly at the PSI as well as PSII. At the PSI, superoxide radical is formed from O_2 by photoreduced Fd, and then it is converted to H_2O_2 by iron-containing superoxide dismutase (FeSOD) at thylakoid membranes via the Mehler reaction (Miller et al. 2010). The superoxide radical generated at the PSII is converted into more toxic ROS like hydroxyl radical via H_2O_2 by the Fenton reaction at the Fe-S centers of the PSII. Hydroxyl radical can harm different cellular components by lipid peroxidation, protein damage, and membrane destruction. At the PSII, singlet oxygen is also produced by the imbalance between light harvesting and energy utilization under environmental stress conditions, as well as by the over-reduction of the PSII. Singlet oxygen can severely damage the PSI and PSII. As described above, chloroplast is a major source of ROS production in plants. To detoxify ROS, AsA presents in millimolar concentrations in

chloroplasts (Smirnoff 2000). In the stroma of chloroplasts, AsA is used as a reductant in a reaction of stromal APX for the reduction of H_2O_2, which is converted by copper-zinc superoxide dismutase (CuSOD) from the superoxide radical generated at the PSI, along with oxidation of AsA to MDHA. The AsA recycling is more essential than AsA biosynthesis to detoxify ROS and to protect from photodamage in chloroplasts because AsA is synthesized in mitochondria (Ostergaard et al. 1997), not in chloroplasts. The photoreduced Fd, which donates electrons to $NADP^+$ in a reaction of Fd-$NADP^+$ reductase, can also donate electrons to MDHA to back to AsA in the stroma as part of the thylakoid scavenging system (Miyake and Asada 1994). In addition to the AsA recycling by Fd, the MDHAR and DHAR with N-terminal targeting sequences are localized in chloroplasts, and they participate in the AsA recycling as part of the AsA-GSH cycle in the stroma of chloroplasts. In the lumen of chloroplasts, to prevent the over-reduction of the PSII, AsA donates electrons to the oxygen-evolving complex in the PSII. And also, AsA is used as a cofactor for violaxanthin de-epoxidase (VDE) (Eskling et al. 1997), which catalyzes the conversion of violaxanthin to zeaxanthin in the xanthophyll cycle. The xanthophyll cycle works for the dissipation of excess absorbed excitation energy during non-photochemical quenching. If not reduced immediately to AsA, MDHA disproportionates to AsA and DHA. Because neither Fd nor MDHAR are localized, MDHA cannot be recycled to AsA in the lumen of chloroplasts. Besides, in the lumen of chloroplasts, the disproportionation of MDHA is fast due to the low pH during light exposure (Asada 1999; Mano et al. 2004). Thus, MDHA produced in the lumen disproportionates to AsA and DHA, then DHA is transported to the stroma and recycled to AsA. In chloroplasts, the MDHAR and DHAR isozymes play important roles in maintaining AsA contents as stromal scavenging system.

Peroxisomes are single membrane spherical organelles and are also a major source of H_2O_2 production by their oxidative metabolism (del Rio et al. 2006; Palma et al. 2009). In peroxisomes, the β-oxidation of fatty acid produces H_2O_2 as a by-product of lipid catabolism. Superoxide radical is also produced at the two different locations of peroxisomes: peroxisomal matrix and membrane. As described above, when the water availability is low and stomata remains closed under stress condition, the ratio of CO_2 to O_2 is reduced considerably, which causes increased photorespiration leading to glycolate formation. The glycolate is oxidized by the glycolate oxidase in peroxisomes to produce H_2O_2 during photorespiration (Noctor et al. 2002). In order to detoxify H_2O_2, peroxisomes possess catalase to convert H_2O_2 to H_2O and O_2 in the peroxisomal matrix. In addition to catalase, peroxisomes possess APX and MDHAR in the peroxisomal membranes, and the APX and MDHAR cooperate with each other on the AsA-dependent electron transfer system to detoxify H_2O_2. H_2O_2 passes freely through membranes, and the H_2O_2 escaping from the peroxisomes is reduced by a peroxisomal membrane-bound APX and MDHAR using AsA as a reductant. In the seedlings of the *Arabidopsis sugar-dependent2* (*sdp2*) mutant, which is deficient in the MDHAR localized to the peroxisomal membrane (Eastmond 2007), the H_2O_2 level was elevated, and the oil body proteins and lipids were oxidized, because of the low AsA contents. Moreover, the oxidation damage in the seedlings of the *sdp2* mutant caused the inactivation of the triacylg-

lycerol (TAG) lipase which is associated with the oil body membranes. In peroxisomes, all of catalase, APX, and MDHAR are essential to the peroxisomal antioxidant system in the seedlings. The main role of MDHAR appears to prevent H_2O_2 from escaping into the cytosol. The H_2O_2 escaping from the peroxisomes appears to lead to the inactivation of TAG hydrolysis in oil bodies and prevents the seedlings from producing energy for initial postgerminative growth. Thus, the MDHAR located in peroxisomes plays a crucial role in growth of seedlings.

Mitochondrion is involved in respiration and photorespiration and is also a source of ROS production as well as chloroplast and peroxisome. Although the ROS production in the mitochondria is less than those in light-exposed chloroplasts or in peroxisomes, mitochondria are the major sources of ROS under the dark, or in non-green tissues (Szarka et al. 2012). The inner membrane of mitochondrion performs oxidative phosphorylation and energy-linked ion translocation. And energy capture, transduction, and utilization are achieved via a number of reactions in the inner membrane. The electron transport chain in mitochondria is the major factor to reduce O_2 to form ROS. The role of manganese-containing superoxide dismutase is well known to protect from ROS induced by oxidative stress in mitochondria. In mitochondria, L-galactono-γ-lactone dehydrogenase presents at the inner mitochondrial membrane and catalyzes the conversion of L-galactono-γ-lactone to AsA, which is the last step of AsA biosynthesis in plants (Ostergaard et al. 1997). AsA, which is synthesized in the mitochondria, is not only transported to other cell compartments to detoxify ROS but also used as an electron donor to the electron transport chain in the mitochondria. In mitochondria, the presence of the AsA-GSH cycle was confirmed in some plants such as *Pisum sativum* (Jimenez et al. 1997) and *Arabidopsis* (Chew et al. 2003). DHA is generated in the intermembrane space by APX, and DHA can be transported to the mitochondrial matrix, where the AsA-GSH cycle is able to recycle DHA to AsA. In order to fuel these reactions, the reductants are supplied in the form of NADH and NADPH from the tricarboxylic acid cycle (Szarka et al. 2013).

In guard cells, ROS serve as secondary messenger for controlling gas exchange in leaves, and H_2O_2 can regulate the opening and closing of the stomatal pores. The signaling of abscisic acid (ABA) induces H_2O_2 production to facilitate the closing of the stomatal pores under water stress conditions. As described above, the photosynthesis process can induce the fluctuation of H_2O_2 contents during day and night along with the movement of the stomatal pores. The AsA redox status is of importance to the movement of the stomatal pores because AsA is used by APX to convert H_2O_2 to H_2O in cells (Gallie 2013). In tobacco, the increase in the AsA redox status and the decrease in the H_2O_2 contents in the guard cells were achieved in the DHAR-overexpression plants (Chen and Gallie 2004). The DHAR-overexpression plants showed a higher percentage of open stomata, an increase in total open stomatal area, and increased transpiration. Also, the guard cells with an increase in the AsA redox status were less responsive to H_2O_2 or ABA signaling, and the plants exhibited greater water loss under drought conditions. On the other hand, the DHAR-suppression plant showed an increase in the H_2O_2 contents in the guard cells and a reduction in total open stomatal area, and consequently, the plant exhibited an

increase of drought tolerance. Thus, DHAR can regulate the opening and closing of stomatal pores mediated by the AsA redox status through the AsA recycling in the guard cells.

5 Contribution of Ascorbic Acid Recycling Enzymes to Environmental Stress Tolerance

In general, salt, drought, cold/freeze, and high light intensity cause oxidative stresses at different compartments in cells (Mittler et al. 2004; Choudhury et al. 2013). And ROS generations such as H_2O_2 are promoted under oxidative stress conditions. The AsA-GSH cycle plays a significant role in oxidative stress tolerance by removing ROS generated in cells (Noctor and Foyer 1998; Mittler et al. 2004; Foyer and Noctor 2005). As shown in Figs. 1 and 3, MDHAR and DHAR are the components of the AsA-GSH cycle and they contribute to scavenging and detoxifying of ROS with AsA and GSH. APX catalyzes the reduction of H_2O_2 with simultaneous oxidation of AsA, which resulted in generation of MDHA as a primary product and followed by oxidation of MDHA to DHA. MDHAR and DHAR reduce two oxidized AsA, MDHA and DHA, respectively, before hydrolyzing to 2,3-diketogulonic acid.

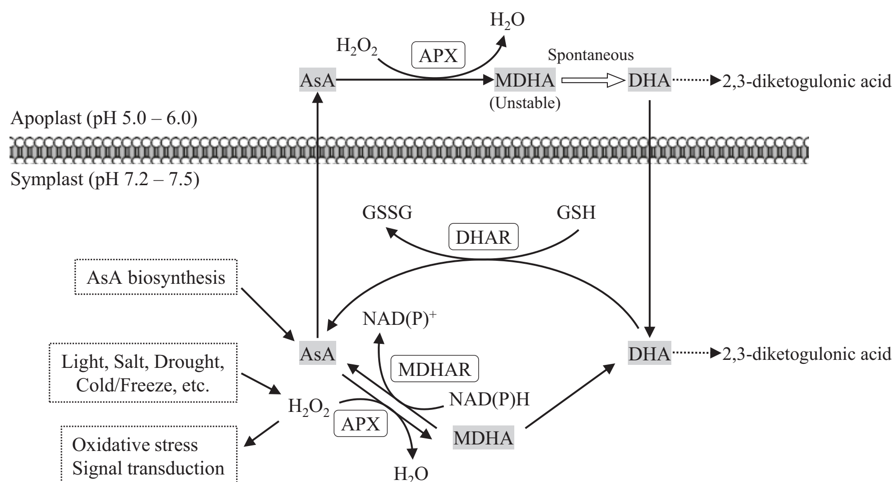

Fig. 3 Overview of ascorbate recycling in plant cells under oxidative stress. Under oxidative stress conditions by light, salt, drought, and cold/freeze, ROS generations such as H_2O_2 are promoted. Ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 with the oxidation of AsA, which resulted in generation of MDHA as a primary product and followed by oxidation of MDHA to DHA. The AsA recycling plays a significant role in oxidative stress tolerance by removing ROS generated in cells. In apoplast, MDHA is rather unstable because of the acidic condition, and MDHA tend to be spontaneously oxidized to DHA. After transporting into the symplast, where DHAR exists, DHAR will reduce DHA to AsA (Adapted from Yin et al. 2010)

Therefore, MDHAR and DHAR are essential enzymes to maintain AsA contents as well as redox status, especially under oxidative stress conditions. As described in the previous section, there are many MDHAR and DHAR isozymes in plants (Leterrier et al. 2005; Lunde et al. 2006; Zhang et al. 2015). And they localize in various cell compartments such as chloroplast, mitochondrion, peroxisome, and cytosol. Thus, MDHAR and DHAR work together to protect from oxidative stress by ROS at different cell compartments. In response to oxidative stresses, the expression levels of MDHAR and DHAR are influenced, although their expression regulations remain unknown. Salt stress is a major limiting factor to plant growth and productivity along with ROS production in plant cells. As described above, MDHAR is the first enzyme getting back oxidized AsA to AsA. In *Avicennia marina* which is a highly salt tolerant species, MDHAR gene was inducibly expressed in salt stressed leaves (Kavitha et al. 2010). Temperature is also one of the factors that not only affects plant growth and development but also causes oxidative stress. Low temperature-induced ROS accumulation has been widely reported in plants (Suzuki and Mittler 2006). And MDHAR activities in tomato fruits were increased by cold stress (Stevens et al. 2008). In addition to oxidative stresses caused by salt and cold, the gene expression of MDHAR in Chinese cabbage was increased by oxidative stress induced by H_2O_2, salicylic acid, paraquat, and ozone (Yoon et al. 2004). The gene expression of DHAR is also induced by oxidative stress. In rice (Urano et al. 2000) and acerola (Eltelib et al. 2011), DHAR activities and expressions were increased under high- and low-temperature conditions. Moreover, the gene expression of DHAR in *Jatropha curcas* was also induced by oxidative stress such as treatments of PEG, NaCl, and H_2O_2 (Chang et al. 2017). The expressions of MDHAR and DHAR in response to oxidative stress in plants suggest that both of MDHAR and DHAR play roles in protecting cell components from oxidative stress through maintaining the AsA redox status. Thus, the overexpressions of MDHAR and DHAR can enhance oxidative stress tolerance. The overexpression of MDHAR in the cytosol of tobacco (Eltayeb et al. 2007, Eltelib et al. 2012) significantly increased AsA contents and AsA redox status as well as MDHAR activities, compared to wild-type tobacco. And the transgenic plants overexpressing MDHAR showed enhanced tolerance to oxidative stress by ozone, salt, and drought. The overexpression of human DHAR in the cytosol of tobacco slightly but significantly increased only AsA redox status but not AsA contents (Kwon et al. 2003). In contrast to human DHAR, the overexpression of rice DHAR in *Arabidopsis* slightly increased only AsA contents but not AsA redox status (Ushimaru et al. 2006). In spite of slight increase of AsA contents and AsA redox status by overexpressing DHAR, the transgenic plants with high DHAR activities enhanced tolerance to oxidative stress such as H_2O_2, methyl viologen, NaCl, and ozone (Kwon et al. 2003; Ushimaru et al. 2006; Chang et al. 2017). Compared to MDHAR, the overexpression of DHAR seems to provide no significant influence on AsA contents and AsA redox status in plants. However, under aluminum (Al) stress leading to accumulation of ROS in roots, tobacco plants overexpressing DHAR in the cytosol showed lower H_2O_2 contents, less lipid peroxidation, and lower level of oxidative DNA damage, compared with transgenic tobacco overexpressing MDHAR and wild-type tobacco (Yin et al.

2010). Al stress causes ROS accumulation mainly in the apoplast of roots. Because of the acidic pH (pH 5–6) where MDHA is unstable in contrast to DHA (Asada 1999), MDHA in the apoplast of roots tend to be spontaneously oxidized to DHA (Fig. 3). Because DHAR is absent in the apoplast, DHA can be reduced to AsA by DHAR after transported into the symplast, where DHAR exists. Thus, the tobacco plants overexpressing DHAR have higher tolerance to Al stress, compared to tobacco overexpressing MDHAR. MDHAR and DHAR play important roles in enhancing stress tolerance against environmental stress according to their localization sites. However, interestingly, there is a report about 2,4,6-trinitrotoluene (TNT) toxicity mediated by MDHAR (Johnston et al. 2015). TNT has a high toxicity and induces ROS accumulation in mitochondria. The suppression of MDHAR targeting to mitochondria leads to enhance tolerance to TNT, which is converted to a nitro radical by MDHAR in mitochondria. Also, the recovery of the MDHAR expression in the mutants reduces the tolerance to TNT.

ROS are accumulated in responses to biotic stress as well as abiotic stress in plants, and cause damage to the cell. However, under biotic stress conditions, ROS act as not only harmful compounds but also valuable substances, because ROS seem to directly kill the invading pathogen and serve as secondary messengers regulating pathogen defense responses (Tripathy and Oelmuller 2012; Choudhury et al. 2013). Thus, the cellular ROS levels may have substantial properties to pathogen defense. It was reported that *Arabidopsis* mutant *vitamin c* (*vtc*), which is deficient in AsA contents, enhanced resistance to pathogens (Barth et al. 2004). When virulent *Pseudomonas syringae* and *Peronospora parasitica* were infected with the *Arabidopsis vtc* mutants, the growth of the bacterial or fungal pathogen was substantially suppressed, compared to wild type. Besides, the expressions of the pathogen-related proteins were strongly induced by the infection of *P. syringae* and *P. parasitica* in the *Arabidopsis vtc* mutants along with high salicylic acid contents. The AsA contents seem to affect defense responses against pathogens in plant cells. Considering functional roles of the AsA-GSH cycles, The AsA-GSH cycles can be a regulator of cellular ROS contents in order to stimulate the redox-regulated plant defense (Noctor and Foyer 1998). Thus, MDHAR and DHAR must be involved in plant–pathogen interactions through maintaining the AsA redox status in plants. *Puccinia striiformis* f. sp. tritici (Pst) causes wheat stripe rust, which is one of the serious diseases in wheat. In wheat (*Triticum aestivum* L.), there are two MDHAR isoforms: TaMDHAR2 and TaMDHAR4. TaMDHAR2 gene is isolated as a target gene of PN-2013, one of microRNAs (miRNAs), in a Pst resistant wheat cultivar. miRNAs are known as regulator of the gene expression of the target gene at the posttranslational level by degrading target mRNA or repressing gene translation (Feng et al. 2014b). When the wheat cultivar was challenged by Pst, the expression of PN-2013 was induced, and then that of TaMDHAR2 was decreased. The negative correlation of PN-2013 expression with the TaMDHAR2 expression suggests that PN-2013 suppress the gene expression of TaMDHAR2 in response to Pst. Similar expression response was reported in the incompatible interaction between wheat and pathogen (Feng et al. 2014a). The expression of TaMDHAR4 was decreased at the early stage of inoculation with a Pst race CYR23 (incompatible interaction),

while no significant change was observed in the compatible interaction with another Pst race CYP31. Based on primary structures, TaMDHAR2 and TaMDHAR4 are expected to exist in cytosol and peroxisomes, respectively. Plant peroxisomes play essential roles in plant–pathogen interaction (McCartney et al. 2005) as well as photorespiration detoxification reaction and plant hormone synthesis (Hayashi and Nishimura 2003; Hu et al. 2012). TaMDHAR2 and TaMDHAR4 function at different cell compartments in plant–pathogen interactions. Besides, the suppression of TaMDHAR2 and TaMDHAR4 showed improved resistance to Pst in wheat (Feng et al. 2014a, b). Thus, MDHAR could contribute to pathogen defense through regulation of ROS metabolism by the AsA-GSH cycles.

6 Conclusion

AsA functions as an antioxidant which is essential for photosynthesis and stress response in order to remove generated ROS. Therefore, AsA contents and its redox status are of importance to health and stress tolerance of plants. And because of its nutrient values for human, which cannot produce AsA by themselves, the AsA contents in vegetables and fruits have been paid attention to for a long time. Although the AsA biosynthesis appears to be a primary factor in determining and regulating the AsA contents in plants, the AsA contents as well as its redox status are the result of the balance between its biosynthesis and recycling. In the AsA-GSH cycles including AsA recycling, two reductases, MDHAR and DHAR, reduce MDHA and DHA to AsA, respectively. In this chapter, at first, the primary structures of MDHAR and DHAR as well as their gene expressions in leaves and fruits are focused. Plants possess the multiple isozymes of MDHAR and DHAR, and the isozymes of MDHAR and DHAR are classified into some groups, based on their primary structures. They localize at different cell compartments: cytosol, chloroplast, mitochondrion, peroxisome, and vacuole. Considering that the overexpression of DHAR localizing in chloroplasts did not increase the DHAR activities in potato tubers, some MDHAR and DHAR isozymes might need to be posttranslationally modified in its localization site. The differences in their cellular localization and posttranslational modification suggest the isozymes function at different cell compartments in plants. In leaves, the gene expression patterns of MDHAR and DHAR suggest they are mainly involved in the diminishment of ROS produced during photosynthesis. MDHAR and DHAR also function during fruit development and ripening. There are mostly two types of the AsA accumulation model during fruit development and ripening. One is that the AsA is accumulated at the highest levels in green fruits and its contents decrease as fruit maturation, while the other shows opposite properties of AsA accumulation. Thus, the contribution and influence of MDHAR and DHAR on the AsA recycling during fruit development and ripening vary among plant species. And their gene expression patterns suggest that they have a complementary relationship in maintaining the AsA redox status.

Aerobic metabolism constantly generates ROS under both normal and stress condition at the different plant cellular compartments, like chloroplasts, mitochondria, and peroxisomes. Chloroplast is a major source of ROS production in plants. To ensure the continuous survival of plants under stress conditions, controlling and scavenging the ROS in the chloroplasts are very essential. AsA present in millimolar concentrations in chloroplasts (Smirnoff 2000). However, AsA is synthesized not in chloroplasts but in the mitochondria (Ostergaard et al. 1997) and transported to other cell compartments to eliminate ROS. Thus, MDHAR and DHAR isozymes are essential to scavenge ROS as part of the AsA-GSH cycles in chloroplasts, mitochondria, and peroxisomes. In addition, the AsA recycling has sometimes greater effect than the AsA biosynthesis especially under environmental stress, because the increase of AsA contents by AsA biosynthesis cannot occur in hours when plants suffer environmental stresses (Bartoli et al. 2006). Therefore, the efficiency of the AsA recycling is of importance to regulate cellular ROS contents under stress conditions. In fact, transgenic plants overexpressing MDHAR and DHAR show enhanced tolerance to oxidative stress, in spite of slight increase of AsA contents. However, ROS acts as not only toxic substances to damage cell components but also signal elements involved in pathogen defense. In pathogen infection, the gene expression of MDHAR was suppressed in the wheat leaves, and the suppression of MDHAR enhanced the resistance to pathogen stress. According to the environmental condition, MDHAR and DHAR contribute to stress tolerance through regulation of ROS metabolism by the AsA-GSH cycles.

In plants, the AsA contents are the results of balance of AsA biosynthesis and its recycling. Thus, the AsA contents can depend on the ability of AsA recycling as well as that of its biosynthesis in plants. The AsA recycling can serve as two integrated means by the effect on the AsA contents in plants, one is to control plant health and development, and the other is engineering of the improving with nutrient values and stress tolerance. The mechanisms of gene expressions of MDHAR and DHAR would be of great interest to manipulating the AsA contents in plants.

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Ascorbic Acid Biofortification in Crops

Gavin M. George, Michael E. Ruckle, Melanie R. Abt, and Simon E. Bull

Abstract L-Ascorbic acid (AsA) is an essential nutrient in the human diet. It is required for growth and repair of tissues, and severe deficiency can lead to debilitating diseases such as scurvy. Although relatively cheap to synthetically produce, ascorbate deficiencies are common throughout developed and developing countries. In plants, AsA is a key component of multiple antioxidant systems, which defend plant cells from biotic and abiotic stresses, and is therefore an attractive target for biofortification in crops. AsA metabolism is complex due to the existence of multiple biosynthetic pathways, which can contribute differing amounts of AsA depending on the plant species, organ, tissue type, and stress conditions. In addition, several pathways of oxidation and recycling of AsA exist, which together allow subcellular compartments to tailor AsA accumulation and turnover. Biotechnological approaches in model and crop species have been successful in increasing AsA concentrations, but these gains have not reached the potential some species of plants have to produce AsA and that could benefit industrial and public stakeholders. Given recent improvements in our understanding of AsA biosynthesis as well as the advancement in novel breeding technology, there is renewed potential to overcome limitations in AsA biofortification. Here we attempt to connect the current biological knowledgebase with novel technologies and crop resources to provide a strategy to improve plant-synthesized AsA in the world food system.

Keywords L-Ascorbic acid · Vitamin C · AsA · Biotechnological improvement · Ascorbate recycling · Smirnoff–Wheeler · D-Mannose/L-galactose · L-Gulose · Pectin · Genome editing · TILLING · Transgenic · Commercial varieties

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Gavin M. George and Michael E. Ruckle contributed equally to this work.

G. M. George (✉) · M. R. Abt · S. E. Bull
Department of Biology, ETH Zurich, Zurich, Switzerland
e-mail: ggeorge@ethz.ch

M. E. Ruckle
Molecular Plant Breeding, Institute of Agricultural Sciences, Zurich, Switzerland

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1 Introduction

L-Ascorbic acid (AsA) or vitamin C has classically been a target for biofortification in crops due to its value in human health, animal nutrition, food processing, and crop stress tolerance. Diets of human ancestors were rich in AsA rendering a mutation in L-gulono-lactone oxidase as neutral allowing it to become fixed in the modern population. Lacking this terminal reaction in the "animal pathway" to synthesise AsA, humans and other primates must acquire it from their diet rendering it an essential micronutrient. The lack of dietary AsA may result in scurvy, a condition with painful and disfiguring symptoms that in severe cases can result in death. AsA has a key role in iron metabolism, in the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (Lane and Richardson 2014). In fact, most of the symptoms associated with scurvy are related to AsA acting as a cofactor in critical biological processes rather than its role as an antioxidant (Camarena and Wang 2016). As an antioxidant, AsA has been shown to be effective against oxidative stress-related diseases such as cancer, ageing, and cardiovascular disease, as well as a general immune booster (Diplock et al. 1998; Padayatty et al. 2003; Levine and Violet 2017). Humans require a minimum of 10 mg day^{-1}, although the Recommended Dietary Allowance (RDA) is approximately 1 mg day^{-1} kg^{-1} body mass (Drouin et al. 2011). Globally, 23% of the population does not obtain the minimum recommended intake of AsA. This deficiency is not limited to developing countries. Even with high public awareness surrounding the benefits of vitamin C, the level of AsA deficiency is unexpectedly high in developed countries. A study in the United States found that between 5 and 17% of people displayed blood serum concentration of vitamin C, which were classified as clinically deficient (Schleicher et al. 2009; Troesch et al. 2012).

Although the development of the Reichstein process in the early 1930s led to cheap dietary supplements of AsA, a majority of the human dietary vitamin C intake is obtained from eating fruits, nuts, tubers, and vegetables. AsA has the largest industrial production volume amongst the 13 essential human vitamins. In 2014, 110,000 metric tons of vitamin C were industrially produced. Approximately 50% of this production is used by the pharmaceutical industry as a dietary supplement for human health and nutrition, 40% is used by the food processing and beverage industry as a preservative, and 10% is used for supplementation into animal feed. The price for industrially produced ascorbic acid has ranged between 4 and 10 USD kg^{-1} over the last 20 years (Pappenberger and Hohmann 2014).

In plants, AsA is produced at varying concentrations in different tissue types and primarily serves as an antioxidant to protect plant cells from reactive oxygen species (ROS) that are produced from metabolic processes such as photosynthesis and respiration (Wheeler et al. 2015). Moreover, as an antioxidant, AsA plays a role in biotic and abiotic induced oxidative stress, such as drought and pathogen attack. Increasing AsA concentrations in plants has been shown to improve tolerance to several oxidative stresses and offers a potential route to maintain productivity in the face of global climate change (Lisko et al. 2014). AsA is also used as a cofactor for biosynthetic reactions, which have roles in cellular elongation, defence, and fruit ripening (Arrigoni and De Tullio 2002; Pastori et al. 2003; Green and Fry 2005a).

Due to the combined benefit to human health and plant productivity, improvement of AsA concentrations has received considerable attention from the plant biology community. Depending on the plant species, plant tissue, and environmental condition, AsA is primarily produced either through the D-mannose/L-galactose biosynthetic pathway, also known as the "Smirnoff–Wheeler pathway", or the D-galacturonate biosynthetic pathway, also known as the "pectin pathway". Over the last few decades, the genes, metabolic intermediates, and enzymes that define these biosynthetic pathways have been increasingly characterized, particularly in the model species *Arabidopsis thaliana*. A majority of the current understanding of the physiological role of AsA has been placed in the context of ROS scavenging in the leaves of Arabidopsis and Arabidopsis mutants. In Arabidopsis, successful attempts to genetically modify and overexpress enzymes in AsA biosynthesis have led to higher AsA levels in plant tissues, and increased oxidative stress tolerance. In crop species, genetic modification leading to increases in AsA concentrations has been reported to have beneficial traits that include higher oxidative, light and osmotic stress tolerance, less post-harvest oxidation, as well as improved photosynthetic rate, and growth (Lisko et al. 2013; Waltz 2015a, b; Nunes-Nesi et al. 2005; Zhang et al. 2011; Lim et al. 2016; Chen and Gallie 2005; Macknight et al. 2017).

Although examples exist where the genetic modification of AsA biosynthesis has resulted in traits that have potential benefit, these modified AsA levels have not reached the degree of improvement required to have a significant impact on human health or the economics surrounding agricultural based AsA production. This book chapter presents the current understanding of AsA metabolism in model species, and places it in the context of the current knowledge of AsA content in crop species, and the ever-expanding genetic molecular toolset that molecular breeders have to improve AsA content in crop plants. Ultimately, the goal is to address the current limitations and recent advances in crop biotechnology for placing potential AsA biofortified crops into the current economic marketplace.

2 Ascorbic Acid Metabolism

2.1 Overview

L-Ascorbate is a carbohydrate derivative that is produced from D-monosaccharides, which are central metabolites in all organisms. Several pathways leading to the production of AsA have been described (Fig. 1), which are generally categorized by their carbohydrate intermediates. The classical D-mannose/L-galactose pathway makes up the major route of biosynthesis in plants (Wheeler et al. 1998). The D-galacturonate pathway provides another route of biosynthesis which shares the final intermediate with the D-mannose/L-galactose pathway: L-galactono-1,4-lactone (L-GalL). The L-gulose pathway is a branch of the galactose pathway but produces a secondary final intermediate: L-gulono-1,4-lactone (L-GulL) (Wolucka and Van Montagu 2003). Two further pathways provide carbon skeleton via

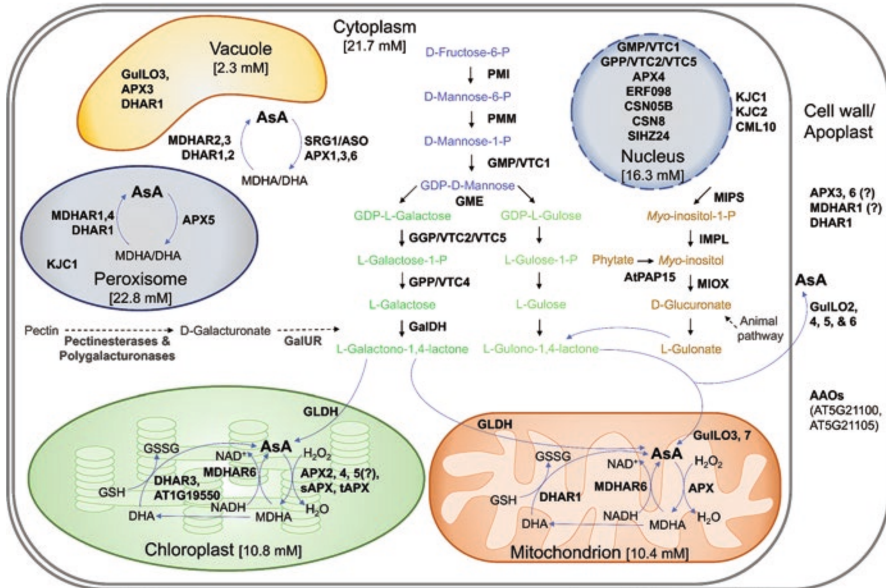

Fig. 1 Overview of the ascorbate biosynthetic and recycling pathways present in plants. Enzyme and protein subcellular localizations, as well as ascorbate concentrations, are representative of those found in Arabidopsis. Unannotated reactions have yet to be conclusively identified

myo-inositol (D-MI) and D-glucuronate (D-GlcU). The latter is regarded as the animal-like pathway and does not appear to contribute to the production of AsA in higher plants. Both of these routes also produce L-GulL as the substrate for L-ascorbate production.

2.1.1 L-galactose Biosynthetic Pathway

In Arabidopsis, most of the primary components of the AsA biosynthetic pathway were proposed based on biochemical experiments by Wheeler et al. (1998). Soon after, the D-mannose/L-galactose pathway was confirmed through reverse genetic screens that identified a series of vitamin C (*vtc*) deficient mutants (Conklin et al. 2000). Alleles of *vtc1* and *vtc2* were first identified from an ethyl-methanesulfonate (EMS) mutagenized population for their increased sensitivity to ozone. More AsA mutants were screened and identified by their reduced concentration of AsA (Conklin et al. 2013). Most of the mutant genes encode enzymes in the D-mannose/L-galactose pathway with the exception of *vtc3*. VTC3 contains both a protein kinase and phosphatase domain, the loss of which prevents accumulation of AsA in response to stresses such as heat and light. The target of this activity is still unknown, although it does not appear to directly interact with other known enzymes in the pathway (according to Arabidopsis.org; string-db.org). Based on the large body of work on Arabidopsis mutants, the primary pathway for AsA biosynthesis was constructed (Fig. 1; Table 1).

Table 1 Arabidopsis enzymes and proteins associated with ascorbate synthesis, recycling, and regulation and their predicted subcellular localization according to TAIR (arabidopsis.org), unless otherwise stated

Name	Substrate	Abbreviation	Alternate name	Locus	Subcellular localization
Phosphomannose isomerase	D-Fructose-6-P	PMI	PMI1	AT3G02570	Cytoplasm
			PMI2, DIN9	AT1G67070	Cytoplasm
Phosphomannomutase	D-Mannose-6-P	PMM		AT2G45790	Cytoplasm
GDP-Mannose pyrophosphorylase	D-Mannose-1-P	GMP	VTC1, CYT1	AT2G39770	Cytoplasm, nucleus
				AT3G55590	Cytoplasm
				AT4G30570	Cytoplasm
GDP-D-Mannose 3′,5′-epimerase	GDP-D-Mannose	GME		AT5G28840	Cytoplasm
GDP-L-Galactose phosphorylase	GDP-L-Galactose	GGP	VTC2	AT4G26850	Cytoplasm, nucleus
			VTC5	AT5G55120	Cytoplasm, nucleus
L-Galactose-1-phosphate phosphatase	L-Galactose-1-P	GPP	VTC4	AT3G02870	Cytoplasm, plasma membrane
L-Galactose dehydrogenase	L-Galactose	GalDH		AT4G33670	Cytoplasm
L-Galactono-1,4-lactone dehydrogenase	L-Galactono-1,4-lactone	GLDH		AT3G47930	Mitochondrion, plastid
L-Gulono-1,4-lactone oxidase	L-Gulono-1,4-lactone	GulLO	GulLO1	AT1G32300	Membrane
			GulLO2	AT2G46750	Extracellular
			GulLO3	AT5G11540	Mitochondrion, vacuole
			GulLO4	AT5G56490	Extracellular
			GulLO5	AT2G46740	Cell wall (Aboobucker et al. 2017)
			GulLO6	AT2G46760	Extracellular
			GulLO7	AT5G56470	Mitochondrion, membrane
myo-Inositol-1-phosphate synthase	D-Glucose-6-phosphate	MIPS	MIPS1	AT4G39800	Cytoplasm, nucleus
			MIPS2	AT2G22240	Cytoplasm
			MIPS3	AT5G10170	Cytoplasm
myo-Inositol oxygenase	*myo*-inositol	MIOX	MIOX1	AT1G14520	Cytoplasm

(continued)

Table 1 (continued)

Name	Substrate	Abbreviation	Alternate name	Locus	Subcellular localization
			MIOX2	AT2G19800	Cytoplasm
			MIOX4	AT4G26260	Cytoplasm
			MIOX5	AT5G56640	Cytoplasm
L-Ascorbate oxidase	L-ascorbate	ASO		AT5G21100	Extracellular
				AT5G21105	Extracellular, plasmodesmata
			SRG1	AT1G17020	Cytoplasm
Ascorbate peroxidase	L-ascorbate	APX	APX1	AT1G07890	Chloroplast, cell wall, cytoplasm
			APX2	AT3G09640	Chloroplast, cytoplasm
			APX3	AT4G35000	Chloroplast, glyoxisome, peroxisome, vacuole, mitochondrion
			APX4	AT4G09010	Chloroplast thylakoid lumen, cytoplasm, nucleus
			APX5	AT4G35970	Chloroplast , peroxisome
			APX6	AT4G32320	Cytoplasm, extracellular
			APXt	AT1G77490	Chloroplast thylakoids
			APXs	AT4G08390	Chloroplast stroma
Monodehydroascorbate reductase	Monodehydroascorbate	MDHAR	MDAR1	AT3G52880	Chloroplast, apoplast, cytosol, peroxisome
			MDAR2	AT5G03630	Chloroplast, cytoplasm
			MDAR3	AT3G09940	Cytoplasm
			MDAR4	AT3G27820	Chloroplast, cytosol, peroxisome
			MDAR5		
			MDHAR	AT1G63940	Cytosol, chloroplast, mitochondrion

Dehydroascorbate reductase	Dehydroascorbate	DHAR	DHAR1, DHAR5	AT1G19570	Chloroplast, cytoplasm, peroxisome, mitochondrion, vacuole, apoplast
			DHAR2	AT1G75270	Cytoplasm, plasma membrane
			DHAR3	AT5G16710	Cholroplast
				AT1G19550	Chloroplast, cytoplasm
Protein kinase/protein phosphatase		VTC3		AT2G40860	Plastid (Conklin et al. 2013)
Ethylene response factor 98		AtERF98		AT3G23230	Nucleus
Ascorbic acid mannose pathway regulator 1		AMR1		AT1G65770	Nucleus
Cop9-signalosome 5b		CSN5B	AJH2	AT1G71230	
Constitutive photomorphogenic 9		CSN8		AT4G14110	Nucleus, cytoplasm
HD-ZIP I—Arabidopsis thaliana homeobox 1		SlHZ24		AT3G01470	Nucleus
KONJAC		KONJAC1		AT1G74910	Cytoplasm, peroxisome
		KONJAC2		AT2G04650	Cytoplasm

The substrates for the production of L-ascorbate through the D-mannose/L-galactose pathway are provided by abundantly available D-monosaccharide pools. Mannose-6-phosphate isomerase (PMI), phosphomannomutase (PMM), and GDP-mannose pyrophosphorylase (GMP/VTC1) act to catalyse the conversion of D-fructose-6-phosphate (F6P) to GDP-D-mannose (GDP-D-Man) (Fig. 1) (Maruta et al. 2008; Qian et al. 2007). GDP-D-mannose-3,5-epimerase (GME) epimerizes a reversible two step reaction providing substrates for both the committed reaction of the D-mannose/L-galactose pathways and the parallel L-gulose pathway, which is an alternative AsA biosynthetic route (Wolucka and Van Montagu 2003). Within the D-mannose/L-galactose pathway, the first reaction considered to be specific to the production of AsA is the conversion of GDP-L-galactose (GDP-L-Gal) to L-galactose-1-phosphate (L-Gal1P) by the GDP-L-galactose phosphorylase (GGP/VTC2/AT4G26850 and GPP/VTC5/AT5G55120). In Arabidopsis, *ggp/vtc2* mutants have only 20% of the total AsA found in wild-type plants (Conklin et al. 2000). VTC2 is, therefore, responsible for 80% of the total GGP activity, while VTC5 is responsible for the remaining 20%. Loss of both enzyme activities in double mutant studies results in growth arrest and bleaching soon after germination (Dowdle et al. 2007). The next step in the pathway is the dephosphorylation of L-Gal1P by L-galactose-1-P phosphatase (GPP/VTC4/AT3G02870) to L-galactose (L-Gal; Conklin et al. 2006). L-Gal is converted to L-galacto-1,4-lactone (L-GalL), which is the final intermediate of the pathway, by galactose dehydrogenase (GalDH/AT4G33670; Gatzek et al. 2002). These reactions occur in the cytoplasm (Fig. 1). The final step to the production of AsA is performed by L-galactono-1,4-lactone dehydrogenase (GLDH/AT3G47930; Ostergaard et al. 1997). GLDH is localized in the chloroplast and in the mitochondrion, in association with the membrane and mitochondrial Complex I (Mapson et al. 1954; Bartoli et al. 2000; Schertl et al. 2012; Wheeler et al. 2015; Subrahmanian et al. 2016).

Within the D-mannose/L-galactose pathway, substrates for the committed reactions are supplied through the activity of four enzymes PMI, PMM, GMP, and GME. In Arabidopsis, synthesis of AsA is partially restricted by the activity of these four enzymes based on overexpression studies. Arabidopsis lines overexpressing PMM resulted in an increase of >10-fold more enzyme activity, but only a 25–33% increase in AsA concentration was observed (Table 2; Qian et al. 2007). Ectopic expression of the Acerola *PMM* in tobacco led to a twofold increase in total AsA concentrations in the leaves (Badejo et al. 2009). Overexpression of *GMP* or *GME* in Arabidopsis resulted in approximately 20 and 50% gains in total AsA concentrations (Li et al. 2016; Ma et al. 2014). The first committed reaction in the L-galactose pathway, however, exerts significantly more control over steady state concentrations of AsA (Bulley et al. 2009; Laing et al. 2007). Overexpression of each individual component of the D-mannose/L-galactose pathway in Arabidopsis leaves alone had some effect from 20–80% increase in total AsA concentration (Zhou et al. 2012). Overexpression of *GGP* in combination with downstream components *GPP* or *GLDH* yielded up to a fourfold increase in AsA concentration. It would appear in Arabidopsis that an increase in the activity of a single enzymatic reaction is not sufficient to result in a meaningful increase in flux through the pathway.

Table 2 Recent successful transgenic interventions in model and crop species

Pathway	Plant	Gene	Gene source	AsA (FC)	Oxidative	Ozone	Salt	Drought	Temperature	Light	Growth	Photosynthesis	Target for modification	Notes	References
L-Galactose	Tobacco	PMM	Acerola	2											Badejo et al. (2009)
	Tobacco	GMP	Acerola	2											Badejo et al. (2008)
	Tomato	GMP	Yeast	1.5–1.7											Cronje et al. (2011)
	Tomato	GMP3	Tomato	1–1.5	•										Zhang et al. (2013)
	Arabidopsis	GMP ΔD27E	Arabidopsis	1.5							↑		✓		Li et al. (2016)
	Rice	GME	Arabidopsis	2			•				↑ Stress				Zhang et al. (2015)
	Tomato	GME	Tomato	2	•		•		•		↑ Stress				Zhang et al. (2011)
	Arabidopsis	GME	Alfalfa	1.5			•				↑ Stress				Ma et al. (2014)
	Rice	GME	Arabidopsis	2							↑ Stress				Zhang et al. (2015)
	Tomato	GGP	Kiwi	2–6							↓ Yield			i	Bulley et al. (2012)
	Potato	GGP	Potato	2–3											Bulley et al. (2012)

(continued)

Table 2 (continued)

Pathway	Plant	Gene	Gene source	AsA (FC)	Oxidative	Ozone	Salt	Drought	Temperature	Light	Growth	Photosynthesis	Target for modification	Notes	References
	Strawberry	GGP	Kiwi	2											Bulley et al. (2012)
	Rice	GGP	Arabidopsis	2–3			•				↑ Stress				Zhang et al. (2015)
	Arabidopsis	GGP + GME	Arabidopsis	1.2											Laing et al. (2015)
	Arabidopsis	GGP (ΔuORF) + GME	Arabidopsis	5									✓	ii	Laing et al. (2015)
	Arabidopsis	GGP + GPP	Arabidopsis	1–4											Zhou et al. (2012)
	Arabidopsis	GGP + GLDH	Arabidopsis	1–3.5											Zhou et al. (2012)
	Tobacco	GLDH	*Rosa roxburghii*	1.5–2	•		•								Liu et al. (2013)
	Lettuce	GLDH	Lettuce	1–1.5										iii	Landi et al. (2015)
L-Gulose and *myo*-inositol	Lettuce and tobacco	GLOase	Rat	2–7											Jain and Nessler (2000)
	Arabidopsis	GLOase	Rat	2–3							↑				Radzio et al. (2003)
	Tobacco cell lines	GulLO1, -2, -3, -5	Arabidopsis	2–3											Matura et al. (2010)

	Arabidopsis	MIOX4	Arabidopsis	1.5–2			•		•		↑				Lisko et al. (2013)
	Arabidopsis	MIOX4	Arabidopsis	2–3, 1.7											Lorence et al. (2004)
	Arabidopsis	MIOX4	Arabidopsis	1.7					•			↑NPQ			Tóth et al. (2011)
	Arabidopsis	MIOX4	Arabidopsis	2–3			•				↑				Lorence et al. (2004)
	Tomato	MIOX4	Arabidopsis	2											Kulkarni (2012)
	Arabidopsis	MIOX4	Arabidopsis	1										iv	Endres and Tenhaken (2009)
	Tomato	MIOX2	Arabidopsis	1										iv	Cronje et al. (2011)
	Arabidopsis	AtPAP15	Arabidopsis	2			•								Zhang et al. (2008)
D-Galacturonate	Arabidopsis	GalUR	Strawberry	2–3											Agius et al. (2003)
	Tomato (hairy roots)	GalUR	Strawberry	2							↓			v	Wevar-Oller et al. (2009)
	Tomato	GalUR	Strawberry	2.5	•		•	•							Lim et al. (2016)
	Tomato	GalUR	Strawberry	1.5–2.5	•		•		•						Cai et al. (2015)
	Potato	GalUR	Strawberry	2			•	•							Hemavathi et al. (2009)

(continued)

Table 2 (continued)

Pathway	Plant	Gene	Gene source	AsA (FC)	Oxidative	Ozone	Salt	Drought	Temperature	Light	Growth	Photosynthesis	Target for modification	Notes	References
	Tomato	GalUR	Strawberry	1–1.4							↑ yield	↑NPQ			Amaya et al. (2015)
Recycling and oxidation	Tobacco and maize	DHAR	Arabidopsis	2		•	•	•							Eltayeb et al. (2006)
	Tobacco	DHAR	Tobacco	3[a]		•						↑ Assimilation[a]			Chen and Gallie (2005)
	Potato	DHAR	Sesame	1.5											Goo et al. (2008)
	Potato	DHAR	Potato	1–1.8										vi	Qin et al. (2011)
	Maize	DHAR	Rice	6										vii	Naqvi et al. (2009)
	Arabidopsis	DHAR	Arabidopsis	2–4	•				•	•					Wang et al. (2010)
	Tomato	DHAR	Tomato	1.6										viii	Haroldsen et al. (2011)
	Tomato	DHAR	Tomato	1–1.5	•		•								Li et al. (2012)
	Tomato	DHAR	Pyrus sinkiangensis	1.5			•		•						Qin et al. (2015)
	Arabidopsis	DHAR	Kiwi	1–1.2											Liu et al. (2015)
	Tomato	MDHAR	Tomato	1.27	•		•		•				✓	ix	Gest et al. (2013)

	Tobacco	Chl-DHAR	Human	1											Kwon et al. (2003)
	Arabidopsis	DHAR	Rice	1–1.5	•		•		•					x	Ushimaru et al. (2006)
	Arabidopsis	DHAR	Arabidopsisi	1–1.2	•										Yin et al. (2010)
	Arabidopsis	Chl-MDHAR	Tomato	1–1.2	•				•		↑	↑A ↑Fm/Fv			Li et al. (2010b)
Alternative/regulation	Tomato	MDH	Tomato	6							↑	↑A ↑rETR			Nunes-Nesi et al. (2005)
	Tomato	ALO	Yeast	1.5											Cronje et al. (2011)
	Tobacco	ALO	Yeast	2–3				•	•						Bao et al. (2016)
	Tobacco	ALO	Yeast	2	•					•					Chen et al. (2015)
	Stylosanthes	ALO + SgNCED	Yeast and stylosanthes	3–4					•			↑Fm/Fv			Bao et al. (2016)
	Tomato	SlHZ24	Tomato	1.5	•									xi	Hu et al. (2016)
	Arabidopsis	AMR1 (mutant)	Arabidopsis	2–3		•							✓	xii	Zhang et al. (2009)
	Arabidopsis	AtERF98	Arabidopsis	1.7			•							xiii	Zhang et al. (2012)
	Arabidopsis	KJC1	Arabidopsis	1.5										xiv	Sawake et al. (2015)
	Tomato	SlDOF22	RNAi silencing	1.2			•						✓		Cai et al. (2016)

(continued)

In each case, the transgene and its source are shown as well as the target plant species. Furthermore, the reported fold change (FC) increase in total ascorbate and stress tolerance conferred is shown

i Increased AsA correlated with loss of seeds/viability
ii An open reading frame with non-cannonical start yields a peptide that regulates GGP translation
iii Reduced post-harvest browning
iv Increased uronic acids in cell wall
v Loss of growth but not correlated with AsA
vi Cytosolic and chloroplastic isoforms expressed - Only cytosolic increased AsA in leaves and tubers, chloroplastic increased leaf only
vii Three interventions combined to increase beta carotene, ascorbate, and folate
viii Increased AsA in fruit but not leaves
ix Expressed in peroxisome and cytosol—negatively correlates with AsA concentrations in light dependent manner
x Expressed in chloroplast; good example of plants having little change in AsA but enhanced tolerance
xi In leaves but less in fruit
xii Negatively regulates Smirnoff-Wheeler pathway
xiii Activates AsA biosynthetic pathway
xiv Stimulates GMP/VTC1 activity

[a]After ozone treatment

2.1.2 The L-Gulose, *Myo*-inositol, and D-Galacturonate Biosynthetic Pathways

Three alternative biosynthetic pathways to the L-galactose pathway have been demonstrated to exist in plants. The L-gulose and *myo*-inositol pathways both produce L-GulL as the substrate for AsA synthesis. The L-gulose pathway results from an epimerization reaction catalysed by GME, which produces GDP-L-gulose (GDP-L-Gul) as well as GDP-L-Gal (Wolucka and Van Montagu 2003). GME catalyses a reversible reaction, thus GDP-L-Gul is typically in equilibrium with GDP-L-Gal and GDP-D-man in a 0.2:0.4:1 ratio. Not all of the components of the L-gulose pathway, however, have been fully characterized. Like the L-gulose pathway, the *myo*-inositol pathway has been proposed to supply L-GulL, but in this case through D-glcU as an intermediate to AsA synthesis (Lorence et al. 2004). It is hypothesized that D-MI is supplied through the action of *myo*-inositol phosphate synthase (MIPS) and *myo*-inositol monophosphatase (IMPL) (Fig. 1). D-MI is then converted to D-GlcU through the action of *myo*-inositol oxygenase (MIOX). Overexpression of *MIOX4* in Arabidopsis leads to increased AsA concentrations (Table 2; Lisko et al. 2013; Lorence et al. 2004; Tóth et al. 2011). In addition, constitutive overexpression of Arabidopsis purple acid phosphatase, *AtPAP15*, which can dephosphorylate *myo*-inositol-1-phosphate (MI1P), increased total AsA by twofold (Zhang et al. 2008). The practical contribution of the *myo*-inositol pathway has not reached full consensus in the field. Constitutive overexpression of *MIOX4* by 30-fold in Arabidopsis was shown to have little effect on AsA concentrations, although it did affect carbon incorporation into the cell wall (Endres and Tenhaken 2009). Similar results in tomato were reported (Cronje et al. 2011). It is likely that if this pathway does contribute to AsA accumulation it may be only conditionally important.

The final reaction of both the *myo*-inositol and L-gulose pathways is catalysed by a suite of L-gulono-1,4-lactone oxidases (L-GulLO) (Maruta et al. 2010). This reaction is distinct from the GLDH-catalysed reaction of the L-GalL precursor-dependent routes. The Arabidopsis L-GulLO5 shows complete specificity for L-GulL as a substrate (Aboobucker et al. 2017). Precursor feeding tobacco cells expressing recombinant L-GulLO results in increased AsA (Matura et al. 2010). In leaves of Arabidopsis and tobacco, AsA concentrations could be increased by tenfold through feeding of L-GulL (Davey et al. 1999; Jain and Nessler 2000). Constitutive expression of the L-gulono-γ-lactone oxidase gene, an ortholog of L-GulLO, from rat (*Rattus norvegicus*) in Arabidopsis displayed a twofold increase in AsA content (Radzio et al. 2003). Transformation of the rat ortholog into the *gmp/vtc1* mutant recovered the AsA concentration to wild-type levels (Radzio et al. 2003). This enzymatic reaction presumably is upstream of both the L-galactose and L-gulose branches of the D-mannose/L-galactose pathway. This suggests that alternate routes, such as through MIOX4, or a hypothesized animal-like pathway, could compensate for the loss of the L-galactose pathway when an L-GulLO is constitutively expressed. In the *ggp/vtc2* mutant background, which only has an active L-gulose branch of D-mannose/L-galactose pathway, more AsA was observed than in wild-type plants transformed with *L-GulLO*. In this case, the L-gulose pathway appears to facilitate increased flux than the wild-type D-mannose/L-galactose pathway (Radzio et al.

2003). Expression of L-GulLO in the cytoplasm may bypass much of the regulation surrounding the D-mannose/L-galactose pathway and allow significant increases in AsA. While conclusive evidence for the subcellular localization of the various L-GulLOs is still required, they are predicted to be targeted to extracellular parts of the cell (Arabidopsis.org), the mitochondrion or the vacuole (Table 1). Indeed, purification of recombinant L-GulLO5 from tobacco required a methodology consistent with a cell wall associated protein (Aboobucker et al. 2017). Taken together, alternate routes to AsA production, which are not under ingrained metabolic constraints or environmental influence, may be more appropriated for AsA biofortification.

The D-mannose/L-galactose alternative pathways are organ or conditionally specific, therefore forward and reverse genetics approaches in Arabidopsis have been less successful in identifying the associated genes. Studies in non-model organisms are furthermore challenging but biochemical feeding studies have proved invaluable in determining alternative pathway contributions. The D-galacturonate pathway is a good example of organ-specific AsA production. During early fruit development, AsA is predominantly supplied via the D-mannose/L-galactose pathway, which converts soluble sugars supplied by source leaves into AsA (Badejo et al. 2012). During fruit ripening, AsA is produced from the breakdown of pectin, and D-galacturonate (D-GalAR) becomes the major substrate for the production of L-GalL and AsA (Di Matteo et al. 2010). This pathway transition during ripening was shown by feeding both green and red tomato fruits with L-Gal and D-GalAR. The L-Gal was able to increase AsA in green and red fruits, while D-GalAR could only increase AsA in the red fruits (Badejo et al. 2012). Genetic evidence for activity of the D-galacturonate pathway in tomato fruit is provided from a study by Di Matteo et al. (2010); The wild species *S. pennellii* (which accumulates AsA) was introgressed into a commercial-bred low-AsA variety of *S. lycopersicum*. Expression analysis of the introgression lines showed the upregulation of pectinesterases and two polygalacturonases, which were hypothesized to supply D-GalAR for the production of AsA.

2.2 Oxidation and Recycling of Ascorbic Acid

The production of reducing equivalents and ATP by the aerobic oxidation of carbon molecules through oxidative respiration, proved to be significantly more efficient than other forms of anaerobic metabolism and thus facilitated the evolution of multicellular life (Raymond and Segre 2006). However, the energy benefit comes at the cost of the production of ROS. ROS are predominantly singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl (HO^-), and perhydroxyl (HO^{2-}) (Halliwell and Gutteridge 1999). ROS can acquire electrons from a broad range of metabolites, carbohydrates, lipids, proteins, nucleic acids, and other cellular components by oxidation, which results in the production of toxic or non-functional products. Each species of ROS differs in its reactivity (Moller et al. 2007). Hydroxyl radicals are amongst the most reactive, and rapidly oxidize all cellular components. Singlet oxygen is highly reactive with polyunsaturated fatty acids and some amino acids but less reactive with carbohydrates. H_2O_2 is more prevalent, has a longer

half-live, and is actively managed to prevent irreversible oxidative damage (Moller et al. 2007). In order to mitigate oxidative intracellular environments generated by aerobic metabolism, antioxidants evolved to freely donate electrons and absorb the oxidative potential of free radicals.

AsA is a fundamental component of the plant antioxidant system. Unlike the functionally related glutathione, AsA is not synthesized in prokaryotes such as *Escherichia coli* (Yew and Gerlt 2002). Therefore, the antioxidant property of AsA appears to have a specific role associated with eukaryote evolution (Gest et al. 2013). As a cellular antioxidant, AsA is energetically cheap to produce. Complete synthesis of AsA requires one glucose, one GTP, and one NAD^+, which produces one GDP, one NADH, and one reduced cytochrome (Smirnoff and Wheeler 2000). Although cheap, plants must carefully limit flux in the direction of AsA to prevent over-utilization of their carbohydrate pools. Approximately 1% of the available glucose is allocated to the production of AsA (Wheeler et al. 1998). The five-carbon ring that AsA carries delocalizes electrons, which can easily be donated to a range of ROS or oxidized cellular components (Niki 1991). Reduced AsA and oxidized monodehydroascorbate (MDHA) display low cellular toxicity (Dumville and Fry 2003). The oxidative potential of MDHA is low, preventing it from acting as a radical itself, and therefore two molecules of MDHA can disproportionate to form one AsA molecule and one dehydroascorbate (DHA) molecule (Bielski 1982; Smirnoff et al. 2001). Finally, oxidized MDHA and DHA can be efficiently recycled back to AsA through the ascorbate-glutathione cycle, which replenishes the AsA pool (Fig. 1) and continues the protection of cellular components (Foyer and Noctor 2011).

ROS are primarily produced in the mitochondrion and chloroplasts of plants (Van Breusegem and Dat 2006) in physiologically significant amounts. In the mitochondria, superoxide is produced by complexes I and III, and up to 5% of the oxygen consumed can become ROS (Moller 2001). Plants face the additional challenge of ROS generation during photosynthesis and photorespiration, which together contribute the majority of ROS in photosynthetically active organs (Foyer and Noctor 2003; Moller et al. 2007). Therefore, AsA concentrations in the leaf increase with light intensity when the demand for oxidative protection is the greatest (Gatzek et al. 2002; Bartoli et al. 2006, 2009; Yabuta et al. 2007; Gao et al. 2011). The increase in demand for AsA during high light periods is signalled by the increasing electron flow through the electron transport chain and not the accumulation of sugars (Yabuta et al. 2007). Thus ROS is an integral signal molecule that integrates energy generation and utilization with responses to the environment (Foyer and Noctor 2003).

AsA is critical for both enzymatic and non-enzymatic detoxification of ROS. It can act directly by donating electrons similar to other antioxidants such as reduced glutathione, carotenoids, flavonoids, phenolics, and α-tocopherol, which are distributed throughout the subcellular compartments of the cell (Miller et al. 2010). Intracellular enzymatic ROS detoxification is predominantly performed by ascorbate peroxidase (APX) enzymes (Fig. 1), which are specifically responsible for the removal of H_2O_2, and produce MDHA (Pandey et al. 2017). Ascorbate oxidases (AAO) use AsA to maintain an oxidizing environment in the apoplast and produce DHA as a by-product (Garchery et al. 2013). Unlike the biosynthetic enzymes of the L-galactose pathway, which are generally encoded by a single gene isoform, APX and

AAO are encoded by multiple isoforms. DHA is recycled by dehydroascorbate reductase (DHAR). Like APX and AAO, ascorbate-recycling enzymes are encoded by multiple isoforms. By having multiple isoforms of the oxidation and recycling enzymes for AsA, the cell can facilitate different subcellular localizations and tailor expression to mitigate stress, and maintain appropriate concentrations of reduced L-ascorbate.

Loss of reduced AsA by APX and AAO is unavoidable due to their central roles in protecting the plant from ROS. APX activity is responsive to both biotic and abiotic stress, but the different isoforms of APX are stress specific (Shigeoka et al. 2002). This specificity is due to their subcellular localization and differential expression. Because different oxidative stresses affect the cell and its cellular compartments differently, each isoform is tailored to mediate specific stresses. Not all isoforms present have characterized subcellular localizations (Pandey et al. 2017; Granlund et al. 2009). There are at least two to four isoforms of APX in the chloroplast (thylakoid: tAPX/APX4; stromal: sAPX/APX2, and APX5). One to three of the isoforms are localized in the cytoplasm (APX1, APX3, and APX6), although APX3 and APX6 may be targeted to the extracellular space through the secretory system (Fig. 1). In crop species, eight isozymes have been found in rice and seven have been found in tomato (Pandey et al. 2017). These isoforms are distributed between the cytosol, mitochondrion, chloroplast, and peroxisome. Within the APX family, there is significant variation in enzyme kinetics, stability, optimal pH, and substrate affinity, which aids in their compartment-specific function. The differences between isoforms also facilitate ROS accumulation when it is used as an important secondary messenger. AAO are targeted to the apoplast and contribute to maintaining a highly oxidized AsA pool, which is generally 5% of the total AsA cellular pool (Veljovic-Jovanovic et al. 2001). Overexpression and silencing of AAO isoforms does not affect the total size of the cellular AsA pool (Garchery et al. 2013). However, silencing AAO does lower the oxidation state of the cellular AsA pool, which leads to improved oxidative stress tolerance (Yamamoto et al. 2005; Frei et al. 2010).

After AsA is oxidized to MDHA or DHA, the ascorbate pool can be replenished by recycling. MDHA and DHA are recycled to their reduced forms through the action of monodehydroascorbate reductase (MDHAR) and DHAR, respectively, by the ascorbate-glutathione (GSH) cycle (Foyer and Noctor 2011). In Arabidopsis, three DHARs (DHAR1, DHAR2, and DHAR3) interact in the ascorbate-glutathione cycle, but DHAR1 constitutes the bulk of the activity in plant leaf extracts (Rahantaniaina et al. 2017). DHAR1 is hypothesized to be localized in most subcellular compartments, but it has been experimentally localized to the cytoplasm and peroxisomes. Similarly, DHAR2 is localized in the cytoplasm while DHAR3 appears to be localized to the chloroplasts (Noshi et al. 2016). *dhar3* exhibits increased sensitivity to high light, demonstrating a potential role in mitigation of chloroplastic stress. MDHARs are similarly distributed throughout the cell; MDHAR1 and MDHAR4 localize to the peroxisomes and membranes, MDHAR2 and MDHAR3 localize to the cytosol, and MDHAR6 localizes to both the chloroplast and mitochondrion (Johnston et al. 2015). The dual targeting of MDHAR6 is conferred by the presence of two transcriptional start sites, which offers the potential for the modification of distribution within the cell though the use of modern molecular breeding tools (Johnston et al. 2015).

2.3 Cellular Localization and Regulation of Ascorbic Acid Metabolism

When Arabidopsis plants are grown under oxidative stress conditions, the peroxisomes and cytosol have the highest concentration of AsA (22 mM), followed by the nucleus (14 mM), the chloroplasts and mitochondria (10 mM), and the vacuole (2.3 mM) (Zechmann et al. 2011). In comparison, tobacco has more AsA in the nucleus, cytosol, and vacuole, and less in the peroxisome under similar oxidative conditions (Zechmann 2011). AsA is present in all subcellular compartments but the distribution and concentrations vary under different environmental conditions. Increasing light intensity induces the accumulation of AsA in the chloroplasts and cytosol, but a fivefold increase in the vacuolar AsA concentrations is also observed. Cellular localization of AsA is also developmentally linked. In pea, AsA was measured in the peroxisomes and mitochondria before and during senescence. During senescence, AsA concentrations were reduced to <1% of non-senescing mitochondrial fractions, but AsA concentrations were maintained during senescence in peroxisomal fractions (Jiménez et al. 1998).

Recently, it was shown that increasing in vivo concentrations of AsA feeds back on the translation of GGP (Laing et al. 2015). It is likely that for this reason, most single transgenic interventions have had limited effect of the total AsA accumulated. In a luciferase-based assay, a fourfold increase in ascorbate leads to a >90% reduction in the translation GGP. This translational repression of GGP occurs via an upstream open reading frame (uORF) contained within the long 5′ untranslated region (UTR) of the mRNA (Laing et al. 2015). The uORF is initiated by a non-canonical start site and produces a *cis* acting polypeptide, which controls the translation of the GGP in a AsA-dependent manner. While the exact mechanism is unclear, presumably this translational mechanism offers a rapid means of adjusting AsA concentrations in response to environmental conditions or cellular signalling events that may need to bypass transcriptional regulation. When GGP is expressed with a mutated uORF, the polypeptide is not produced and ascorbate accumulates to nearly twice the level of wild-type plants (Laing et al. 2015). With the feedback regulation removed, AsA concentrations are limited by other reactions of the biosynthetic pathway. When GME is overexpressed with the GGP-uORF mutant, AsA concentrations are fivefold higher than in wild-type plants. The regulatory GGP uORF is highly conserved in dicotyledonous species such as kiwi, apple, and tobacco, and moderately conserved in monocots. This uORF is therefore an attractive biotechnological target for AsA biofortification in crop species.

A negative regulator of the D-mannose/L-galactose pathway was identified by T-DNA activation tagging in Arabidopsis (Zhang et al. 2009). The insertion activated the expression of the ascorbic-acid mannose pathway regulator 1 (*AMR1*/AT1G65770). The activated expression of *AMR1* resulted in increased ozone sensitivity similar to that described for *vtc* mutants (Conklin et al. 2000). A deleterious insertion mutant, *amr1*, has the opposite phenotype with increased transcription of several genes of the D-mannose/L-galactose pathway, which includes *GMP*, *GME*, *GGP*, *GPP*, and *GLDH,* but not *MIOX4*. Therefore, AMR1 appears to be specific to

the regulation of AsA biosynthesis. The *amr1* mutant also had a twofold increase in AsA concentration compared to wild-type. Expression of *AMR1* is suppressed by high light intensities and also increases with age, both factors correlate with the observed AsA accumulation pattern in wild-type plants. AMR1 contains a N-terminal F-box domain and a C-terminal conserved DUF295 domain. While homologues exist in crop species, the relatively common nature of both domains complicates accurate identification solely by informatic approaches. Positive regulation of the D-mannose/L-galactose pathway is conferred through the ethylene response factor, AtERF98 (Zhang et al. 2012). Overexpression of the *AtERF98* results in increased expression of *GMP/VTC1*, *GGP/VTC2,* and *GalDH* in the biosynthetic pathways to AsA. AtERF98 also upregulates the expression of *MIOX4*, *MDHAR3* and two *DHAR* isoforms (Zhang et al. 2012). Expression of *AtERF98* is rapidly induced in response to ethylene, salt, and H_2O_2 treatments suggesting an integral role in mitigating oxidative stress, which requires more AsA production and recycling due to the increase in the oxidative load in the cell. It was found that AtERF98 promotes the expression of *VTC1* through direct binding to a dehydration-responsive element (DRE) found in the promoter of the gene (Zhang et al. 2012).

In addition to AMR1 and AtERF98, several other regulators of the D-mannose/L-galactose pathway exist in Arabidopsis. These include the photomorphogenic factor COP9 signalosome subunit 5B (CSN5B), KONJAC1 and KONJAC 2 (KJC1 and KJC2) (Li et al. 2016; Sawake et al. 2015). KJC1 and KJC2 were investigated because of their structural similarity to GDP-mannose pyrophosphorylases, but have little-to-no GMP activity (Sawake et al. 2015). Instead, they act to stimulate the activity of GMP/VTC1. Mutants of *kjc1* have a GMP activity similar to *vtc1* mutants, and have low total AsA concentrations. Overexpression leads to a >2-fold increase in GMP activity, but only a small increase in AsA concentration is observed in overexpression lines (Sawake et al. 2015; Table 2). Signalled through the CSN5B subunit of the COP9 complex, the 26S proteasome-mediated degradation of GMP/VTC1 is triggered in the dark (Li et al. 2016). A single mutation in the GMP resulting in ΔD27E prevents the interaction with CSN5B and enhanced VTC1 stability at night (Li et al. 2016). GMP ΔD27E mutants have higher AsA concentrations than wild-type and GMP overexpression lines. The CSN5B interaction domain is also conserved between several citrus species as well as tomato, suggesting that it may be a potential target for crop improvement.

3 Improvement of Ascorbic Acid Metabolism in Crops

Many studies have employed transgenic approaches in an attempt to increase the total concentration of AsA in crops (Table 2; Lorence and Nessler 2007; Macknight et al. 2017). Successful increases by a factor of two or more were reported, but these gains are relatively low considering the capacity of some plants to accumulate AsA naturally in their fruits and leaves (Table 3; Fig. 2). AsA content is highly variable (Table 3), with concentrations as low as 1.7 mg 100 g^{-1} fresh mass (FM) in some

Table 3 Ascorbate content of a range of crop plants

Crop	Common name/ type	Species	AsA (mg/100 g FM)
Acerola	West Indian cherry	*Malpighia emarginata*	1677.6
Alpine snowbell[a]		*Soldanella alpina*	792.5
Rose hips		*Rosa rugosa*	426
Guava		*Psidium guajava*	228.3
Blackcurrant		*Ribes nigrum*	181
Pepper	Green	*Capsicum annuum*	80.4
	Yellow		183
	Red		127
Mustard spinach	Komatsuna	*Brassica rapa*	130
Kiwi		*genus Actinidia variety of species*	92.7
Broccoli		*Brassica oleracea*	89
Brussels sprouts		*Brassica oleracea*	85
Lychee		*Litchi chinensis*	71.5
Papaya		*Carica papaya*	60.9
Turnip greens		*Brassica rapa subsp. Rapa*	60
Strawberry		*Fragaria × ananassa*	58.8
Orange		*Citrus × sinensis*	53.2
Lemon		*Citrus limon*	53
Clementine		*Citrus × clementina*	48.8
Cauliflower		*Brassica oleracea*	48.2
Peas		*Pisum sativum*	40
Melon	Cantaloupe	*Cucumis melo var. cantalupo*	36.7
Grapefruit		*Citrus × paradisi*	34.4
Spinach		*Spinacia oleracea*	28.1
Tangerine		*Citrus tangerina*	26.7
Tomato	Red	*Solanum lycopersicum*	13.7
	Green		23.4
Potatoes		*Solanum tuberosum*	19.7
Rocket	Arugula	*Eruca sativa*	15
Radish		*Raphanus raphanistrum subsp. Sativus*	14.8
Cassava	Yuka	*Manihot esculenta*	13.2
Fennel		*Foeniculum vulgare*	12
Banana		*Musa species*	8.7
Corn		*Zea mays*	6.8
Peach		*Prunus persica*	6.6
Endive		*Cichorium endivia*	6.5
Carrot		*Daucus carota subsp. Sativus*	5.9
Apple		*Malus pumila*	4.6

(continued)

Table 3 (continued)

Crop	Common name/ type	Species	AsA (mg/100 g FM)
Pear		*Pyrus species*	4.3
Lettuce	Iceburg	*Lactuca sativa*	2.8
	Butterhead	*Lactuca sativa*	3.7
	Cos	*Lactuca sativa L. var. longifolia*	4
Celery		*Apium graveolens*	3.1
Coconut	Milk	*Cocos nucifera*	2.8
	Meat		3.3
Broad bean	Fava beans	*Vicia faba*	1.4
Rice		*Oryza sativa*	ND[a]
Barley		*Hordeum vulgare*	ND[a]

Reported content is that found in the edible organs of the plants and was obtained from the United States Department of Agriculture database (ndb.nal.usda.gov), with the exception of *Soldanella alpine* (Streb et al. 2003)

[a]These values represent measurements made in processed grain as would be received by a consumer

mosses (Sun et al. 2010) but in Acerola or West Indian Cherry, it is more than 1.6 g 100 g^{-1} FM, or 1% of its fruit FM, is AsA (United States Department of Agriculture: usda.gov). Within the Rosids clade, rose hips, guava, and blackcurrant accumulate high concentrations of AsA; however, peaches, pears and apples produce 50-fold less than these high AsA producers (Interactive Tree of Life—iTOL: Letunic and Bork 2016). Within the Asterids, the concentration of AsA is also variable; yellow peppers can accumulate up to 183 mg 100 g^{-1} FM, 30-fold more than an apple. Similarly, the non-crop alpine plant *Soldanella alpina* has the capacity to contain nearly 1 g 100 g^{-1} FM of AsA when grown at high altitudes (Streb et al. 2003). Tomato, potato and spinach contain only 20–30 mg 100 g^{-1} FM, but despite their relatively low AsA concentrations compared to other plants, their prevalence and importance in the food system means these crops are generally associated with having moderate to high ascorbate content. In comparison, cereal grains that are valued for their high calorie content contain almost no ascorbate. The prevalence of AsA-rich plant species could therefore serve as a resource for both improving our understanding of ascorbate biosynthesis and to translate that knowledge into biotechnological strategies for crop improvement.

3.1 Improvement of L-Galactose Biosynthetic Pathway in Crops

As in Arabidopsis, the D-mannose/L-galactose pathway is the predominant AsA biosynthetic route in fruits and the focus for several transgenic approaches (Table 2). Feeding experiments performed in a number of species show that L-Gal and L-GalL are efficient at increasing AsA concentrations (Baig et al. 1970). For example,

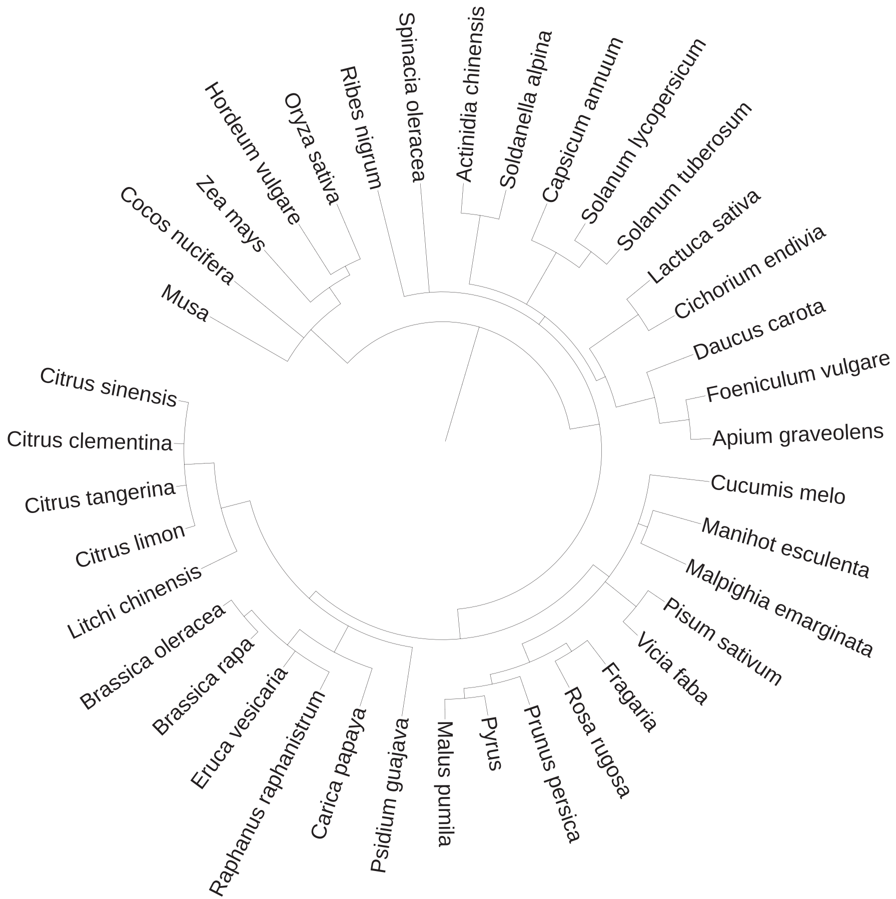

Fig. 2 Cladogram showing the relationships between major crop species and those that accumulate large amounts of ascorbate according to the Interactive Tree of Life (Letunic and Bork 2016)

feeding strawberry plants with either L-GulL or L-GalL increased production of AsA (Baig et al. 1970; Lisko et al. 2014). Peach plants fed with L-Gal or L-GalL yielded higher AsA concentrations, whereas feeding L-GulL did not (Imai et al. 2009). Understanding the role of recently characterized Arabidopsis L-GulLO3 and L-GulLO5, which catalyse the conversion of L-GulL to AsA, in fruit tissues could be incorporated into strategies to increase AsA in plant tissues utilizing this L-GulL substrate pool (Aboobucker et al. 2017). In most fruits, the periods of rapid AsA accumulation correlate with increased expression of the enzymes of the D-mannose/L-galactose pathway. In peach and kiwi fruits, increased transcription of *GME* and *GGP* correlate with the development stage, where AsA accumulation is the greatest (Imai et al. 2009; Bulley et al. 2009). Similar results have been reported in apples and blueberry (Mellidou et al. 2012b; Liu et al. 2015).

In tissues of crops that accumulate high levels of AsA, there is evidence that the expression and activities of the enzymes in the D-mannose/L-galactose pathway are not substrate repressed or limited. *PMM* expression in Acerola fruits is lower than that observed in tomato, while its AsA content is more than tenfold higher (Badejo et al. 2009). On the other hand, in Acerola, *GMP* is expressed at three to fourfold higher than in tomato fruits and tenfold more than in Arabidopsis leaves (Badejo et al. 2008). Overexpression of a yeast *GMP* in tomato under a constitutive promoter led to a 70% increase in AsA in the leaves, and 50% increase in green fruits, but only a 35% increase in red fruit (Cronje et al. 2011). Similar increases were reported in tomato plants overexpressing *GMP3* (Zhang et al. 2013). Overexpression of *GME* in the leaves of rice and tomato also yields moderate increases in AsA levels (Zhang et al. 2011, 2015). In both cases, despite only moderate AsA concentration increases, the plants have improved tolerance to oxidative stress (Zhang et al. 2011, 2015; Ma et al. 2014). In other crop species, the expression and regulation of GMP and GME does not always directly correlate with an increase in AsA concentrations, particularly in species that accumulate extremely high amounts (Mellidou and Kanellis 2017). This mirrors observations in Arabidopsis, and the most significant gains in AsA concentrations in crops species come from the overexpression of the committed reactions of the L-galactose pathway. Ectopic expression of the kiwi *GGP* in strawberry and tomato fruits resulted in a two to sixfold increase in AsA concentrations (Table 2, Bulley et al. 2012). A twofold increase was also observed in potato tubers overexpressing a native *StGGP* (Bulley et al. 2012).

3.2 Improvement of Alternative Ascorbic Acid Biosynthetic Pathways in Crops

Fruits have one of the greatest potentials for improvement and biofortification of AsA, because in many crop species AsA concentrations in fruiting bodies are much higher than in leaves or other plant tissues. One explanation for this phenomenon is that during early fruit development AsA is predominantly synthesized via the D-mannose/L-galactose pathway, but during ripening, AsA is synthesized via the D-galacturonate pathway (Di Matteo et al. 2010). Genetic evidence for the presence of both pathways in developing fruit was provided by the characterization of the introgression wild germplasm into elite breeding lines, which demonstrated that pectinesterases and two polygalacturonases were upregulated and they could supply D-GalAR for AsA biosynthesis (Di Matteo et al. 2010). The D-galacturonate pathway appears to be important for AsA synthesis in strawberry (Badejo et al. 2012). Indeed, when a pectin lyase was silenced in strawberry fruits, AsA in the red fruit was reduced (Agius et al. 2003). Overexpression of D-galacturonate reductase (GalUR) from tomato in Arabidopsis also led to an increase in AsA concentrations (Table 2; Agius et al. 2003). Expression of this *GalUR* in potato tubers also led to a nearly twofold increase in AsA concentration while conferring resistance to stresses such as drought and salinity (Hemavathi et al. 2009). GalUR was overexpressed in

tomato by multiple independent studies, which demonstrated that constitutive expression in the leaves and fruits of tomato, under the control of a 35S promoter, led to increases in the total amount of AsA (Amaya et al. 2015; Cai et al. 2015; Lim et al. 2016). The *GalUR* overexpression lines displayed improved non-photochemical quenching (NPQ) in the leaves and improved fruit number and yield over control lines. In two similar studies, improved resistance to cold, oxidative stress, and salt tolerance were also observed (Cai et al. 2015; Lim et al. 2016).

Plants can also produce AsA when fed with L-GulL and this reaction is proposed to be catalysed by a suite of GulLOs (Maruta et al. 2010). In leaves, this pathway appears to be conserved in crop species, as constitutive expression of the *GulLO* gene from rat in tobacco and lettuce leaves resulted in up to a sevenfold increase in leaf-ascorbate concentrations in both plant species (Jain and Nessler 2000). Although only recently identified in Arabidopsis, crop homologues of *At*GulLO3 and *At*GulLO5 are also potential targets for biotechnological non-transgenic improvement (Aboobucker et al. 2017). In fruits, the contribution of alternative pathways is proposed to be species and developmental stage specific. Feeding with L-GulL, D-MI, and L-Gul precursors leads to increased concentrations of L-gulose pathway intermediates and increased AsA concentrations in tobacco leaves and blackcurrant flowers (Lisko et al. 2014; Hancock et al. 2007). Feeding peas, kiwi, and papaya fruits with L-GulL has a relatively small (25–50%) effect on AsA concentrations (Pallanca and Smirnoff 1999; Li et al. 2010a; Barata-Soares et al. 2004). In young and mature apple fruit, no change in AsA concentrations was observed in analogous feeding experiments (Li et al. 2008). Feeding D-MI also resulted in no change in AsA in apple and kiwi fruits (Li et al. 2008, 2010a). However, in tomato, feeding D-MI to green or red fruits increased AsA by 12% and 49%, respectively (Mellidou et al. 2012a). Although there appears to be some similarity in the role the L-gulose pathway plays in organ and tissue types, across different plant species a considerable diversity is observed in the degree the L-gulose and MIOX pathways contribute to total AsA concentrations.

3.3 Improvement of Oxidation and Recycling of Ascorbic Acid in Crops

Although improvement of AsA concentrations in crops would be beneficial for human and animal consumption, it is also an essential component of the plant's protection against oxidative stress. In crops, a number of agronomic traits are connected to oxidative stress or cellular oxidation, such as post-harvest degradation. For such traits, tailoring AsA oxidation and recycling systems in plants may be more effective to improve an agronomic trait than increasing the AsA concentration. Protection from cellular oxidation by intracellular and intraorganellar concentrations of AsA does not only depend on the biosynthesis of AsA, but also on AsA degradation, recycling, and transport (Smirnoff et al. 2001; Green and Fry 2005b; Chen et al. 2003; Horemans et al. 2000). Nearly all biotic and abiotic challenges

result in rapid accumulation of ROS in plants, which must be properly managed through a range of detoxification systems. Reported examples of these stresses include pathogen infection in barley (Vanacker et al. 1998a), powdery mildew in oat (Vanacker et al. 1998b), copper and cadmium induced oxidative stress (Ratkevicius et al. 2003; Chamseddine et al. 2009; Collin et al. 2008), high light (Bartoli et al. 2006; Giacomelli et al. 2006), ozone (Conklin and Barth 2004), UV (Gao and Zhang 2008), hypoxia and anoxia (Cheeseman 2006) and in varied species such as tomato (Li et al. 2012), potato (Hemavathi et al. 2009), and tobacco (Chen and Gallie 2005).

Many studies have overexpressed AsA-recycling enzymes and these transgenic plants contain more AsA (Table 2). More importantly, a majority of these transgenic plants have significantly improved tolerance to stress. In Arabidopsis, DHAR overexpression resulted in a two to fourfold increase in total AsA, and increased oxidative stress tolerance (Wang et al. 2010). Overexpression in tomato led to an increase in AsA by 50% in the fruits, but also improved oxidative, salt, and temperature stress tolerance (Haroldsen et al. 2011; Li et al. 2012; Qin et al. 2015). In tobacco, overexpression of DHAR resulted in plants that are better able to tolerate acute and sustained ozone treatments. Overexpression of DHAR in potato increases the total pool size of AsA in tubers, but not to the same degree as in other crop species (Goo et al. 2008; Qin et al. 2011). QTL analysis of tomatoes has suggested that increased MDHAR activity in the fruits leads to a decrease in firmness after chilling (Stevens et al. 2008). Transgenic strategies also show that stress tolerance is conferred by the overexpression of MDHAR in tomato (Gest et al. 2013).

3.4 Improvement of Regulation of Ascorbic Acid Metabolism in Crops

In tomato, a protein was identified that binds to the promoter region of the *SlGMP3*. *Sl*HZ24 is a HD-Zip I type transcription factor that positively regulates the transcription of *GMP* in both leaves and immature fruit (Hu et al. 2016). Transcription of *SlHZ24* is light dependent, as its expression decreases in the dark and increases in the light, which is consistent with a role in modulating AsA production. Overexpression of the transcription factor led to an increase in the transcription of *SlGME2* and *SlGGP*, which produce enzymes that control the first committed reaction of AsA biosynthesis. Overexpression of *SIHZ24* represses *SlMDHAR* and *SlDHAR* expression, while increasing APX expression, which should lead to less recycling of oxidized AsA. In addition, *SI*HZ24 strongly represses the transcription of *SlGGP1* and *2*, *SlGLDH*, and *SlMIOX* (Hu et al. 2016). Although early reactions in AsA are transcriptionally upregulated, later reactions are repressed by the overexpression of *Sl*HZ24, but ultimately total AsA content increased. Another transcription factor in tomato, *Sl*DOF22, was shown to interact with components of the salt overly sensitive (SOS) response pathway (Cai et al. 2016). Silencing of the *SlDOF22*

resulted in increased expression of the *SlGPP1* and *SlGPP2*, *SlGalDH*, *SlGLDH*, *SlMDHAR*, and an *SlAPX*. It remains to be experimentally demonstrated if this transcription factor directly negatively regulates AsA biosynthesis and recycling.

4 Modern Technologies for Improving Ascorbic Acid Content in Crops

4.1 Phenotyping Platforms for Screening Ascorbic Acid Concentration in Crops

The toolbox of genomic and molecular techniques, as well as high-throughput phenotyping platforms, has accelerated the emergence and characterization of traits in crops that were previously intangible. However, the varied physiological roles, tissue localizations and concentrations of AsA complicate the capacity to easily increase AsA yield. For example, AsA performs a complex physiological role that is essential for normal plant metabolism in leaves, whereas in fruiting bodies it has the non-essential role of a frugivore attractant. The breeding of complex traits can be significantly improved using advanced phenotypic selection strategies, in comparison to highly defined biological functions. Unfortunately, AsA biofortification of crops remains challenging using classic phenotypic selection strategies for two main reasons. The first is that AsA concentrations are closely connected to energy metabolism and oxidative stress, which are strongly influenced by environmental conditions (Bulley et al. 2012; Yabuta et al. 2007). Only a few studies have addressed the phenotypic distribution and heritability of AsA concentrations within breeding germplasms of different crops (Stevens et al. 2007; Di Matteo et al. 2010), or the genotype-by-environment interactions that exist in either controlled or field environments (Stevens et al. 2008). The second challenging factor is that accurate determination of AsA content is more labour- and resource-intensive when compared to visual traits such as fruit colour, size, or disease resistance. Successful breeding strategies require the characterization of a trait from a large population grown in multiple locations and environments. High-throughput metabolite screens using robotic systems have been designed for characterizing multiple primary metabolites, such as starch and sugar content (Ménard et al. 2013). High-throughput AsA determination is relatively more challenging due to more difficult isolation, stability, and interfering pigments. Established high-throughput methods are usually based on enzyme-linked or colormetric/fluorescence assays, which are being adapted for determining AsA concentrations (Ueda et al. 2013; Vislisel et al. 2007). However, in tissues with relatively high AsA concentrations and high variability between genotypes, spectral scanning or imaging could be an efficient tool for preliminary screening of AsA concentrations (Moeslinger et al. 1995; Markarian and Sargsyan 2011; Lane and Lawen 2014; Zechmann et al. 2011).

4.2 Development of Genome Selection for Trait Improvement

Identifying the genetic basis for a trait in a breeding population can help to reduce sampling, especially for traits where phenotypic selection is challenging. This strategy usually involves a genome-wide association study (GWAS), where either a specifically designed mapping population or a subset of the breeding population is both genotyped and phenotyped to determine quantitative trait loci (QTL) . Genetic markers based on polymorphisms near these loci can then be used for marker assisted selection (MAS), where laborious phenotyping of traits is replaced by genomic section (GS) pipelines. However, the complexity of AsA biosynthesis could be challenging for GS approaches, as seen in tomato, where relatively high AsA concentrations in the fruit have gradually been reduced through modern selection for traits associated with higher pectin content (Di Matteo et al. 2010). This pectin content leads to higher market value due to improved firmness and prolonged shelf life of the fruit, as well as resistance to pests (Kramer et al. 1992). This and other complex traits, including grain and biomass yield in cereals, have not profited as much as expected from GS strategies (Hickey et al. 2017). To gain a better understanding of these complex systems, researchers are increasingly combining GWAS with other multi-omics approaches, such as metabolomics and transcriptomics to identify mQTL and eQTL (Arbona et al. 2013). Such studies can then be placed in the context of oxidative stress tolerance to identify QTL that couple the metabolite content with growth or stress by placing traits in the proper ecophysiology (Hammer et al. 2016). Multi-omics approaches have helped identify a novel QTL and the inverse relationship between starch and growth (Sulpice et al. 2009). And also glutathione metabolism was identified as a causal QTL for stress (Wentzell et al. 2007). Although the usefulness of such QTL analysis in GS has yet to be determined, the understanding of how metabolic pathways interact on a genomic level with complex traits could aid in improving selection strategies.

4.3 Mutational Breeding and Genome Editing for Trait Improvement in Crops

Mutational breeding is one of the most powerful techniques to rapidly produce new varieties with novel traits. It has been successfully used for biofortification and to substantially increase antioxidants concentrations in crops, most notably anthocyanin concentration (Chiu et al. 2010; Passeri et al. 2016). Mutants with higher anthocyanin content generally display a disruption of regulatory genes targeting the anthocyanin biosynthetic pathway (Rubin et al. 2009). The non-committed reactions of the L-galactose pathway also supply substrates for cell wall biosynthesis and glycoproteins (Smirnoff and Wheeler, 2000), thus the overexpression of single genes in the pathway can lead to a reallocation of carbon from these essential processes leading to pleiotropic defects (Bulley et al. 2012). In such cases, where AsA

synthesis is physiologically coupled to other biological processes, mutations in regulators of the Smirnoff–Wheeler pathway or pectin pathways, such as AMR1 or the uORF of *GGP*, could result in high concentrations of AsA without causing these pleotropic defects (Zhang et al. 2009; Laing et al. 2015). Targeted Induced Local Lesions IN Genomes (TILLING) is one of the most powerful reverse genetic tools to identify mutations in genes, where a phenotype is described in other systems (Till et al. 2004). It has been successfully applied to the identification of AsA mutants in tomato but has yet to capture an AsA accumulating allele (Baldet et al. 2013). Although TILLING has been crucial in screening for traits to date, it does have several limitations. For example, the target gene sequence must either be identified in the target crop species, or large populations are required for screening (Till et al. 2004). TILLING is also challenging in outcrossing species and highly heterozygous populations (Weil 2009; Wang et al. 2012).

The rapid advance in genome editing technologies could enable generation of crops with enhanced AsA levels. Genome editing offers several advantages over conventional mutational breeding and TILLING. Primary amongst them is the capacity to generate specific alterations in the genome due to the targeted design of Sequence Specific Nucleases (SSN) rather than highly mutagenized populations. At the forefront of genome editing techniques is the CRISPR-Cas9 system—a synthetic version of the prokaryotic adaptive immune system (Jinek et al. 2012). The simplest form of the CRISPR-Cas9 system has been widely adopted and adapted for genomic engineering of various organisms. There are several excellent reviews addressing Cas-based genome editing and the myriad of modified systems for use in crop research (Baltes et al. 2017; Scheben et al. 2017). In the context of AsA, the targeted mutagenesis tool could be applied to at least two biosynthetic checkpoints. For example, it has recently been demonstrated that the mRNA of GDP-L-galactose phosphorylase (GGP)—an important enzyme in the biosynthetic pathway of AsA—carries an unusual, conserved uORF (Laing et al. 2015). This uORF is preferentially translated if high AsA levels are prevailing, and its translation seems to hinder translation of the actual *GGP* CDS, presumably by ribosome stalling. Thus, high ascorbic acid levels reduce GGP protein abundance and thereby ascorbic acid biosynthesis rates, providing an effective negative feedback loop. In addition, Laing et al. (2015) demonstrated that mutating the uORF's presumptive start codon, in addition to changing conserved amino acids encoded by the uORF, abolish this effect. Targeting this uORF using genome editing would thus be an obvious approach to potentially increase GGP and thus AsA content. Another potential target is the single D27E amino acid mutation in GMP (Li et al. 2016). In darkness, the key AsA biosynthetic enzyme GMP is downregulated. This decrease in GMP levels is mediated by the interaction of GMP with CSN5B, a subunit of the Cop9 signalosome (Li et al. 2016; Wang et al. 2013). A single D27E amino acid mutation in GMP abolishes the interaction with CSN5B and increases its stability in darkness (Li et al. 2016). Both sites would be good candidates to edit, since the uORF start site or the GMPs' aspartate could be targeted by a single Cas9-induced DSB followed by non-homologous end joining (NHEJ). The power of genome editing has phenomenal potential, especially

in crop species whose breeding is hampered by genetic heterogeneity, clonal propagation, or poor fertility, such as the staple food and commercially important crop cassava (*Manihot esculent*a Crantz).

5 Current Economic Marketplace for AsA

In developed countries, a majority of the AsA required to maintain human health comes from our recommended dietary intake of fruits and vegetables. This natural source of AsA is also supplemented by chemically synthesized AsA. Approximately 50% of the global chemically synthesized AsA production is used for the pharmaceutical supplementation of dietary AsA. With an annual global production of 110 ktons per year and an average market price between 4 and 8 USD per kilo, the value of AsA as a commodity is approximately 800 million USD (Pappenberger and Hohmann 2014). Non-traditional crops that have exceptionally high AsA levels in their fruits or leaves could be eaten to replace chemically synthesized dietary supplements and have significant market potential. One breeding target would be to increase AsA of crops like acerola, rose hips, guava, and blackcurrant, which can provide the RDA of AsA with only a few grams of fruit. Because of their high concentration (Table 3), they could replace pharmaceutical products that offer relatively high doses of AsA. Although the current market for these specialized crop species is relatively small, in developed countries agricultural markets can readily adapt novel crops into the food system, such as has been seen with the trends of "superfoods" such as Aronia, where sales have grown significantly and have similarly been marketed for its high antioxidant concentration (Brand 2010). In developing countries, consumers often favour food based on its calorie rather than nutritional value (Abrahams et al. 2011). Fresh vegetable consumption in sub-Saharan Africa makes up an alarmingly low proportion of the diet at approximately 1.18% of the total caloric intake (Chauvin et al. 2012). In this sense, several targets discussed above may be of critical importance in the context of nutritional supplementation in poverty stricken regions, such as multivitamin corn or AsA-augmented potatoes (Naqvi et al. 2009; Bulley et al. 2012). However, biotechnological efforts to improve the AsA concentration in food face two significant challenges: public perception and post-harvest deterioration (Lucht 2015). In a survey of US citizens, it was found that genetically engineered (GE) products were less desirable when they were to be consumed fresh, as is the case for apples (Lusk et al. 2015). Regarding the desirability of processed foods' ingredients, however, fewer differences were found between GE and non-GE variants. In this context, AsA is a problematic target as processing leads to significant losses in the final content once it reaches the consumer. Improvement of crops for developing countries should, therefore, be chosen with care, so that the consumers will receive the intended benefit.

5.1 Ascorbate as an Additive Compared to Engineering Plants with Higher Content

Beyond the potential human and animal health benefits of increasing AsA in crops, there is also an accessory benefit to agriculture in terms of prevention of post-harvest degradation. AsA content of fruits and vegetables has been implicated as a significant factor which prevents post-harvest browning of fruits and vegetables (Bottino et al. 2009). Post-harvest browning leads to significant loss of value in agricultural products due to the ease with which it can be detected by a consumer. This is especially evident in fresh-cut produce which retains the ability to respire while being processed, shipped, and sold (Brecht et al. 2004). Browning occurs primarily due to the accumulation of polymerized quinones produced enzymatically by polyphenol oxidases and other peroxidases acting on phenolic compounds released from the vacuole (Degl'Innocenti et al. 2007). Browning can be mitigated through the exogenous application of AsA which is a component of commercial anti-browning agents, and has been applied successfully to a range of fruits to extend shelf life (Gorney et al. 2002; Rupasinghe et al. 2005). Indeed, upwards of 40% of synthesized AsA is used in the food and beverage processing industry as an antioxidant and preservative. The European Food Safety Authority designates L-ascorbate as E300, L-ascorbate salts as E301-303, and L-ascorbate esters as E304 (Varvara et al. 2016). These are used in the preservation of colour, texture, and flavour of fruit juices, jams, wine, breads, and meats (Bauernfeind and Pinkert 1970).

It would be more economically and socially desirable for crops to contain higher endogenous concentrations of AsA to prevent post-harvest degradation, rather than supplying it exogenously. Recent approval of two GE crops, Arctic® apples and Innate®potatoes, which address post-harvest browning through silencing of polyphenol oxidases, provides encouraging proof that regulators and consumers are prepared to accept such modified foods (Waltz 2015a, b). Lettuce, which contains little AsA in the leaves, is particularly susceptible to post-cut browning compared to other species such as rocket or spinach, which have higher endogenous concentrations (Degl'Innocenti et al. 2007; Bottino et al. 2009). Cut lettuce will start to brown within the first 24 h, while in rocket and spinach this effect is almost undetectable for up to 3 days. The potential for increased endogenous AsA to mitigate browning was recently confirmed in lettuce overexpressing L-galacto-1,4-lactone dehydrogenase (Landi et al. 2015). Similar to studies in other plants, the greatly increased expression resulted in a small increase, 30%, in AsA but significantly reduced the browning of the leaves in test conditions. This provides a direct link between AsA accumulation or supply with the prevention of post-harvest degradation in a crop. Other efforts to increase AsA concentrations in leafy greens have focused on providing specific lighting conditions, which induces AsA production in the leaves under controlled growth conditions, such as those used by Aerofarms® (Zaraska 2017). In addition to preventing post-harvest degradation, increasing endogenous concentrations of AsA would counteract the loss due to long-term storage and preparation of foods (Grudzinska et al. 2016). AsA itself is particularly susceptible to

post-harvest degradation (Lee and Kader 2000). In modern agriculture, several foods sold as fresh have in fact been stored for long periods of time before arriving at the consumer resulting in a loss of the AsA and its nutritional value.

6 Conclusion

Characterization of the AsA biosynthetic pathway by Wheeler and Conklin in the late 1990s significantly advanced our knowledge of AsA metabolism and with it the possibility for trait modification in crops (Wheeler et al. 1998; Conklin et al. 2000). A majority of the biosynthetic enzymes, alternative pathways, and their relative contribution are now well understood in both model and crop species. In addition, a number of biotechnological advances have demonstrated that (1) increasing AsA concentrations by two to sevenfold is possible; (2) increases in AsA concentrations lead to improved tolerance to oxidative, biotic, and abiotic stress; and (3) biofortification of AsA can enhance consumer-preferred characteristic of crops. To date, however, these improvements have not been implemented into the food system. Although multiple explanations exist, the reliance on the transgenic technology to improve AsA concentration has been limited by the number of crop species that are amenable to genetic transformation, have extensive breeding resources, or established laboratory protocols, such as tomato. Improving AsA concentration in tomato and other fruits, to combat severe cases of AsA deficiency in humans, may not be the optimal choice, as consumers who choose and have access to fresh vegetables are less prone to such dietary shortfalls. Moreover, regulatory constraints and public perception associated with transgenic crops have historically been a significant barrier to market success. Notwithstanding, market approval has been given to small biotech firms for Arctic® apple and Innate® potatoes. Unlike other transgenic crops that target traits for farmers, these target the visual appeal of the consumer product directly. Both products have recently been subjected to successful trial marketing campaigns (Hallerman and Grabau 2016).

AsA metabolism has been an unfavourable target for non-transgenic approaches due to the relative complexity to quantitate AsA in crops. However, with recent advances in both high-throughput phenotyping and molecular breeding tools, including genome editing, there is a renewed effort to unlock the potential of AsA metabolism in non-traditional crops. Several plant species, such as Acerola and Alpine snowbell, accumulate naturally high concentrations of AsA and the application of a new set of breeding tools could be used to leverage improvements in crops and thus make them better suited for integration into the food system. Fundamental research into these high-yielding AsA species would also be important for understanding how plant physiology can modulate high AsA flux and concentrations. With a detailed understanding of not just the physiology of AsA, but also the demand for it in multiple sectors of industry, this knowledge could be applied for the enhancement of food quality and contribute to overcoming several challenges of modern agriculture.

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Evolution of the Metabolic Network Leading to Ascorbate Synthesis and Degradation Using *Marchantia polymorpha* as a Model System

Jarrod Creameans, Karina Medina-Jiménez, Tanya Gómez-Díaz, Viankail Cedillo-Castelán, Dulce Flores-Martínez, Adolfo Aguilar-Cruz, Omar Altehua-Lopez, Grecia Lopez-Ramirez, Ana E. Dorantes-Acosta, John L. Bowman, Argelia Lorence, and Mario A. Arteaga-Vazquez

Abstract In plants, L-ascorbic acid (AsA) is a functional enzyme cofactor, a major antioxidant, and a modulator of several biological processes including photosynthesis, photo-protection, cell wall growth and expansion, tolerance to environmental stresses, and synthesis of other molecules. One of the major roles of AsA in plants is detoxifying reactive oxygen species (ROS) such as singlet oxygen or peroxide radicals. ROS are produced when plants undergo biotic or abiotic stresses and if accumulated in high concentrations, can cause damage to macromolecules such as nucleic acids, membrane lipids, and proteins. Until now, little study has been done on ascorbate metabolism in liverworts. Bryophytes (liverworts, hornworts, and mosses) comprise the earliest diverging land plant lineages that came about approximately 360–450 million years ago between the Ordovician and Devonian periods. The ancient liverwort *Marchantia polymorpha* is an emergent model system specifically suited to use in the study of the evolution of different biosynthetic pathways.

J. Creameans
Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

K. Medina-Jiménez · T. Gómez-Díaz · D. Flores-Martínez · A. Aguilar-Cruz
O. Altehua-Lopez · G. Lopez-Ramirez · A. E. Dorantes-Acosta · M. A. Arteaga-Vazquez (✉)
Instituto de Biotecnologia y Ecologia Aplicada (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico
e-mail: maarteaga@uv.mx

V. Cedillo-Castelán
Instituto de Biotecnologia (IBT), Universidad Nacional Autonoma de Mexico (UNAM), Cuernavaca, Morelos, Mexico

J. L. Bowman
School of Biological Sciences, Monash University, Melbourne, VIC, Australia

A. Lorence (✉)
Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

Department of Chemistry and Physics, Arkansas State University, Jonesboro, AR, USA
e-mail: alorence@astate.edu

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In this chapter, basal levels of both reduced and oxidized AsA in *M. polymorpha* are reported. Comparative and functional genomics experiments in combination with precursor feeding experiment are also discussed in order to provide valuable insights on the evolution of the AsA biosynthetic pathways.

Keywords Marchantia · Liverworts · Vitamin C · Ascorbic acid · Ascorbate pathways · Pathway evolution

1 Introduction

There are many roles that L-ascorbic acid (AsA, a.k.a. vitamin C) plays in the biochemistry of plants and of them, two are especially important to study in modern plant biotechnology: (1) The antioxidant properties of AsA and (2) AsA's contributions as a modulator of biological processes including photosynthesis, photoprotection, cell wall growth and expansion, tolerance to environmental stresses, and synthesis of other molecules (Smirnoff and Wheeler 2000; Gest et al. 2013). The former of these important functions is in detoxifying reactive oxygen species (ROS), both in enzymatic and nonenzymatic detoxification.

Reactive oxygen species are produced in response to various stresses, biotic and abiotic alike, and serve as messengers for plants to indicate that a change in plant biochemistry is necessary. ROS play a key role in cell signaling processes such as growth, development, response to biotic and abiotic stresses, and apoptosis; however, increased levels cause excessive oxidative stress to macromolecules such as membrane lipids, proteins, and nucleic acids, eventually leading to cellular damage (Bailey-Serres and Mittler 2006). This role in cell signaling is especially apparent in periods with prevalent drought and salinity stress as ROS have a dual role in sensing cellular redox state and in retrograde signaling (Golldack et al. 2014). It has been proposed that different abiotic stresses result in different ROS signatures that determine the specificity of the acclimation response and help tailor the plant to the stress situation (Choudhury et al. 2017).

Liverworts, known collectively with hornworts and mosses as Bryophytes, are the earliest diverging land plant lineages arising approximately 360–450 million years ago between the Ordovician and Devonian periods (Bowman et al. 2016). Species of the genus *Marchantia* are liverworts that have recently emerged as excellent model systems specifically suited to study the evolution of different biosynthetic pathways, including the various routes to AsA. The genus *Marchantia* has been used as a model for almost two centuries (Bowman 2016) and was used in early genome sequencing of chloroplasts (Ohyama et al. 1986) and mitochondria (Oda et al. 1992). *Marchantia* has also been used for sex chromosome sequencing in plants with haploid systems, including gene organization of the Y chromosome (Yamato et al. 2007) and in the study of sex differentiation and determination (Oda et al. 1992).

The interest in *Marchantia* could be accredited in part to its relatively small genome size (230 Mb) with only 20,000 protein coding genes and the fact that gene

families present on it consist of mostly fundamental components (Berger et al. 2016). Liverworts are also being utilized as bioindicators in an increasing number of environmental monitoring programs due to their tolerance to abiotic stresses in different environments (Paciolla and Tommasi 2003). There is more than enough rationale warranting further study on the evolution of biosynthetic pathways in *Marchantia polymorpha*, especially in the context of abiotic stress on plants.

In this chapter, basal levels of both reduced and oxidized AsA were reported in *M. polymorpha*. Bioinformatics approaches to confirm the presence of genes and transcripts of ascorbate biosynthetic and recycling genes in the *Marchantia* genome and transcriptome are also reported. Precursor feeding studies in *Marchantia* in vitro cultures are discussed. We show evidences suggesting that L-galactose, *myo*-inositol, and L-gulono-1,4-lactone are precursors of ascorbate in *Marchantia*. Once combined, this data indicates that *Marchantia* possesses the metabolic machinery to synthesize ascorbate using more than one pathway.

2 Effect of Exogenous Ascorbate in the Phenotype of *Marchantia* Cultures

Levels of ascorbate in plants and animals vary greatly over several orders of magnitude (Pauling 1970; Herrero-Martínez et al. 1998; Gest et al. 2013; Akram et al. 2017). In plants, concentrations of AsA as low as 0.1–0.6 micromol per gram fresh weight (μmol/g FW) have been reported in the moss *Hypnum plumaeforme* (Sun et al. 2010) while levels as high as 170 μmol/g FW have been found in Camu Camu (*Myrciaria dubia)* fruits (Justi et al. 2000; Gest et al. 2013). Variation in AsA levels is also observed within members of the same genus or species. For example, *Solanum pennellii* contains five times more AsA than its domesticated relative *Solanum lycopersicum* (Stevens et al. 2007; Gest et al. 2013).

Information on the role of AsA in basal land plant lineages is very scarce and dominated mainly by reports on the determination of concentration levels for a reduced number of species. In the case of *M. polymorpha*, concentration levels of AsA fall within the low range 0.3 μmol/g FW (Paciolla and Tommasi 2003). Experimental evidence indicates that AsA in *M. polymorpha* is involved in the removal of hydrogen peroxide but the pool of AsA levels upon desiccation decline rapidly (Paciolla and Tommasi 2003). Interestingly, it has been reported that *M. polymorpha* methanol extracts exhibit antioxidant properties and can reduce the formation of free radicals, ROS, and oxidative stress in HEK293 human embryonic kidney cell lines exposed to lead (Saputra et al. 2016).

In order to characterize the effects of exogenous AsA on the development of *M. polymorpha* gametophytes, we established hydroponic cultures that allowed us to provide a constant supply of AsA over the course of the experiments. Gemmae from in vitro cultured *M. polymorpha* plants (accession Takaragaike-1) were grown in hydroponic cultures (half-strength Gamborg media supplemented with 1% sucrose,

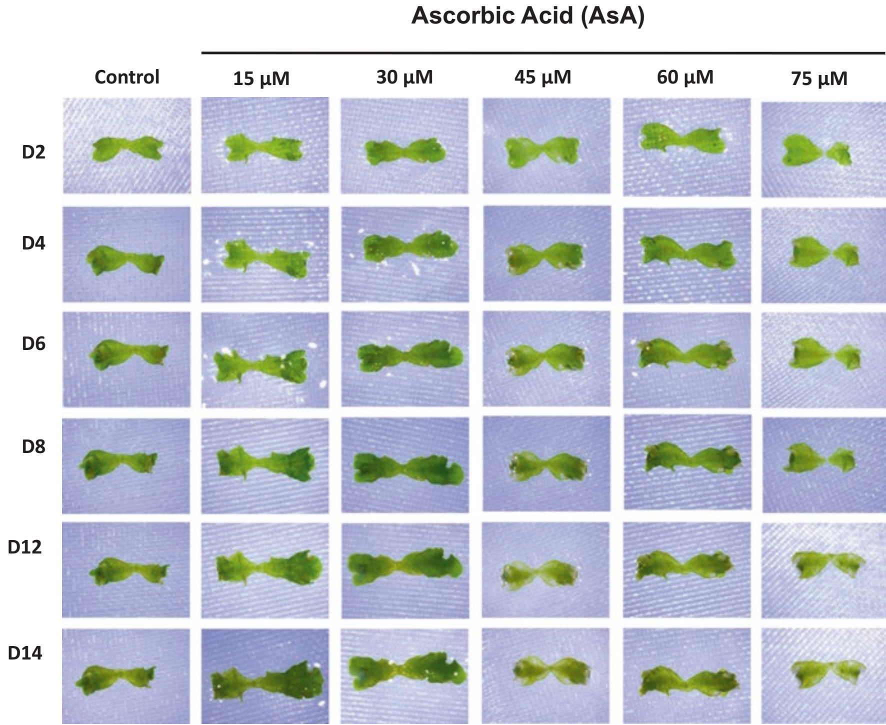

Fig. 1 Exogenous AsA induces dramatic effects on *Marchantia polymorpha* development. *Marchantia* gemmae germinated in hydroponic cultures were grown for 7 days under optimal conditions and then fed with increasing concentrations of AsA during (15, 30, 45, 60, and 75 μM) 14 days (14D). AsA was added to the hydroponic cultures every 24 h and development of thalli was recorded every 24 h. Relevant phenotypes at 2 (D2), 4 (D4), 6 (D6), 8 (D8), 12 (D12), and 14 (D14) days are shown. Phenotypically noticeable effects were observed in plants grown under 45 μM of AsA for 4 days (D4) as evidenced by the arrest of growth, the presence of brown patches around the apical notches and subsequent chlorosis. In sharp contrast, plants exposed to 15 and 30 μM showed increased growth relative to control plants grown under the same conditions but in the absence of AsA

1% agar, and different concentrations of AsA) in a growth chamber at 22 °C under an 18 h light/6 h dark photoperiod regime. First, gemmae were grown in hydroponic cultures without AsA for 7 days in order to allow for the establishment of the dorsoventral pattern, the germination of rhizoids, and the proper development of thalli. On the eighth day after culture, the media was supplemented with different concentrations of AsA that were applied at the same time every 24 h. We documented the development of each individual plant upon exposure to five different concentrations (15, 30, 45, 60, and 75 μM) of AsA. Concentrations as low as 45 μM induced cell death as evidenced by the presence of brown patches located around the apical meristems of the gemmae (also known as apical notches) (Fig. 1). In addition to cell death, exposure to higher concentrations of AsA (from 60 μM to above) induces photo-bleaching and a reduction in growth rate. In sharp contrast, 15 and

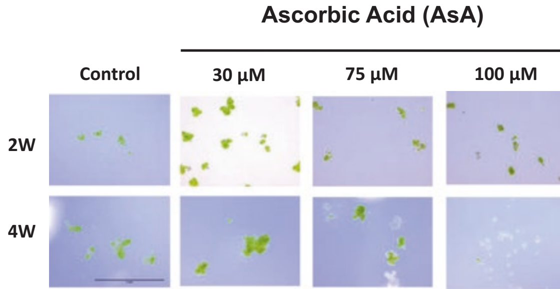

Fig. 2 *Marchantia* sporelings tolerate higher concentrations of exogenous AsA relative to developing thalli. *Marchantia* spores germinated directly in hydroponic cultures supplemented with increasing concentrations of AsA (30, 75, and 100 μM) during 4 weeks (4 W). Development of sporelings was recorded every 24 h and relevant phenotypes are shown after 2 (2 W) and 4 (4 W) weeks. Spores germinated in 75 μM show germination rates and growth patterns at 2 W and 4 W similar to that observed in control spores grown under the same conditions but in the absence of AsA. Similar to that observed in developing thalli, developing spores showed increased growth relative to control plants 2 W after exposure. Spores grown at 100 μM of AsA did germinate and develop for 2 W but ultimately turned chlorotic and died at 4 W

30 μM AsA treatments had positive effects on plant growth. We performed similar experiments on developing sporelings and observed that sporelings are more tolerant to AsA (Fig. 2).

3 Interrogating the *Marchantia polymorpha* Genome to Identify Ascorbate Biosynthetic and Recycling Genes

In order to gain insight into the evolutionary history of the genetic machinery involved in the biosynthesis and recycling of AsA in land plants, we used the well-characterized genetic framework from *Arabidopsis thaliana* as a template to interrogate publicly available Embryophyte genomes and transcriptomes from the Phytozome database (Goodstein et al. 2012), including the recently released *Marchantia polymorpha* genome (phytozome.org) (Bowman et al. 2017). First, we used the repertoire of AsA-related genes from *A. thaliana* to screen the *M. polymorpha* genome and transcriptomes available from Phytozome, the Joint Genome Institute genome-sequencing project (http://www.jgi.doe.gov/) and the Sequence Read Archive (SRA) from the National Center for Biological Information (NCBI) (https://www.ncbi.nlm.nih.gov/sra), using a combination of reciprocal BLAST (Altschul et al. 1990; Gish and States 1993) and conserved domains-based sequence similarity searches using Pfam (Finn et al. 2016) and HMMER (Finn et al. 2011). For phylogenetic inference we employed the Maximum Likelihood (ML) criterion and selected the substitution model that best fit our data from the Akaike Information Criterion (AIC) given its close proximity to the ML method (Anisimova and Gascuel 2001). The AIC estimates the expected distance between the model and the True

Value (deLeeuw 1992) and therefore considers how well the model does fit to the data and its associated variance. The AIC value was calculated using the Prottest software (Darriba et al. 2011) which gave us the best phylogenetic model. With the smallest AIC, the best model of amino acid replacement to infer protein evolution of our data is Whelan and Goldman, which uses an approximate maximum-likelihood method. Branch support was calculated with the approximate Likelihood Ratio Test (aLRT) and the SH (Shimodaira–Hasegawa) correction (Shimodaira 2002) with the software PhyML 3.0 (Guindon et al. 2010). While aLRT calculates verisimilitude logarithms almost as conventional LRT, the branch support made with aLRT-SH has the advantage of being faster and requiring less computational time relative to Bootstrap. The results could differ between both approaches as a consequence of small samples or different levels of divergence (Anisimova and Gascuel 2006). Manual inspection and editing of trees was performed with Geneious version 10.2.3. Based on our phylogenetic inferences, we found homologs for all Arabidopsis genes involved in the biogenesis and recycling of AsA except for two regulators of the pathway, namely *ASCORBATE MANNOSE/GALACTOSE PATHWAY REGULATOR 1* (*AMR1*-AT1G65770) and *ETHYLENE RESPONSE FACTOR 98* (ERF98-AT3G23230) (reviewed in Lisko et al. 2014) (Fig. 3).

Similar to what has been reported for other gene families, the great majority of homologous AsA-related gene families present in the *Marchantia* genome exhibit a reduced number of members per family relative to most of the sequenced genomes from land plants (Fig. 3). In order to explore the evolution of the AsA pathway in land plants, we used the incredible collection of genomic information deposited in Phytozome for the identification of homologous genes through BLAST and a subsequent analysis of the Family History and Gene Ancestry views that rely on the analysis of relationships through Inparanoid analysis and a combination of Smith–Watermann alignments (based on BLOSUM45, gap opening and extension penalty of −12 and −2, respectively, up to a gap length of 50 aa, with zero extension cost after), for each gene in the pathway (Goodstein et al. 2012). The presence and number of genes (including isoforms) are shown in Fig. 3.

3.1 The D*-Mannose/*L*-Galactose (Man/Gal) Pathway*

We identified single copy genes for all enzymes involved in the D-mannose/L-galactose (Man/Gal) pathway. Mapoly0082s0088: phosphomannose isomerase (*PMI1*-At3g02570) and *PMI2* (At1g67070); Mapoly0004s0121: phosphomannose mutase (*PMM*-At2g45790); Mapoly0034s0043: GDP-D-mannose pyrophosphorylase (*VITAMIN C1*-*VTC1*-At2g39770) and *VTC1*-like (At3g55590); Mapoly0101s0064: GDP-D-mannose-3,5-epimerase (*GME*-At5g28840); Mapolv31013666m: GDP-L-galactose phosphorylase (*VTC2*-At4g26850 and *VTC5*-At5g55120); Mapoly0002s0010: L-galactose-1-phosphate phosphatase (*VTC4*-At3g02870); Mapoly0002s0285: L-galactose dehydrogenase (*GalDH*- At4g33670); and Mapoly0077s0021: L-galactono-1,4-lactone dehydrogenase (GLDH- At3g47930) (Fig. 4).

			D-Mannose / L-Galactose Pathway											Master regulators		D-Galacturonate Pathway	L-Gulose Pathway	Myo-Inositol Pathway					Recycling	
			PMI1	PMI2	PMM	VTC1	PUT-VTC1	GME	VTC2	VTC5	VTC4	GalDH	GLDH	AMR1	ERF98	GalUR	GME	PAP15	MIOX	GlcUR	GNL	Gul LO	MDAR	DHAR
	Non vascular	*Marchantia polymorpha*	1	1	1	1	1	1	1	1	1	1	1			10	1	1	1	10	3	3	3	2
Bryophytes		*Physcomitrella patens*	2	2	1	1	1	1	2	2	3	2	1			3	1	4	1	3	10	2	4	2
		Sphagnum fallax	1	1	1	1	1	1	1	1	2	1	1		1	9	1	3	1	9	10	1	6	2
		Selaginella moellendorffii	1	1	1	2	2	9	3	3	2	2	1		7	14	9	3	1	14	8	19	3	4
		Amborella trichopoda	1	1	1	3	3	1	2	2	1	1	1	1	5	9	1	3	2	9	3	3	4	2
		Aquilegia coerulea	1	1	1	2	2	1	1	1	1	1	1	146	3	13	1	2	3	13	10	2	3	2
		Ananas comosus	1	1	1	3	3	2	5	5	1	1		11	4	6	2	3	2	6	4	1	3	2
		Musa acuminata	1	2	2	5	5	4	6	6	1	2	1	12	13		4	1	5	13	3	8	3	4
		Amaranthus hypochondriacus	2	2	1	2	2	2	2	2	1	1		8	9	5	2	1	1	5	4	1	2	3
		Daucus carota	2	2	1	3	3	2	2	2	1	1	1	22	12	13	2	2	2	13	13	3	5	2
		Mimulus guttatus	2	2	1	1	1	2	2	2	2	1	1	23	6	9	2	1	4	9	8	2	4	2
		Solanum lycopersicum	2	2	3	2	2	2	2	2	3	1	1	13	6	12	2	1	2	12	13	2	3	2
		Solanum tuberosum	2	2	3	2	2	2	2	2	3	1	1	40	7	14	2	1	2	14	15	3	3	2
		Kalanchoe fedtschenkoi	2	2	1	1	1	1	1	1	2	1	1	6	5	8	1	2	2	8	7	2	2	2
		Kalanchoe laxiflora	2	2	2	1	1	2	1	1	5	2	1	6	8	14	2	3	6	14	10	3	3	3
		Eucalyptus grandis	1	1	1	1	1	2	2	2	1	2	1	59	9	34	2	5	3	34	21	3	3	3
		Vitis vinifera	2	2	1	1	1		3	3	1	1	1	1	5	17		3	2	17	20	2	3	2
		Linum usutatissimum	5	5	2	8	8	4	1	1	2	2		14	17	17	4	7	3	17	17	8	4	5
		Manihot esculenta	2	2	2	2	2	3	4	4	1	2	1	12	8	22	3	5	3	22	10	2	4	2
		Populus trichocarpa	2	2	1	3	3	3	3	3	3	2	2	13	9	10	3	4	3	10	10	3	3	3
		Ricinus communis	2	2	1	1	1	1	3	3	1	1	1	10	5	13	1	4	2	13	8	3	3	3
Eudicots		*Salix purpurea*	3	3	2	3	3	5	3	3	2	1	1	9	10	10	5	5	5	10	10	2	3	4
		Citrus sinensis	2	2	1	3	3	2	3	3	1	1	1	11	6	15	2	4	2	15	9	3	3	2
		Citrus clementina	2	2	1	2	2	2	3	3		1	1	13	8	16	2	3	2	16	17	3	3	2
		Carica papaya	1	1	2	1	1	1	2	2	1	1	1	5	4	12	1	3	1	12	7	2	3	2
		Gossypium raimondii	2	2	2	2	2	2	3	3	2	1	1	7	8	13	2	4	4	13	9	3	4	3
		Theobroma cacao	1	1	1	1	1	1	2	2	1	1	1	6	2	7	1	2	3	7	7	3	3	2
		Arabidopsis halleri	2	2	1	2	2	1	2	2	2	1	1	55	4	6	1	3	4	6	14	4	5	3
	Vascular	*Arabidopsis lyrata*	2	2	1	2	2	1	2	2	1	1	1	60	5	10	1	4	5	10	13	6	5	3
		Arabidopsis thaliana	2	2	1	3	3	1	2	2	1	1	1	40	4	10	1	3	4	10	15	6	5	3
		Boechara stricta	2	1	1	3	3	1	2	2	1	1	1	23	4	9	1	3	5	9	16	3	5	3
		Brassica oleracea			2	4	4		2	2		2	1	16	2	7		4	3	7	24	6	4	3
		Brassica rapa	3	3	2	5	5	2	3	3	1	2	1	39	7	11	2	4	5	11	22	6	6	5
		Capsella grandiflora	2	2	1	2	2	1	2	2	1	1	1	42	3	8	1	3	4	8	13	4	5	3
		Capsella rubella	2	2	1	2	2	1	2	2	1	1	1	48	4	9	1	3	4	9	16	5	5	3
		Eutrema salsugineum	2	2	1	3	3	1	2	2	1	1	1	37	5	12	1	3	4	12	14	5	4	3
		Cucumissativus	2	2	1	1	1	1	1	1	2	1	2	8	7	14	1	1	2	14	5	1	3	2
		Fragaria vesca	2	2	1	1	1	1	4	4	1	1	1	77	6	14	1	3	4	14	10	3	3	4
		Glycine max	4	4	2	5	5	5	4	4	3	2	2	5	22	26	5	4	6	26	17	4	6	4
		Malus domestica	5	5	3	2	2	3	3	3	3	2	2	43	15	30	3	5	7	30	10	5	2	10
		Medicago truncatula	1	1	1	3	3	2	2	2	1	1	1	65	8	19	2	4	2	19	19	4	3	2
		Phaseolus vulgaris	1	1	1	2	2	2	2	2	2	1	1	2	11	18	2	3	7	18	8	3	3	2
		Prunus persica	2	2	2	1	1	2	2	2	1	1	2	29	5	13	2	3	5	13	7	2	4	2
		Trifolium pratense	1	1		2	2	2	2	2		1	2	13	13	23	2	3	4	23	10	2	3	4
		Brachypodium distachyon	2	2	1	2	2	2	4	4	1	1	1	45	5	10	2	3	1	10	15	6	5	2
		Brachypodium stacei	2	2	1	3	3	2	4	4	1	1	1	38	5	8	2	4	1	8	14	6	5	2
		Oryza sativa	3	3	1	3	3	2	3	3	1	1	1	88	5	15	2	2	1	15	18	5	5	2
		Oropetium thomaeum	2	2	1	2	2	2	2	2		2		9		6	2	2	1	6	9		4	3
Monocots		*Panicum hallii*	2	2	1	2	2	3	3	3	1	1	2	36	4	21	3	2	1	21	18	5	6	2
		Panicum virgatum	7	7	2	4	4	6	6	6	3	2	4	89	9	26	6	6	2	26	25	8	10	4
		Setaria italica	2	2	1	3	3	3	3	3	1	1	2	42	6	21	3	2	1	21	26	7	5	2
		Setaria viridis	2	2	1	2	2	3	3	3	1	1	2	42	6	20	3	2	1	20	26	7	5	2
		Sorghumbicolor	2	2	2	2	2	2	3	3	1	1	1	44	5	19	2	2	1	19	17	4	5	3
		Zea mays	3	3	1	3	3	2	2	2	1	1	1	15	4	17	2	2	1	17	9	3	3	4

Fig. 3 Conservation of gene families involved in the biogenesis and recycling of AsA in land plants. Sequence similarity searches using BLAST, HMMER, and pfam combined with a phylogenetic analysis allowed us to identify homologous genes involved in the biogenesis and recycling of AsA in *Marchantia*. The number of homologous genes (including isoforms) for each gene family in land plants is indicated inside each colored box and was obtained from the gene ancestry and gene family analysis in phytozome.org. Absence of homologous sequences is represented by blank boxes without a number

3.2 The D-Galacturonate (GalUR) Pathway

For the GalU pathway, we identified 11 *M. polymorpha* genes (Mapolv31014624m, Mapolv31005742m, Mapolv31011830m, Mapolv31011827m, Mapolv31023497m, Mapolv31002560m, Mapolv31005838m, Mapolv31002670m, Mapolv31023489m, Mapolv31011434m, Mapolv31008564m, Mapolv31019915m, Mapolv31011412m, Mapolv31021855m, and Mapolv31004812m) homologous to both copies of the Arabidopsis D-galacturonate reductase (*GalUR*-At1g59950 and At1g59960). This is quite interesting as in this case the number of members in the family in *Marchantia* is higher than that present in Arabidopsis. Undergoing functional genomic approaches

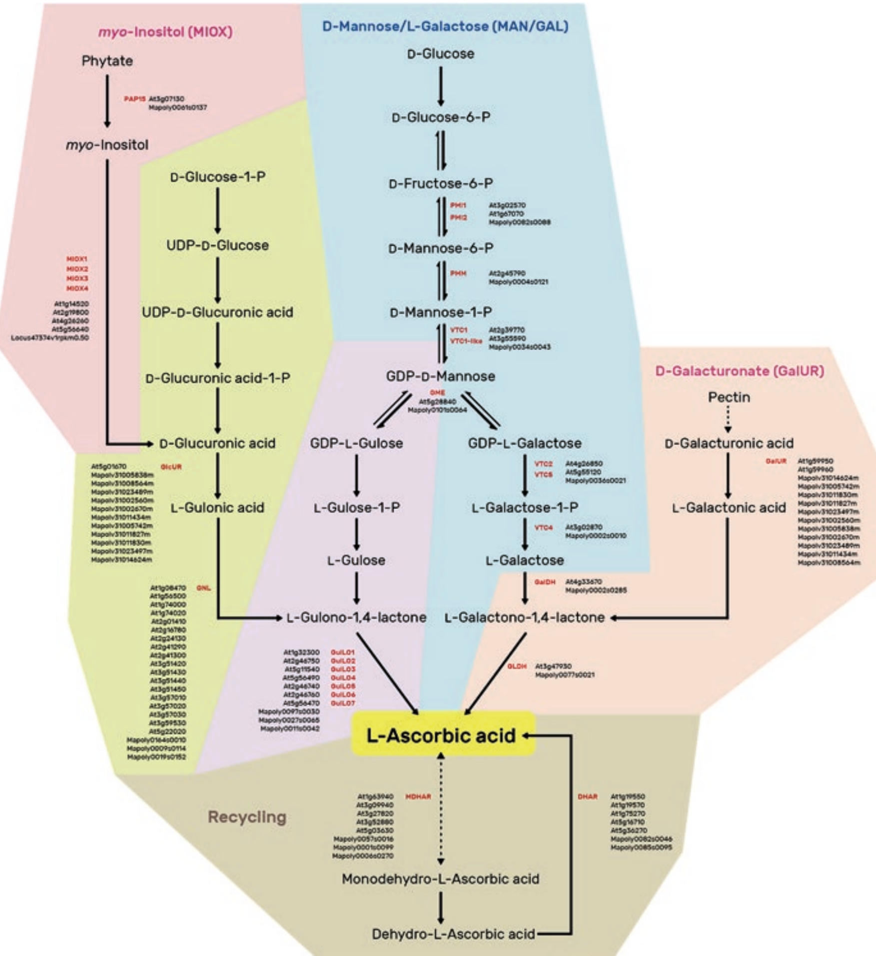

Fig. 4 Pathways involved in the biogenesis and recycling of AsA in *Arabidopsis thaliana* and *Marchantia polymorpha*. The four tested metabolic paths leading to the production of AsA in *Arabidopsis* are depicted. Abbreviated loci names of experimentally identified components for each path are highlighted in red. Each path is shown with a different color. *Arabidopsis* and *Marchantia* loci identifiers are shown next to the abbreviated loci names.

as part of a collaboration between the Lorence and the Arteaga laboratories through forward (genetic screens of mutant plants resistant and oversensitive to AsA) and reverse genetics (CRISPR-Cas based genome edition) will help us unravel the functions and contributions of each member of the family to the pathway (Fig. 4).

3.3 The myo-Inositol (MIOX) Pathway

In the case of the MIOX pathway, we identified a single homolog (Mapoly0061s0137) for the PAP15 (At3g07130) phytase. We were not able to find a homolog of the *myo*-inositol oxygenase gene in *Marchantia* in the publicly available version of the genome but we were able to identify a homolog in a publicly available transcriptome (Sharma et al. 2014) from immature antheridiophores and in an unpublished transcriptome from mixed tissues (Bowman et al., unpublished). The deduced open reading frame (ORF) from the composite transcriptional units (Locus47374v1rpkm0) is homolog to all four Arabidopsis *myo*-inositol oxygenase genes (MIOX1-At1g14520, MIOX2-At2g19800, MIOX4-At4g26260, and MIOX5-At5g56640).

The next enzyme in the inositol pathway to AsA is D-glucuronate reductase (GlcUR). The Lorence Laboratory has characterized the enzyme encoded by At5g01670 and confirmed that it is a functional reductase of uronic acids with no substrate preference between D-glucuronate and D-galacturonate. Arabidopsis plants overexpressing this gene possess enhanced ascorbate, while knockouts have diminished content of this antioxidant (Yactayo-Chang 2011). While the D-glucuronate reductase (*GlcUR*- At5g01670) family in *Marchantia* is also large with 11 members (Mapolv31005838m, Mapolv31008564m, Mapolv31023489m, Mapolv31002560m, Mapolv31002670m, Mapolv31011434m, Mapolv31005742m, Mapolv31011827m, Mapolv31011830m, Mapolv31023497m, Mapolv31014624m), the gluconolactonase (*GNL*-At1g08470, At1g56500, At1g74000, At1g74020, At2g01410, At2g16780, At2g24130, At2g41290, At2g41300, At3g51420, At3g51430, At3g51440, At3g51450, At3g57010, At3g57020, At3g57030, At3g59530, At5g22020) family in *Marchantia* shows a remarkable reduction with only three members (Mapoly0164s0010, Mapoly0009s0114, Mapoly0019s0152) (Fig. 4). The Lorence Laboratory has characterized the enzyme encoded by At1g56500 and confirmed that it is functional gluconolactonase (GNL). This GNL isoform is localized in chloroplasts. Knockouts on this gene have lower AsA content compared to wild-type controls. Arabidopsis overexpressers and complemented lines (knockouts overexpressing the functional gene) have higher AsA than wild type, enhanced tolerance to high light stress, improved photosynthetic efficiency, and higher seed yield (Yactayo-Chang 2016; Yactayo-Chang and Lorence 2016).

The last enzyme that participates in the intersect between the *myo*-inositol and the L-gulose pathways to ascorbate is L-gulono-1,4-lactone oxidase (GulLO). The *GulLO* gene family in *Marchantia* is composed by three members (Mapoly0097s0030, Mapoly0027s0065, Mapoly0011s0042) that correspond to seven members in Arabidopsis (GulLO1-At1g32300, GulLO2-At2g46750, GulLO3-At5g11540, GulLO4-At5g56490, GulLO5-At2g46740, GulLO6-At2g46760, GulLO7-At5g56470). The Lorence Laboratory has recently characterized GulLO5 and has confirmed that this enzyme possesses oxidase activity towards L-GulL (Aboobucker et al. 2017).

Wheeler et al. proposed that GulLO was lost in all photosynthetic eukaryotes and functionally replaced with GLDH (Wheeler et al. 2015). However, genetic and biochemical evidence from different groups indicates that the *Arabidopsis* genome

does contain genes encoding GulLO enzymes (Maruta et al. 2010; Aboobucker et al. 2017). Interestingly, overexpression of the rat GulLO enzyme in Arabidopsis can functionally rescue vitamin C (*vtc*) mutants with a concomitant increase in AsA levels (Radzio et al. 2003), and evidences from different groups have demonstrated the effective conversion of L-Gul into AsA in different plant species (Baig et al. 1970; Davey et al. 1999; Pallanca and Smirnoff 1999; Jain and Nessler 2000; Radzio et al. 2003; Davey et al. 2004; Imai et al. 2009; Li et al. 2010; Mellidou et al. 2012; Aboobucker et al. 2017). Based on these evidences and taking into account that irrespective of the considerations inherent to any overexpression experiment and to the potential substrate specificity, the data indicates that plants fed with a specific substrate for GulLO do produce AsA and that AsA levels are increased when increasing the expression of GulLO (reviewed in Lisko et al. 2014). Given there are seven GulLO genes in Arabidopsis, genetic redundancy is hard to overcome in order to functionally characterize the GulLO gene family in that model; however, taking into account there are only three *GulLO* genes in *Marchantia* and the feasibility of generating mutant edited alleles, we are focusing our efforts to functionally test the involvement of GulLO in the biosynthesis of AsA in *Marchantia*.

3.4 Ascorbate Recycling

The monodehydroascorbate reductase (MDHAR-At1g63940, At3g09940, At3g27820, At3g52880, At5g03630) and dehydroascorbate reductase (DHAR-At1g19550, At1g19570, At1wg75270, At5g16710, At5g36270) gene families that regulate the rapid regeneration of reduced AsA in plants are represented in *Marchantia* by three (Mapoly0057s0016, Mapoly0001s0099, Mapoly0006s0270) and two (Mapoly0082s0046 and Mapoly0085s0095) members, respectively (Fig. 4).

4 Precursor Feeding Studies as a Proxy to Test the Operation of Ascorbate Pathways in *Marchantia*

Based on the fact that we detected the presence of *Marchantia* genes and transcripts with significant homology to the genes involved in AsA metabolism, next we interrogated the function of the various pathways to AsA by doing feeding studies with nonradioactive precursors. For this purpose, six ascorbate precursors were added to *Marchantia* cultures growing in B5 growth media (Fig. 5). The tissue with media supplemented with AsA showed the lowest *in planta* AsA concentration of any of the samples, followed by the control, which included no precursors added into the media. This result indicates that feedback inhibition took place, causing decreased AsA synthesis. D-Galacturonate and L-gulose feeding led to modest ascorbate increases but were not found to be significant when statistical analysis was done in comparison to the control. Three precursors, however, did cause

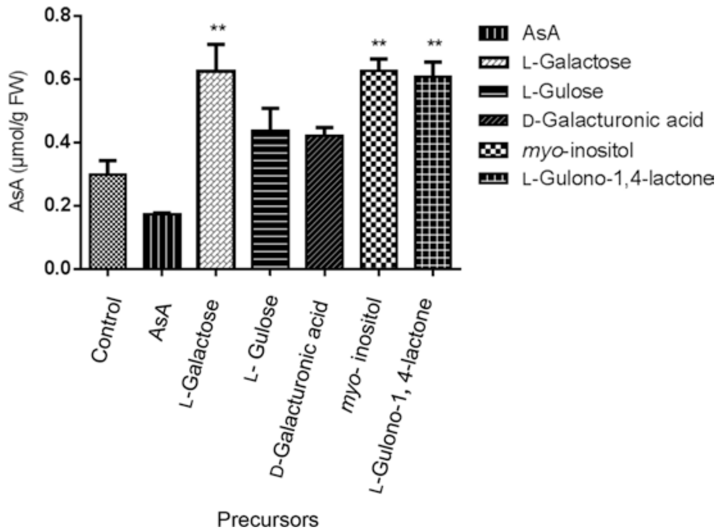

Fig. 5 L-Galactose, *myo*-inositol, and L-gulono-1,4 lactone feeding significantly increased the ascorbate content in *Marchantia*. In vitro *Marchantia* cultures were grown on B5 media; once enough biomass was obtained, cultures were transferred to B5 media supplemented with various ascorbate precursors. Tissue samples were collected and flash frozen 48 h after feeding. Reduced, oxidized, and total ascorbate were determined using an enzyme-based spectrophotometric method. One-way ANOVA and Tukey's post hoc test were performed at significance level of 0.05. p-value < 0.01 was indicated by **. Five biological replicates were used in these assays

significant changes in ascorbate content after analysis using the one-way ANOVA and Tukey's post hoc statistical tests. These precursors were L-galactose, *myo*-inositol, and L-gulono-1,4-lactone. The precursor that resulted in the highest levels of AsA in the thalli was L-galactose, followed by *myo*-inositol and L-gulono-1,4-lactone. These results indicate the operation of at least two of the proposed ascorbate pathways to AsA in *Marchantia*.

5 Conclusions

In summary, *M. polymorpha*, one of the earliest diverging land plants, contains homologous genes to both classical and alternative pathways for the biosynthesis and recycling of AsA and given its characteristics as a powerful model for functional genomics it will surely help aid current efforts to understand how evolution has shaped the biosynthetic pathways of AsA and its role during plant development and responses to the environment in land plants.

Feeding studies with nonradioactive (a.k.a. cold) precursors indicate the functionality in *Marchantia* of both the D-mannose/L-galactose and the *myo*-inositol pathways to ascorbate. Follow-up studies will shed light into the role of specific pools of AsA in the response and adaptation of basal plants to specific stresses.

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Ascorbic Acid in Processed Plant-Based Foods

Sze Ying Leong, Tingting Liu, Indrawati Oey, and David J. Burritt

Abstract Vitamin C (ascorbic acid or L-ascorbic acid) is an essential vitamin for humans and is vital for maintaining good health. As humans are unable to synthesise ascorbic acid, they are dependent upon its presence in their diet to meet their daily ascorbic acid needs. Plants are a good source of ascorbic acid and so it is not difficult for humans to obtain an adequate daily supply of ascorbic acid from a wide range of plant-based foods. However, the ascorbic acid content in plant tissues is variable and levels in fresh product can be affected from the time of harvest until ingestion. In addition, the increasing consumption of processed plant-based foods, rather than fresh product, has led to a considerable amount of research into the most appropriate processing methods to convert raw plant materials into plant-based foods while retaining high levels of ascorbic acid in the final food product. In this chapter, we discuss the impacts of conventional food processing techniques, such as blanching, frying, and freezing on ascorbic acid levels and how novel processing techniques, such as pulsed electric field and high hydrostatic pressure, could be used to improve the retention of ascorbic acid in plant-based foods. The importance of selecting appropriate food processing techniques to maintain both the levels and bioactivity of vitamin C in a range of different plant-based foods is critically evaluated.

Keywords Ascorbic acid · Plants · Food · Processing · Stability · Bioactivity

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S. Y. Leong · I. Oey
Department of Food Science, University of Otago, Dunedin, New Zealand

Riddet Institute, Palmerston North, New Zealand

T. Liu
Department of Food Science, University of Otago, Dunedin, New Zealand

Riddet Institute, Palmerston North, New Zealand

Department of Botany, University of Otago, Dunedin, New Zealand

D. J. Burritt (✉)
Department of Botany, University of Otago, Dunedin, New Zealand
e-mail: david.burritt@otago.ac.nz

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1 Introduction to Ascorbic Acid in Plants and its Importance for Human Health

Vitamin C (ascorbic acid or L-ascorbic acid) is an important multifunctional molecule in plants, with a diverse array of functions including regulating gene expression, acting as a cofactor for key enzymes and as a substrate for oxalate and tartrate biosynthesis, modulating plant growth via interactions with plant hormones, and can also act as an antioxidant and a regulator of intracellular redox state (Gest et al. 2013). Ascorbic acid also has a multifunctional role in animals where it is important as an antioxidant and regulator of intracellular redox state, promotes iron absorption, helps to promote immune responses, strengthens blood vessels, and reduces cholesterol concentrations (Figueroa-Méndez and Rivas-Arancibia 2015). Ascorbic acid also promotes collagen formation and cartilage development and helps to facilitate wound healing (Figueroa-Méndez and Rivas-Arancibia 2015). Because of these properties, ascorbic acid has also been shown to help prevent diseases such as arteriosclerosis-related cardiovascular diseases (e.g. hypertension) and cancer (Figueroa-Méndez and Rivas-Arancibia 2015).

Humans, unlike most animals, cannot synthesise ascorbic acid and so must obtain their daily requirement from dietary sources. Ascorbic acid deficiency can result in the development of several diseases including scurvy, a disease associated with the breakdown of the connective tissues. While it is widely acknowledged that the best source of ascorbic acid for humans is fresh fruit and vegetables, increasingly humans are relying on processed foods to meet their daily nutritional needs, including plant-based products (Weaver et al. 2014).

It is well known that the ascorbic acid contents of plant tissues are highly variable, with levels in fresh produce being sensitive to change from the time of harvest until consumption. Numerous studies have shown that the choice of processing method can greatly influence ascorbic acid stability and hence its levels in processed plant-based foods. In addition, how ascorbate/vitamin C levels are reported in the published literature also varies. Some studies report both the levels of ascorbic acid and ascorbic acid + dehydroascorbic acid (total vitamin C), while others only report ascorbic acid or total vitamin C levels. For the remainder of this chapter, we will use the terms ascorbic acid and total vitamin C as defined above.

In the following sections, we provide an overview of conventional food processing techniques, including those most commonly used to stabilise/protect ascorbic acid from enzyme-catalysed oxidation e.g. blanching, and combined blanching and freezing, and discuss how novel processing techniques could be used to improve the retention of biologically active ascorbic acid in processed plant-based foods. The importance of selecting appropriate food processing techniques to maintain both the levels and bioactivity of ascorbic acid, in a range of different plant-based foods, is critically evaluated.

2 An Overview of Food Processing Techniques for Fruit and Vegetables

Most fruit and vegetables are perishable after harvest and undergo a progressive loss of quality-related characteristics including reduced nutritional value and changes in organoleptic properties, e.g. colour, taste, appearance, flavour, texture, and palatability. This often results in the post-harvest loss of produce and hence food wastage. The application of food processing technologies to fruit and vegetables is becoming increasingly important as processing can reduce waste and produce plant-based foods of consistent quality. Processing fruit and vegetables can retard the activity of microorganisms that cause spoilage of fresh produce, help to inhibit biochemical reactions that are associated with reduced quality in fresh produce, create product variation in order to improve customer satisfaction, make nutritious plant-based foods that are more convenient for consumers, and enable consumers to access seasonal commodities all-year round.

With respect to processing, fruit and vegetables can be washed, cut, sliced, shredded, juiced or pureed, and then further processed to produce canned, frozen, dried, or preserved products. Depending on the physical characteristics of the fruit or vegetables, the extent of the quality-related changes caused by processing is variable, with some fruit and vegetables being suitable for most food processing techniques, while for others suitable processing techniques are more limited. For example, apples can be processed to produce fresh-cut or dried slices, juice, puree, cider, jam, chutney, marmalade, chips, and canned or frozen products. In contrast, bananas are best when eaten fresh, in a pureed form or as a dried chip, but are not suitable for juicing, canning, or freezing. The high water contents of leafy vegetables e.g. spinach, lettuce, and cucumber mean that they are not suitable for freezing due to the loss of cell turgor and the resultant textural changes that occur upon thawing.

Traditionally, thermal processing techniques such as blanching, canning, and pasteurisation are used by food industries to inactivate enzymes that can cause undesirable quality-related changes and to eliminate microbes that could compromise the safety of processed food products. However, thermal processing may have negative effects on plant-based foods including unwanted changes in colour, softening, loss of flavour, and degradation of heat-sensitive compounds, including ascorbic acid. Since blanching at high temperatures for short times is very effective for enzyme inactivation, fruit and vegetables are often blanched before they are processed into dried or frozen products, which have longer shelf lives. As most fresh fruits and vegetables have high water contents, that can limit their storage life, freeze-drying can also be used to remove water from the plant tissues with minimal degradation of heat-sensitive components. However, frozen food products can experience degradation of some nutrients and texture loss upon thawing.

To better preserve some of the inherent characteristics of fruit and vegetables, novel processing technologies such as pulsed electric fields (PEF) and high hydrostatic pressure processing (HPP) have been developed. These technologies involve non-thermal processing, as they most often involve processing at ambient

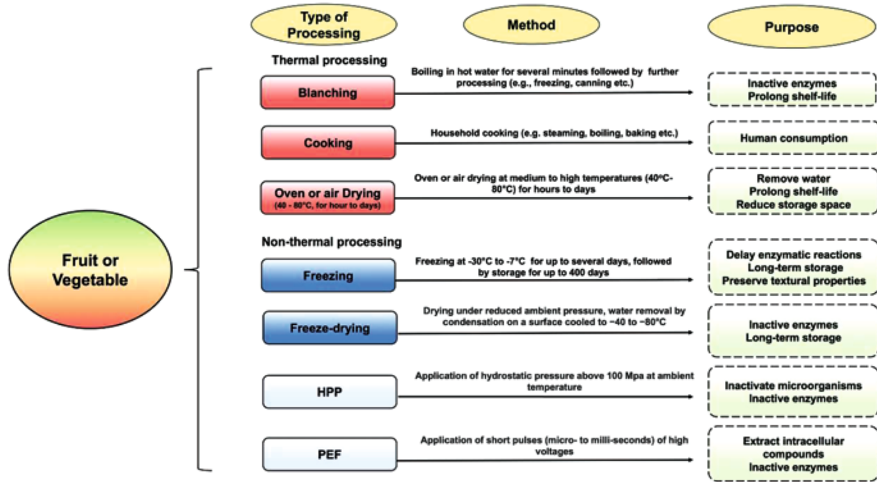

Fig. 1 Summary diagram of the most commonly used processing technologies

temperatures, and have the potential to minimise quality-associated degradation of plant-based foods while still inactivating microorganisms.

In the context of the ascorbic acid content of plant-based foods, it can be expected that each processing step involved in the production of the final product can have consequences on the ascorbic acid content. A processing technique that may appear to be suitable for maintaining the levels of ascorbic acid in a specific fruit or vegetable could exert a negative impact on the ascorbic acid content of a different fruit or vegetable. Therefore, ongoing evaluation of the effects of conventional and novel food processing technologies on the ascorbic acid contents of a wide range of plant-based foods is important. Figure 1 provides a summary of the main processing technologies used in the food industry.

3 The Impact of Conventional Processing on the Levels of Ascorbic Acid in Plant-Based Foods

Ascorbic acid is a labile molecule and the concentration of ascorbic acid often decreases in fruit and vegetables during conventional processing. Tables 1 and 2 summarise the ascorbic acid and total vitamin C contents of selected fresh and processed fruits and vegetables. Food processing techniques are often harsh and the first step in processing usually requires a reduction in size or damage to the fruit or vegetable, e.g. peeling, cutting, or other size-reduction operations. As a result, enzymes are released from their cellular compartments and come into contact with their substrates. This kind of enzyme–substrate interaction can bring about ascorbic acid oxidation and degradation.

Table 1 Ascorbic acid and total vitamin C (mg/kg fresh weight) contents of selected fresh and processed fruits

Fruit	Fresh or processed	Processing parameters	Country	Ascorbic acid (retention %)[a]	Total vitamin C (retention %)	With (+) or without (−) skin and or/ seeds	References
				mg/kg fresh weight unless specified			
Apple	Fresh		China	<50	60	+/+	Szeto et al. (2002)
			China	–	45–48	+/−	Zhang and Zhang (2014)
			UK	–	60	+/+	Proteggente et al. (2002)
			Nigeria	218	–	−/+	Achinewhu (1983)
			Germany	–	108	Unknown	Taiwo et al. (2001)
			Canada	–	1124.3[b]	+/−	Joshi et al. (2011)
	Juice	Wash, slice, and then press	Korea	67	–	–	Kim et al. (2012)
	Juiced + heated	Fresh pressed juice heat at 85 °C for 1 min	Korea	6 (9%)	–	–	Kim et al. (2012)
	Blanched	60 °C for 5 min	Germany	–	ND	+/−	Taiwo et al. (2001)
	Dried	Air dried at 47 °C for 7 h	Canada	–	555.3[b] (50%)[a]	+/−	Joshi et al. (2011)
		Oven dried at 70 °C for 10 h		–	781.4[b] (70%)	+/−	
		Vacuum dried at 20 °C for 24 h		–	1109.1[b] (99%)	+/−	
	Microwave treated	90 W for 25 s	China	–	43–45 (95–97%)	+/−	Zhang and Zhang (2014)

(continued)

Table 1 (continued)

Fruit	Fresh or processed	Processing parameters	Country	Ascorbic acid (retention %) [a]	Total vitamin C (retention %)	With (+) or without (–) skin and or/seeds	References
				mg/kg fresh weight unless specified			
Apple	Frozen	−28 °C, overnight	Germany	–	41 (38%)	+/–	Taiwo et al. (2001)
	HPP	400 MPa for 10 min at 25 °C	Germany	–	103 (95%)	+/–	Taiwo et al. (2001)
	Juice + HPP	Fresh pressed juice treated at 500 MPa, 25 °C for 3 min	Korea	64 (96%)	–	–	Kim et al. (2012)
	PEF	1.4 kV/cm, specific energy 154 J/kg	Germany	–	74 (69%)	+/–	Taiwo et al. (2001)
Avocado	Fresh		Nigeria	260	–	–/–	Achinewhu (1983)
	Roasted	5 min	Nigeria	146 (56%)	–	–/–	
	Boiled	5 min	Nigeria	109 (42%)	–	–/–	
Apricot	Fresh		New Zealand	10[b]	40[b]	–/–	Leong and Oey (2012b)
	Heated	98 °C, 1:5 food/water, for 10 min	New Zealand	0[b] (0%)	60[b] (150%)	–/–	
	Frozen	Frozen in liquid nitrogen, then frozen at −20 °C	New Zealand	10[b] (100%)	90[b] (225%)	–/–	
	Freeze-dried	Frozen in liquid nitrogen, then freeze-drying for 48 h	New Zealand	10[b] (100%)	20[b] (50%)	–/–	

Banana (ripe)	Fresh		UK	–	100	–/–	Proteggente et al. (2002)
			USA	153	190	–/–	Vanderslice et al. (1990)
			China	–	110	–/–	Szeto et al. (2002)
			Malawi	–	130	–/–	Masamba et al. (2013)
	Dried	40–45 °C, humidity (25–45%), 10–16 h	Malawi		37.4 (29%)	–/–	Masamba et al. (2013)
		60 °C for 10 min	Nigeria	100.5 (NA)	–	–/–	Taiwo and Adeyemi (2009)
	Blanched + dried	60 °C blanching + 60 °C drying	Nigeria	99.2 (NA)	–	–/–	Taiwo and Adeyemi (2009)
Banana (unripe)	Fresh		Unknown	17–23	–	–/–	Belayneh et al. (2014)
	Fried	170 °C for 4 min	Unknown	11–17 (65–74%)	–	–/–	
Cherry	Fresh		New Zealand	50^b	90^b	–/–	Leong and Oey (2012b)
	Heated	98 °C, 1:5 food/water, for 10 min	New Zealand	10^b (20%)	120^b (133%)	+/–	
	Frozen	Frozen in liquid nitrogen, then frozen at −20 °C	New Zealand	10^b(20%)	210^b (233%)	+/–	
	Freeze-dried	Frozen in liquid nitrogen, then freeze-drying for 48 h	New Zealand	0^b (0%)	60^b (67%)	+/–	

(continued)

Table 1 (continued)

Fruit	Fresh or processed	Processing parameters	Country	Ascorbic acid (retention %)[a]	Total vitamin C (retention %)	With (+) or without (−) skin and or/ seeds	References
				mg/kg fresh weight unless specified			
Lemon	Fresh		China	420	580	−/−	Szeto et al. (2002)
			Romania	–	410–470	−/−	Simion et al. (2008)
	Oven dried	30 °C for 5 h	Romania	–	420 (95%)	−/−	Simion et al. (2008)
		80 °C for 5 h		–	74 (17%)	−/−	
	Frozen + thawed	−6 °C for 2 days	Romania	–	432 (98%)	−/−	Simion et al. (2008)
	Freeze-dried	Unknown	Romania	–	411 (93%)	−/−	Simion et al. (2008)
Blue Berry	Fresh		Unknown	–	7650[b]	+/+	Arancibia-Avila et al. (2012)
			Chile	210[b]	–	+/+	López et al. (2010)
	Oven heated	100 °C for 60 min	Poland	–	4330[b] (57%)	+/+	Arancibia-Avila et al. (2012)

Blue Berry	Dried	80 °C until constant weight achieved	Chile	17[b] (8%)	–	+/+	López et al. (2010)
	Juice	Washed, pressed, centrifuged at 4000 × *g* for 15 min, fresh	Germany	163	–	–	Barba et al. (2012)
		Washed, pressed, centrifuged at 4000 × *g* for 15 min, stored at 4 °C for 56 days	Germany	81 (50%)	–	–	
	Juice + HPP	HPP: (600 MPa, for 5 min, from 25 to 45 °C	Germany	155 (95%)	–	–	Barba et al. (2012)
		HPP: (600 MPa, for 5 min, from 25 to 45 °C); stored at 4 °C for 56 days	Germany	112 (69%)	–	–	
	Juice + PEF	PEF: (36 kV/cm, 100 μs, pulse width 3 μs)	Germany	158 (97%)	–	–	Barba et al. (2012)
		PEF: (36 kV/cm, 100 μs, pulse width 3 μs) stored at 4 °C for 56 days	Germany	82 (50%)	–	–	
Kiwi fruit	Fresh		China	520	590	−/+	Szeto et al. (2002)
			Italy	–	300–500	−/+	Tavarini et al. (2008)
			Turkey	–	2321.8	−/+	Kayaa et al. (2010)
	Dried	35 °C, humidity 85%	Turkey	–	1176.5 (51%)	−/+	Kayaa et al. (2010)
		65 °C, humidity 40%		–	274.47 (12%)	−/+	

(continued)

Table 1 (continued)

Fruit	Fresh or processed	Processing parameters	Country	Ascorbic acid (retention %) [a]	Total vitamin C (retention %)	With (+) or without (−) skin and or/ seeds	References
				mg/kg fresh weight unless specified			
Strawberry	Fresh		China	540	770	+/+	Szeto et al. (2002)
			UK	–	610	+/+	Proteggente et al. (2002)
			Ireland	6331[b]	–	+/+	Patras et al. (2009a, b)
	Air-dried	48.9 °C for 88 h	USA	36–53 (NA)	–	+/+	Asami et al. (2003)
	Blanched	70–80 °C	Ireland	4691[b] (74%)	–	+/+	Patras et al. (2009a, b)
	Frozen	−25 °F for 10 min	USA	271–326 (unknown %)	–	+/+	Asami et al. (2003)
	Freeze-dried	−45 °C, 20–22 h	USA	98–144 (unknown %)	–	+/+	Asami et al. (2003)
	HPP	600 MPa	Ireland	5991[b] (95%)	–	+/+	Patras et al. (2009a, b)
Mango	Fresh		China	210	370	−/−	Szeto et al. (2002)
			Nigeria	980	–	−/−	Achinewhu (1983)
			Malawi	–	260	−/−	Masamba et al. (2013)
	Dried	40–45 °C, humidity (25–45%), 10–16 h	Malawi	–	50.8 (20%)	−/−	Masamba et al. (2013)

Pineapple	Fresh		China	100	325	−/−	Szeto et al. (2002)
			Nigeria	335	–	−/−	Achinewhu (1983)
			Malawi	–	325	−/−	Masamba et al. (2013)
	Dried	40–45°C, humidity (25–45%), 10–16 h	Malawi	–	65.2 (20%)	−/−	Masamba et al. (2013)
Green Grape	Fresh		China	20	30	+/−	Szeto et al. (2002)
			UK		20	+/+	Proteggente et al. (2002)
Red Grape	Fresh		China	<10		+/−	Szeto et al. (2002)

(continued)

Table 1 (continued)

Fruit	Fresh or processed	Processing parameters	Country	Ascorbic acid (retention %) [a]	Total vitamin C (retention %)	With (+) or without (−) skin and or/ seeds	References
				mg/kg fresh weight unless specified			
Orange	Fresh		China	330	540	−/−	Szeto et al. (2002)
			UK	–	460	−/+	Proteggente et al. (2002)
			Nigerian	483	–	−/−	Achinewhu (1983)
			USA	547–750	630–830	−/−	Vanderslice et al. (1990)
			Nigeria	–	611.8	−/−	Bello and Fowoyo (2014)
	Juice	Fresh pressed	Spain	–	563	–	Elez-Martínez and Martín-Belloso (2007)
		Fresh pressed	Spain	409	444	–	Sánchez-Moreno et al. (2005)
	Juice + PEF	15 kV/cm, 200 Hz, 4 μs length	Spain	–	(98.2%)	–	Elez-Martínez and Martín-Belloso (2007)
		35 kV/cm, for 750 μs	Spain	378 (92%)	413 (93%)	–	Sánchez-Moreno et al. (2005)
	Juice + HPP	400 MPa, 40 °C, 1 min	Spain	36.9 (90%)	42.8 (96%)		Sánchez-Moreno et al. (2005)
Guava (white)	Fresh		Nigeria	675	–	+/+	Achinewhu (1983)

Guava (white)	Puree	Fresh pressed	China	1800	–	–	Yen and Lin (1996)
	Puree + heated	Heat: 88–90 °C for 24 s	China	1800 (100%)	–	–	Yen and Lin (1996)
	Puree + HPP	HPP: 600 MPa, 25 °C, for 15 min	China	1800 (100%)	–	–	Yen and Lin (1996)
Pear	Fresh		China	<10	60	+/–	Szeto et al. (2002)
			UK		30	+/+	Proteggente et al. (2002)
Peach	Fresh		UK	–	60	+/+	Proteggente et al. (2002)
Papaya	Fresh		Nigeria	518	–	+/+	Achinewhu (1983)
Chinese pear	Fresh		China	<10	–	+/–	Szeto et al. (2002)
Raspberry	Fresh		UK	–	260	+/+	Proteggente et al. (2002)
			Spain	–	311	+/+	de Ancos et al. (2000)
Raspberry	Frozen	−80 °C for 15 min	Spain	–	320 (103%)	+/+	de Ancos et al. (2000)
		−80 °C for 15 min; then −20 °C for 90 days		–	243 (76%)	Unknown	
		−80 °C for 15 min; then −20 °C for 180 days		–	192 (60%)	Unknown	

(continued)

Table 1 (continued)

Fruit	Fresh or processed	Processing parameters	Country	Ascorbic acid (retention %) [a]	Total vitamin C (retention %)	With (+) or without (−) skin and or/seeds	References
				mg/kg fresh weight unless specified			
Grapefruit	Fresh		UK	–	520	−/+	Proteggente et al. (2002)
			China	390	360	−/−	Szeto et al. (2002)
			USA	213	240	−/−	Vanderslice et al. (1990)
Persimmon	Fresh		China	–	80	+/−	Szeto et al. (2002)
Mandarin	Fresh		China	–	240	−/−	Szeto et al. (2002)
Cantaloupe	Fresh		USA	313	340	−/−	Vanderslice et al. (1990)
Watermelon	Fresh		USA	80	100	−/−	Vanderslice et al. (1990)
	Juice	Chopped and filtered using a steel sieve with an approximate mesh of 2 mm	Spain	26	–	−/−	Oms-Oliu et al. (2009)
Watermelon	Juice + PEF	Chopped and filtered using a steel sieve with an approximate mesh of 2 mm; juice treated at 35 kV/cm, 7 μs pulse length, 200 Hz	Spain	19 (72%)	–	−/−	Oms-Oliu et al. (2009)

Peppers, green	Fresh		USA	1290	1340	+/–	Vanderslice et al. (1990)
			NA	885.0	–		Castro et al. (2008)
			Japan	767	708	+/–	Chuah et al. (2008)
	Blanched	98 °C, 2.5 min	NA	440–460 (50–52%)	–	+/–	Castro et al. (2008)
	Boiled	30 min	Japan	~400 (52%)	–	+/–	Chuah et al. (2008)
	Stir-fried	5 min	Japan	~550 (72%)	–	+/–	Chuah et al. (2008)
	Microwave	30 min	Japan	~640 (83%)	–	+/–	Chuah et al. (2008)
	HPP	200 MPa, 20 min	NA	720–750 (81–84%)	–	+/–	Castro et al. (2008)

(continued)

Table 1 (continued)

Fruit	Fresh or processed	Processing parameters	Country	Ascorbic acid (retention %) [a]	Total vitamin C (retention %)	With (+) or without (–) skin and or/ seeds	References
				mg/kg fresh weight unless specified			
Peppers, red	Fresh		USA	1510	1550	+/–	Vanderslice et al. (1990)
			NA	1074	–	+/–	Castro et al. (2008)
			Japan	1446	–	+/–	Chuah et al. (2008)
	Blanched	98 °C, 2.5 min	NA	620–650 (58–61%)	–	+/–	Castro et al. (2008)
	Boiled	30 min	Japan	~850 (59 %)	–	+/–	Chuah et al. (2008)
	Stir-fried	5 min	Japan	~1240 (86%)	–	+/–	Chuah et al. (2008)
	Microwave	30 min	Japan	~1250 (86%)	–	+/–	Chuah et al. (2008)
	HPP	200 MPa, 20 min	NA	1230–1250 (115–116%)	–	+/–	Castro et al. (2008)

Tomato	Fresh		UK	–	180	+/+	Proteggente et al. (2002)
			Israel	120–160	–	+/+	Stevens et al. (2008)
			USA	–	133	+/+	Dewanto et al. (2002)
			USA	106	140	+/+	Vanderslice et al. (1990)
			USA	4339[b]	–	+/+	Horuz et al. (2017)
			Italy	185[b]	–	+/+	Zanoni et al. (1998)
			China	~700[b]		+/+	Chang et al. (2006)
	Heated	80 °C, 30 min	USA	–	95(71%)	+/+	Dewanto et al. (2002)
	Microwave	120 W, until constant weight	Turkey	1209.7[b] (28%)	–	+/+	Horuz et al. (2017)
	Dried	80 °C, air flow 1.5 m/s, 190 min	Italy	106[b] (57%)		+/+	Zanoni et al. (1998)
		110 °C, air flow 1.5 m/s, 190 min		Not detected (0%)		+/+	
	Freeze-dried	−50 °C, 5 Pa, 24 h	China	~630[b] (90%)		+/+	Chang et al. (2006)

(continued)

Table 1 (continued)

Fruit	Fresh or processed	Processing parameters	Country	Ascorbic acid (retention %) [a]	Total vitamin C (retention %)	With (+) or without (−) skin and or/ seeds	References
				mg/kg fresh weight unless specified			
Tomato	Puree	Vacuum mixed at 500 rpm, 1 °C	Ireland	2048	–	+/+	Patras et al. (2009a, b)
	Puree + thermal	Vacuum mixed at 500 rpm, 1 °C; then heat at 70 °C for 2 min	Ireland	1250 (61%)	–	+/+	Patras et al. (2009a, b)
	Puree + HPP	Vacuum mixed at 500 rpm, 1°C; then treat at 600 MPa at 20 °C for 15 min	Ireland	1921 (94%)	–	+/+	Patras et al. (2009a, b)
	Juice	Blending at high speed 10 s	China	186	210	+/+	Hsu et al. (2008)
		Chopped and then filtered through steel sieves 2 mm in diameter	Spain	152	–	+/+	Odriozola-Serrano et al. (2007)
	Juice + thermal	Fresh prepared juice treated at 92 °C for 2 min, then 98 °C for 15 min, cooled at 0 °C for 2 min, stored at 4 °C for 0 h	China	70 (38%)	88 (42%)	+/+	Hsu et al. (2008)
		Fresh prepared juice treated at 92°C for 2min, then 98°C for 15 min, cooled at 0 °C for 2 min, stored at 4°C for 28 days		55 (30%)	70 (33%)	+/+	
	Juice + HPP	Fresh prepared juice treated at 500 MPa, 25 °C for 10 min, stored at 4 °C for 0 days	China	142 (76%)	151 (72%)	+/+	Hsu et al. (2008)
		Fresh prepared juice treated at 500 MPa, 25 °C for 10 min, stored at 4 °C for 28 days		137 (74%)	147 (70%)	+/+	

Tomato	Juice + PEF	Fresh pressed juice treated at 35 kV/cm, 1 μs pulse duration, 250 HZ	Spain	137 (90%)	–	+/+	Odriozola-Serrano et al. (2007)

HPP high pressure processed, *PEF* pulsed electric field processed
[a]Data in bracket represent the value of the retention of ascorbic acid/total vitamin C after processing
[b]Data were presented as mg of ascorbic acid/vitamin C per kg of dried samples

Table 2 Ascorbic acid and total vitamin C (mg/kg fresh weight) contents of selected fresh and processed vegetables

Vegetables	Fresh/processing	Processing parameter	Country of the study conducted	Ascorbic acid (retention %)[a]	Total vitamin C (retention %)	Reference
				mg/kg fresh weight unless specified		
Broccoli	Fresh		USA	890	970	Vanderslice et al. (1990)
			Poland	1160	–	Lisiewska and Kmiecik (1996)
			Spain	–	1120	Murcia et al. (2000)
			UK	–	450	Proteggente et al. (2002)
			Turkey	–	681.8	Tosun and Yücecan (2008)
			Romania	–	344.5	Balan et al. (2016)
			Italy	1500[b]	–	Severini et al. (2016)
			Italy	8020[b]	–	Pellegrini et al. (2010)
	Blanched	96 °C for 170 s	Turkey	–	551.6 (78%)	Tosun and Yücecan (2008)
		92–96 °C for 150 s	Spain	–	550 (49%)	Murcia et al. (2000)
		100 °C for 30 s	Italy	1250[b] (83%)	–	Severini et al. (2016)
		100 °C for 60 s		850[b] (57%)	–	
		100 °C for 180 s		620[b] (41%)	–	
	Blanched + frozen	Blanching at 98 °C for 3 min, frozen at 30 °C for 12 months	Poland	569 (49%)	–	Lisiewska and Kmiecik (1996)

Broccoli	Steamed	Unknown	Italy	5755^{b} (68%)	–	Miglio et al. (2007)
		100 °C for 30 s	Italy	1150^{b} (77%)	–	Severini et al. (2016)
		100 °C for 60 s		980^{b} (65%)	–	
		100 °C for 180 s		700^{b} (47%)	–	
		Oven steaming, 13 min	Italy	6435^{b} (80%)	–	Pellegrini et al. (2010)
	Boiled	5 min	USA	370 (42%)	400 (41%)	Vanderslice et al. (1990)
		10 min	Romania	–	161.8 (49%)	Balan et al. (2016)
		Unknown	Italy	4376^{b} (52%)	–	Miglio et al. (2007)
		1:5, food/water; 8 min	Italy	6450^{b} (80%)	–	Pellegrini et al. (2010)
	Canned	121 °C for 30 min	Spain	–	180 (16%)	Murcia et al. (2000)
	Microwave	2450 MHz, 900 W for 40 s	Italy	1760^{b} (117%)	–	Severini et al. (2016)
		2450 MHz, 900 W for 60 s		1400^{b} (93%)	–	
		2450 MHz, 900 W for 80 s		1600^{b} (107%)	–	
		2450 Hz, 300 W for 17 min	Italy	133 (2%)		Pellegrini et al. (2010)
		High heat, 6 min	USA	1110 (124%)	1160 (119%)	Vanderslice et al. (1990)

(continued)

Table 2 (continued)

Vegetables	Fresh/processing	Processing parameter	Country of the study conducted	Ascorbic acid (retention %)[a]	Total vitamin C (retention %)	Reference
				mg/kg fresh weight unless specified		
Broccoli	Fried	Frying at 170 °C	Italy	1133[b] (13%)	–	Miglio et al. (2007)
	Frozen + boiled	Boiling for 5 min	USA	210 (23%)	230 (23%)	Vanderslice et al. (1990)
	Frozen	6 months	Turkey	–	532.6 (78.1%)	Tosun and Yücecan (2008)
		Unknown	USA	537 (60%)	560 (58%)	Vanderslice et al. (1990)
Brussels sprouts	Fresh		Italy	10969[b]	–	Pellegrini et al. (2010)
			Argentina	964	–	Vina et al. (2007)
	Blanched	100 °C, for 1 min	Argentina	964 (100%)	–	Vina et al. (2007)
		100 °C, for 4 min		732 (76%)	–	
		50 °C, for 5 min		780 (89%)	–	
	Boiled	1:5, food/water; 10 min	Italy	6077[b] (55%)	–	Pellegrini et al. (2010)
	Steamed	Oven steaming, 17 min	Italy	8370[b] (76%)	–	Pellegrini et al. (2010)
	Microwave	2450 Hz, 300 W for 18 min	Italy	3851[b](35%)	–	Pellegrini et al. (2010)
		700 W for 5 min	Argentina	1156 (120%)		Vina et al. (2007)
Cucumber	Fresh		USA	103	140	Vanderslice et al. (1990)
			Japan	–	98	Matsufuji et al. (2009)

Cabbage	Fresh		USA	423	420	Vanderslice et al. (1990)
			Poland	725	–	Podsędek et al. (2008)
	Boiled	15 min	USA	244 (58%)	240 (58%)	Vanderslice et al. (1990)
		1:2 food to water ratio, for 20 min	Poland	237.4 (33%)	–	Podsędek et al. (2008)
		1:1 food to water ratio, for 20 min		336.1 (46%)	–	
	Steamed	100 °C, for 5 min	Poland	615 (85%)	–	Podsędek et al. (2008)
		100 °C, for 20 min		563 (78%)	–	
Carrots	Fresh		USA	47	70	Vanderslice et al. (1990)
			Japan	–	22	Matsufuji et al. (2009)
	Boiled	Unknown	Italy	281[b] (91%)	–	Miglio et al. (2007)
	Steamed	Unknown	Italy	192[b] (62%)	–	Miglio et al. (2007)
	Fried	Frying at 170 °C	Italy	Not detected	–	Miglio et al. (2007)
Celery	Fresh		Japan	–	59	Matsufuji et al. (2009)

(continued)

Table 2 (continued)

Vegetables	Fresh/processing	Processing parameter	Country of the study conducted	Ascorbic acid (retention %)[a]	Total vitamin C (retention %)	Reference
				mg/kg fresh weight unless specified		
Cauliflower	Fresh		UK	–	150	Proteggente et al. (2002)
			USA	540	630	Vanderslice et al. (1990)
			Poland	647	–	Lisiewska and Kmiecik (1996)
			Italy	11945[b]	–	Pellegrini et al. (2010)
	Boiled	1:5, food/water; 10 min	Italy	6919[b] (58%)	–	Pellegrini et al. (2010)
	Steamed	Oven steaming, 17 min	Italy	5865[b] (49%)	–	Pellegrini et al. (2010)
	Microwave	2450 Hz, 300 W for 30 min	Italy	629[b](5%)	–	Pellegrini et al. (2010)
	Blanched + frozen	Blanching at 98 °C for 3 min; frozen at 30 °C for 12 months	Poland	440 (68%)	–	Lisiewska and Kmiecik (1996)
Corn	Frozen	−26 °F	USA	21–35 (AN)	–	Asami et al. (2003)
	Freeze-dried	−45 °F for 20–24 h	USA	ND	–	
	Air-dried	48.9 °C for 20–25 h	USA	ND	–	
Beans	Fresh		USA	100	120	Vanderslice et al. (1990)
	Boiled	Boiled for10 min	USA	67 (67%)	80 (67%)	
	Frozen	Unknown	USA	190 (NA)	220 (NA)	
	Frozen + boiled	Boiled for 10 min	USA	93 (93%)	120 (100%)	

Fennel	Thermal + extracted Juice	90 °C, for 80 min	France	~135		El-Belghiti et al. (2008)
	PEF + extracted Juice	0.4 kV/cm, 450 pulses	France	~335		
Green beans	Fresh		Turkey	–	115	Tosun and Yücecan (2008)
			Romania	–	152.0	Balan et al. (2016)
	Blanched	94–96 °C for 2.5 min	Turkey	–	84.1 (73%)	Tosun and Yücecan (2008)
	Boiled	10 min	Romania	–	84.5 (56%)	Balan et al. (2016)
Leek	Fresh		UK	–	160	Proteggente et al. (2002)
Lettuce	Fresh		UK	–	<20	Proteggente et al. (2002)
			USA	33	60	Vanderslice et al. (1990)
			Japan	–	44	Matsufuji et al. (2009)
Mustard green	Fresh		USA	362	360	Vanderslice et al. (1990)
	Boiled	100 °C for 1 h	US	48 (13%)	50 (14%)	
Onion	Fresh		UK	–	60	Proteggente et al. (2002)
			Japan	–	36	Matsufuji et al. (2009)

(continued)

Table 2 (continued)

Vegetables	Fresh/processing	Processing parameter	Country of the study conducted	Ascorbic acid (retention %)[a]	Total vitamin C (retention %)	Reference
				mg/kg fresh weight unless specified		
Peas	Fresh		UK	–	220	Proteggente et al. (2002)
			Turkey	–	286.3	Tosun and Yücecan (2008)
			Romania	–	212.0	Balan et al. (2016)
	Boiled	10 min	Romania	–	107.6 (51%)	Balan et al. (2016)
	Blanched	94–96 °C for 2.5 min	Turkey	–	208 (73%)	Tosun and Yücecan (2008)
	Frozen	Unknown	Turkey	–	203.1 (71%)	Tosun and Yücecan (2008)
Potato	Fresh		USA	77	130	Vanderslice et al. (1990)
			Turkey	–	224.9	Tosun and Yücecan (2008)
	Peeled		Turkey	–	199.9 (89%)	Tosun and Yücecan (2008)
	Washed		Turkey	–	168.8 (75%)	Tosun and Yücecan (2008)
	Blanched	94–96 °C for 2.5 min	Turkey	–	132.9 (59%)	Tosun and Yücecan (2008)
	Boiled	25 min	USA	83 (107%)	100 (76%)	Vanderslice et al. (1990)
	Baked	240 °C for 1 h	USA	97 (126%)	240 (185%)	Vanderslice et al. (1990)

Spinach	Fresh		UK	–	70	Proteggente et al. (2002)
			USA	520	520	Vanderslice et al. (1990)
			Turkey	–	1108.7	Tosun and Yücecan (2008)
			Romania	–	353.7	Balan et al. (2016)
	Washed		Turkey	–	936.1 (85%)	Tosun and Yücecan (2008)
	Blanched	95 °C for 140 s	Turkey	–	766.8 (69%)	Tosun and Yücecan (2008)
	Boiled	1 min	US	196 (38%)	200 (38%)	Vanderslice et al. (1990)
		5 min	Romania		206.9 (58%)	Balan et al. (2016)
	Microwave	3–5 min	USA	483 (93%)	540 (104%)	Vanderslice et al. (1990)
	Frozen	Unknown	USA	220 (42%)	250 (48%)	Vanderslice et al. (1990)
		−18 °C for 3–6 months	Turkey	–	705.3 (64%)	Tosun and Yücecan (2008)
Indian Spinach	Fresh		Nigeria	–	224	Babalola et al. (2010)
	Sun dried	Drying under the sun until crispy texture achieved	Nigeria	–	208 (93%)	
Water leaf	Fresh		Nigeria	5470[b]	–	Adefegha and Oboh (2011)
	Steamed	10 min	Nigeria	1982[b] (36%)	–	
Wild basil	Fresh		Nigeria	8420[b]	–	Adefegha and Oboh (2011)
	Steamed	10 min	Nigeria	6384[b] (76%)	–	

(continued)

Table 2 (continued)

Vegetables	Fresh/processing	Processing parameter	Country of the study conducted	Ascorbic acid (retention %)[a]	Total vitamin C (retention %)	Reference
				mg/kg fresh weight unless specified		
Ewuro-odo	Fresh		Nigeria	–	522	Oboh (2005)
	Boiled	1:1 food/water for 5 min	Nigeria	–	169 (32%)	
Efinrin	Fresh		Nigeria	–	520	Oboh (2005)
	Boiled	1:1 food/water for 5 min	Nigeria	–	273 (52%)	
Water cress	Fresh		Portugal	367	–	Gonçalves et al. (2009)
	Blanched	95 °C, for 20 s	Portugal	406	–	
	Frozen	Frozen at −40 °C until the food reach −25 °C, then stored at −7 °C for 400 days	Portugal	20 (5%)	–	
		Frozen at −40 °C until the food reach −25 °C, then stored at −15 °C for 400 days		28 (7%)	–	
		Frozen at −40 °C until the food reach −25 °C, then stored at −30 °C for 400 days		97 (24%)	–	

HPP high pressure processed, *PEF* pulsed electric field treated
[a]Data in bracket represent the value of the retention of ascorbic acid/total vitamin C after processing
[b]Data were presented as mg of ascorbic acid/vitamin C per kg of dried samples

During processing, the oxidation of biological-active L-ascorbic acid (L-AA) can be enhanced by enzyme catalysis, with ascorbic acid oxidase (AAO, EC 1.10.3.3) and ascorbic acid peroxidase (APX, EC 1.11.1.11) being the main families of enzymes involved in L-AA oxidation (Hancock and Viola 2005). AAO catalyses the oxidation of L-AA in the presence of oxygen (De Tullio et al. 2007), while APX catalyses the reduction of hydrogen peroxide with L-AA functioning as a cofactor (Noctor and Foyer 1998). Both enzymatic reactions result in the transfer of an electron from L-AA, leading in the formation of the partially oxidised monodehydro-L-ascorbic acid (MDHA) radical. MDHA then disproportionates spontaneously to form the fully oxidised molecular species dehydro-L-ascorbic acid (DHA). DHA is relatively unstable and can rapidly undergo irreversible hydrolysis to form 2,3-diketo-L-gulonic acid (2,3-DKG), with a consequent loss of potential biological activity. Therefore, it is important to minimise and prevent the oxidation of L-AA to DHA during food processing through efficient inactivation of both AAO and APX.

3.1 Heat Treatment

Heat treatment is the most widely applied food processing technology. Heat treatment helps to ensure long-term storage and stabilises some of the quality attributes of fresh fruit and vegetable products, by killing microorganisms and through the inactivation of endogenous enzymes. Since ascorbic acid is known to be the most labile and temperature-sensitive vitamin (Lee and Kader 2000), it is necessary to optimise any heat treatment conditions to sufficiently inactivate ascorbic acid degrading enzymes while ensuring optimum L-AA retention in plant-based foods. Conventional heat treatments are used for a wide range of fruit and vegetables and include domestic cooking, blanching, and drying.

3.1.1 Cooking

Domestic cooking can result in the loss of ascorbic acid, the extent of which depends upon the cooking method used. A study on the effect of three common cooking methods (boiling, steaming, and frying) on carrots and broccoli showed that frying caused the greatest loss of ascorbic acid (>80%), while boiling and steaming resulted in losses of up to 10% and 40% ascorbic acid, respectively (Miglio et al. 2007). With respect to boiling, reducing the volume of cooking water by half can minimise the amount of ascorbic acid leaching out of the plant produce (Podsędek et al. 2008), and reducing the cooking time results in better preservation of ascorbic acid (Castro et al. 2008; Severini et al. 2016). Steaming has been recognised as the gentlest cooking method and leads to minimal ascorbic acid loss in most vegetables, e.g. broccoli (Severini et al. 2016), cabbage (Podsędek et al. 2008), and tropical green leafy vegetables (Adefegha and Oboh 2011).

Microwave ovens have gained popularity for domestic cooking as they provide convenience, with more homogeneous heating (a greater penetration depth) and shorter cooking times than conventional ovens (Chandrasekaran et al. 2013). However, the impact of microwave cooking on plant produce has been shown to be variable. Microwaved peppers and apples retain more ascorbic acid than stir-fried or boiled peppers (Chuah et al. 2008; Zhang and Zhang 2014), while in contrast microwave cooking resulted in less ascorbic acid retention than conventional cooking for broccoli, cauliflower, and Brussels sprouts with the loss of 98% of the ascorbic acid found in raw produce (Pellegrini et al. 2010). This effect was attributed to water loss during microwave heating that resulted in the concurrent loss of water-soluble nutrients, including ascorbic acid (Pellegrini et al. 2010).

3.1.2 Blanching

Blanching, exposure to boiling hot water for a short time, is a relatively mild heat treatment often used during the processing of fruits and vegetables. Blanching is highly effective at inactivating enzymes and is commonly used as a step in the processing line for the production of frozen, and canned fruit and vegetable products. Blanching can greatly reduce (by at least 50%) the ascorbic acid content of various fruits and vegetables (Tables 1 and 2), including strawberries (Patras et al. 2009a), broccoli (Severini et al. 2016), and several green leafy vegetables (Oboh 2005). The lower levels of ascorbic acid and total vitamin C in fruits and vegetables after blanching is predominantly due to thermal degradation of temperature-sensitive ascorbic acid, but can also be due to leaching of ascorbic acid from the plant tissues into the blanching medium. Ascorbic acid losses can be minimised by blanching with steam instead of immersion in hot water (Severini et al. 2016), or by microwave blanching (Vadivambal and Jayas 2007).

By carefully optimising both temperature and blanching time, it is possible to better preserve the ascorbic acid contents of a wide range of fruit and vegetables, e.g. optimal conditions for maximal retention of ascorbic acid in red peppers are 70 °C for 1 min (Castro et al. 2008), carrots are 95 °C for 5 min (Shivhare et al. 2009), and Brussels sprouts are 100 °C for 1 min (Vina et al. 2007). Blanching fruits and vegetables at optimised temperature and time combinations is very effective at inactivating L-AA oxidising enzymes, e.g. AAO, and minimises the thermal degradation of ascorbic acid and total vitamin C (Munyaka et al. 2010; Wawire et al. 2011; Leong and Oey 2012a). In addition, optimised blanching treatments can be used to inactivate other enzymes, such as peroxidases and catalases, which can negatively influence product quality (Shivhare et al. 2009).

3.1.3 Drying

Dehydrated fruits and vegetables can be produced by drying fresh produce under the sun or by using a hot air dryer, or conventional or microwave oven (Santos and Silva 2008). As fruit and vegetables have high water contents (80% or more) and

when fully hydrated are metabolically active, removal of most of this water stops metabolic activity, extends shelf life, and can reduce storage space and packaging costs. Drying can also be used to produce products with a crispy texture. However, many nutrients are sensitive to heat, light, and oxygen, and experience oxidative degradation and heat damage during the dehydration process. Drying at high temperatures, for a long time, with constant exposure to oxygen causes degradation of ascorbic acid in fruit and vegetables (Sablani 2006). However, degradation can be reduced by optimising the drying temperature, duration of drying and airflow (Santos and Silva 2008).

For blueberries (López et al. 2010), tomatoes (Zanoni et al. 1998), and red bell peppers (Di Scala and Crapiste 2008), slow drying results in more ascorbic acid degradation than rapid drying. Ascorbic acid oxidation during drying can also be minimised by pre-soaking fruit or vegetables in a concentrated sugar or salt solution. This drying method is known as osmotic dehydration and has been widely used for processing fruit, as it helps to prevent oxygen penetrating into the tissues during drying (Torreggiani and Bertolo 2001). Vacuum drying is a method that can be used to reduce oxidation of nutrients as drying is conducted in a reduced oxygen environment. Vacuum drying has been shown to prevent ascorbic acid loss in apple slices dried for 24 h at 20 °C (Joshi et al. 2011). Pre-drying treatments, such as blanching or freezing, of fresh produce prior to drying can be used to inactivate oxidative enzymes and can also help to minimise ascorbic acid losses during drying (Lewicki 1998).

3.2 Freezing

Freezing can delay the enzymatic reactions that affect the stability of nutrients and is well suited for the long-term storage of many fruits and vegetables. In general, the freezing process has only a slight effect on total vitamin C levels in fruit and vegetables, with losses dependent upon the freezing rate. Most total vitamin C losses in frozen fruit and vegetables occur during prolonged storage and during thawing, due to oxidative breakdown. Factors that influence breakdown include storage duration, temperature fluctuations during storage, thawing rate, and thawing method. For example, frozen raspberry fruits stored for 180 days at −20 °C retain only 40% of initial ascorbic acid levels (de Ancos et al. 2000). The rate of ascorbic acid and total vitamin C loss is often dependent upon the fruit or vegetable, e.g. leafy vegetables such as spinach that have large surface areas and lose more ascorbic acid than frozen green peas and okra that have lower surface area to volume ratios (Giannakourou and Taoukis 2003). To improve ascorbic acid retention in frozen products, destined for long-term storage, a pre-blanching step is often used prior to freezing to inactivate oxidative enzymes and ensure better preservation of nutrients. Blanching watercress for 20 s at 95 °C prior to freezing and storage (400 days, at temperatures of −7, −15, and −30 °C) has been reported to stabilise ascorbic acid levels (Gonçalves et al. 2009).

3.2.1 Freeze-Drying

Dehydration by freeze-drying is widely used for fruit and in contrast to drying at elevated temperatures is based upon the direct phase transition of ice to water vapour (sublimation), without an intermediate liquid phase. Due to the lack of a liquid phase and the low temperatures required for the drying process, undesirable enzymatic reactions can be avoided and hence the ascorbic acid contents of freeze-dried fruit are generally higher than in heat dried fruit. Previous studies have shown that freeze-dried tomatoes (Chang et al. 2006) and summer fruits (Leong and Oey 2012b) have ascorbic acid levels similar to those found in fresh fruit.

4 The Potential for Novel Processing Techniques to Maintain Ascorbic Acid Levels in Plant-Based Foods

Recently interest in adopting novel food processing technologies for the preservation of fruit and vegetables has increased due to high consumer demand for healthy, safe foods, without preservatives and additives, and with properties more similar to those of fresh produce. The wider use of non-thermal food processing techniques could enable increased production of healthy and safe plant-based foods high in nutrients. Non-thermal processing technologies such as PEF and HPP have been used on various fruit and vegetables, and have been shown to improve the retention of nutrients, including biologically active ascorbic acid.

4.1 Pulsed Electric Fields Processing

PEF processing involves the application of short electric pulses, typically in the range of micro- to milli-seconds, of high voltage across a food product (preferably in semi-solid or liquid form) placed between two conducting electrodes. This process induces cell electroporation and the formation of permanent holes in cell membranes, thus increasing cell permeability and allowing the leakage of the cell contents to take place (Knorr et al. 1994). This can improve the release of intracellular compounds, especially in plant-based foods.

With respect to ascorbic acid, it has been shown that PEF (electric field strength of 0.4 kV/cm for 450 rectangular pulses) facilitated aqueous extraction of fennel bulbs produced extracts containing more ascorbic acid than extracts obtained by thermal extraction at 90 °C (El-Belghiti et al. 2008). In addition to improving extraction, PEF can also be used as an alternative to conventional pre-treatment for the production of plant-based foods high in ascorbic acid. PEF pre-treatment (electric field strength of 2 kV/cm and 400 μs pulse width) of red bell peppers was found to be an effective replacement for a freezing pre-treatment step, enhancing the efficiency of the subsequent osmotic dehydration process and promoting a faster rate of water loss from the peppers during convective air drying (Ade-Omowaye et al.

2003). PEF pre-treated peppers showed a 11–24% loss in ascorbic acid after convective drying while a freezing pre-treatment resulted in a 24% loss in ascorbic acid, compared to fresh samples.

Other important feature of PEF, when applied at high electric field strengths (E = 25–40 kV/cm), is the ability to inactivate microbes and also to achieve at least an 80–90% inactivation of undesirable oxidative enzymes. For these reasons, the use of PEF as a potential preservation technology to replace conventional thermal treatments for the production of fruit and vegetable juices has been investigated. Studies on oranges (Elez-Martínez and Martín-Belloso 2007; Sánchez-Moreno et al. 2005), tomatoes (Odriozola-Serrano et al. 2007), and watermelon (Oms-Oliu et al. 2009) have shown that PEF can minimise the degradation of ascorbic acid and maintain up to 98% of the total vitamin C content of fresh juice. In addition, the PEF-treated juices have shelf lives of between 30 and 70 days at 4 °C without substantial depletion of ascorbic acid.

One plausible explanation for the better retention of ascorbic acid in PEF-treated produce is the inactivation of ascorbic acid degrading enzymes. Recent work on carrots showed that carrot AAO was susceptible to PEF treatment (Leong et al. 2015). In this respect, future research should consider optimising appropriate combinations of PEF processing parameters (electric field strength and specific energy input) for a wide range of plant produce in order to reduce the adverse effect of AAO, while maintaining the stability and improving the retention of ascorbic acid. Overall, there is strong evidence to indicate that PEF has the potential to help maintain high ascorbic acid levels in plant-based foods/juices and has numerous potential applications for plant-based food products.

4.2 High Hydrostatic Pressure Processing

HPP involves the application of hydrostatic pressures above 100 MPa at ambient temperatures to inactivate microbes and inhibit the activities of oxidative enzymes, while retaining the inherent quality attributes of the food material (Oey et al. 2008). Food products, in the form of liquids or semi-solids, are pre-packed and loaded into a HPP chamber vessel and the vessel is then closed and filled with a pressure-transmitting medium such as water or food-grade solutions (e.g. castor oil, silicone oil, sodium benzoate, ethanol, and glycol). The food products are then held inside the vessel under pressure for a predefined duration, then the system is depressurised, the vessel is opened, and the food products are unloaded (Tao et al. 2014).

HPP has considerable potential for the preservation of high quality plant-based foods, either in the form of pulps, purees, or juices, and could replace conventional thermal treatments (e.g. blanching or pasteurisation). Previous studies have demonstrated that HPP (applied between 400 and 600 MPa for 5–10 min) can improve the retention of ascorbic acid in apple juice (Kim et al. 2012), guava puree (Yen and Lin 1996), strawberry and blackberry purees (Patras et al. 2009a, b), and tomato puree (Patras et al. 2009b), compared to thermally processed products. In general, HPP-processed food products have similar ascorbic acid contents to that of their fresh or

untreated counterparts, which is thought to be mostly due to the fact that HPP food products are not exposed to high temperatures during HPP processing. HPP-processed food products have also demonstrated better ascorbic acid stability during refrigerated storage, particularly at lower storage temperatures (Tewari et al. 2017). The shelf life of refrigerated (4 °C) HPP-treated juice can be extended up to 28 days for tomato juice (Hsu et al. 2008), up to 35 days for pomegranate juice (Varela-Santos et al. 2012), up to 56 days for blueberry juice (Barba et al. 2012), and up to 60 days for grape juice (Daoudi et al. 2002), without substantial loss of ascorbic acid. It is, however, important to note that some HPP plant materials may show a slight enhancement of ascorbic acid degradation, under both aerobic and anaerobic conditions (Tewari et al. 2017). This is possibly caused by the presence of endogenous pro-oxidants, such as metal ions and ascorbic acid degrading enzymes, in the food matrix.

During HPP, the pressure applied to foods is usually instantaneously and uniformly distributed, and so the size and shape of food products are not greatly affected, unless the product has a very porous structure (Oey et al. 2008). HPP also increases cell permeability and hence the movement of molecules, including water, out of plant cells. HPP can therefore improve the process of osmotic dehydration by increasing the rate of cellular water loss and sugar uptake in HPP-treated plant materials, compared to other pre-treatments. Freezing or blanching is often used in the food industry to produce dehydrated frozen food products including apple slices. However, apple slices pre-treated with HPP (400 MPa for 10 min) had a higher ascorbic acid content, firmer texture, and brighter colour than slices produced using freezing or blanching pre-treatments (Taiwo et al. 2001).

It is clear that HPP can be a suitable alternative to thermal processing to prolong the shelf life while improving the retention of ascorbic acid in plant-based foods and has now been adopted as a cold-pasteurisation technique by several food companies in Europe and the USA to preserve fruit juices.

5 Conclusions and Future Perspectives

We live in a world where an increasing global population is set against a background of climate change, and where difficulties associated with sustainable food production and distribution mean that eliminating food waste is becoming increasingly important. Efficient, low energy food processing technologies that can be used to preserve fruit and vegetables and produce plant-based foods of consistent quality, while maintaining their nutritional value, are becoming increasingly important. An adequate daily intake of ascorbic acid is a requirement for a healthy diet and while a diet high in fresh fruit and vegetables is ideal, increasingly humans are relying on processed plant products in their daily diets to supply their nutritional needs. Greater use of novel, energy efficient non-thermal processing technologies, such as PEF and HPP, has the potential to aid in the preservation of biologically active ascorbic acid in plant-based foods. However, as fresh plant produce is highly variable and hence

the raw materials used to produce processed plant-based foods are also highly variable, much more work is required to understand how non-thermal processing technologies change the raw plant material used to produce plant-based foods, and how any changes might affect the nutritional and storage attributes of the final product. The development and optimisation of low cost and energy efficient processing methods that retain high levels of key nutrients, such as ascorbic acid, and can be used for a wide range of plant-based food products are critical areas for future research in order to help ensure global food security in the future.

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Ascorbate Metabolism and Nitrogen Fixation in Legumes

Manuel A. Matamoros, David A. Dalton, and Manuel Becana

Abstract Ascorbic acid (AsA) is a major antioxidant and redox buffer in plants that acts as a direct scavenger of reactive oxygen species (ROS), substrate of ascorbate peroxidase (APX), and cofactor of key enzymes involved in cell wall expansion and hormone biosynthesis. N_2-fixing root nodules of legumes contain high levels of AsA (1–2 mM), APX (0.9% of the total soluble protein), and the other enzymes of the AsA-glutathione pathway. The AsA content and their associated enzyme activities are strongly correlated with N_2 fixation, providing adequate protection of nodules against ROS generated by, among other processes, the oxidation of nitrogenase and leghemoglobin. These antioxidant defenses are concentrated in the infected zone and in the endodermis/nodule parenchyma, where they may be part of the O_2 diffusion barrier that restricts entry of O_2 into the nodule interior to avoid nitrogenase inactivation. AsA can enhance N_2 fixation fourfold to fivefold when supplied by stem infusion to soybean plants or when added, along with APX, to an in vitro reconstitution system. GDP-D-mannose 3,5-epimerase (GME) and GDP-L-galactose phosphorylase (GGP) catalyze critical limiting steps in AsA biosynthesis. Consequently, studies are underway to produce transgenic N_2-fixing plants that overexpress GME and GGP. Preliminary results have indicated that these plants have higher rates of N_2 fixation but with a metabolic cost, resulting in slightly smaller plants. Additionally, since AsA oxidation and decline are early symptoms of nodule aging, a high AsA content might delay nodule senescence and extend the period of N_2 fixation.

Keywords Ascorbate biosynthesis · Ascorbate peroxidase · Leghemoglobin · Nitrogen fixation · Nodules · Oxygen diffusion barrier · Symbiosis

M. A. Matamoros · M. Becana (✉)
Estación Experimental de Aula Dei, CSIC, Zaragoza, Spain
e-mail: becana@eead.csic.es

D. A. Dalton
Biology Department, Reed College, Portland, OR, USA

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1 Introduction

All organisms produce reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide (H_2O_2), as well as reactive nitrogen species (RNS), such as nitric oxide (NO) and nitrosothiols. Antioxidants keep the concentrations of ROS and RNS under tight control to allow intracellular redox signaling while preventing nitro-oxidative damage of vital cellular components.

Ascorbic acid (AsA) or vitamin C is a major water-soluble antioxidant and redox buffer in plant and animal cells. At physiological pH, AsA is mostly found (>99.5%) as ascorbate, the deprotonated form. Humans and some other primates, bats, and guinea pigs cannot synthesize AsA and need to incorporate it in their diet. In humans, diets deficient in AsA cause scurvy, evidenced by gum swelling and severe joint pain, among other symptoms, due to incorrect collagen hydroxylation (Myllylä et al. 1978).

In plants, AsA performs a dual function as antioxidant, by directly scavenging ROS (Buettner and Jurkiewicz 1996) and by being the substrate of ascorbate peroxidase (APX), a critical enzyme of the AsA-glutathione or Foyer-Halliwell-Asada pathway for H_2O_2 detoxification (Foyer and Noctor 2011). This pathway is fully operative in leaves, roots, and nodules (Noctor and Foyer 1998; Dalton et al. 1986). Although the infected region of N_2-fixing legume nodules is nearly anaerobic, there is still potential for ROS production by the oxidation of leghemoglobin (Lb), ferredoxin, and nitrogenase, all of them essential proteins for N_2 fixation (Becana et al. 2010). Consequently, AsA and the associated antioxidant enzymes are particularly crucial for proper functioning of nodules. Several studies strongly suggest that increasing AsA in nodules can result in increased N_2 fixation and delayed senescence. The stage is set for ongoing studies to produce transgenic plants with elevated AsA in hopes of generating plants with enhanced N_2 fixation and tolerance of abiotic stresses.

2 Overall Roles of AsA in Plants

The antioxidant actions of AsA stems from its ability to directly scavenge ROS and from its pivotal role in the AsA-glutathione pathway. In this pathway, four enzymes participate in concert to reduce H_2O_2 to water by using, ultimately, NADH and NADPH as electron donors. APX catalyzes the reduction of H_2O_2 by AsA, which is oxidized to monodehydroascorbate (MDHA) and dehydroascorbate (DHA). These are reduced back to AsA by NADH-dependent monodehydroascorbate reductase (MDHAR) and glutathione-dependent dehydroascorbate reductase (DHAR). In turn, reduced glutathione is regenerated by NADPH-dependent glutathione reductase. In legumes, homoglutathione (γGlu-Cys-βAla) may partially or completely replace glutathione (γGlu-Cys-Gly) as reductant for DHAR (Becana et al. 2010). Besides its antioxidant functions, AsA plays a critical metabolic role as cofactor of

numerous enzymes, including violaxanthin de-epoxidase, prolyl-hydroxylase, and dioxygenases involved in photoprotection, cell wall glycoprotein hydroxylation, and in ethylene, gibberellin, and abscisic acid biosynthesis (Arrigoni and De Tullio 2002; Müller-Moulé et al. 2003).

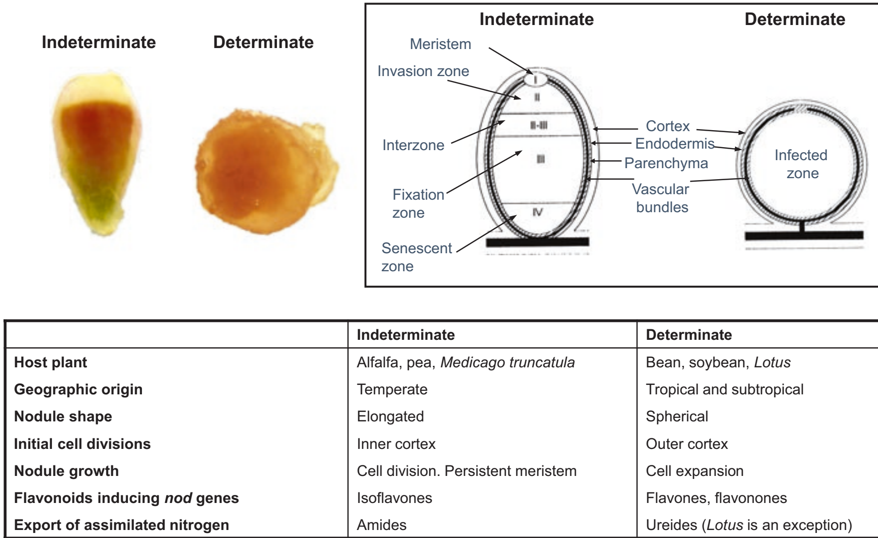
The functions of AsA in plants have been investigated using *vitamin C (vtc)* mutants of *Arabidopsis thaliana* that contain only 10–30% of the AsA levels of wild-type plants (chapter "Chemistry and Metabolism of Ascorbic Acid in Plants"). Such experiments have demonstrated that AsA is essential for normal growth and development (Veljovic-Jovanovic et al. 2001; Dowdle et al. 2007). The *vtc1* mutants, deficient in GDP-mannose pyrophosphorylase (VTC1/GMP), are more sensitive to ozone (Conklin et al. 1997) and salt stress (Huang et al. 2005). On the other hand, the *vtc2* mutants, deficient in GDP-L-galactose phosphorylase (VTC2/GGP), show reduced capacity to dissipate as heat the excess of absorbed light energy (Müller-Moulé et al. 2003). The low-AsA mutants also show up-regulation of defense genes and are more resistant to bacterial and fungal pathogens (Pastori et al. 2003; Barth et al. 2004; Pavet et al. 2005). In tomato plants, silencing of the gene encoding the last enzyme of AsA biosynthesis, mitochondrial L-galactono-1,4-lactone dehydrogenase (GalLDH), caused a strong reduction in leaf and fruit size due to diminished cell expansion (Alhagdow et al. 2007).

As could be anticipated, the enzymes that regenerate AsA are also important in the response to abiotic stress. An *A. thaliana* mutant in cytosolic DHAR showed lower AsA/DHA ratio and exhibited increased ozone sensitivity (Yoshida et al. 2006). Tobacco plants overexpressing MDHAR or DHAR in the cytosol showed higher levels of reduced AsA and enhanced stress tolerance (Eltayeb et al. 2006, 2007). Similarly, tomato seedlings overexpressing chloroplastic MDHAR showed increased resistance to low or high temperatures, whereas antisense transgenic lines were more sensitive to the same stress conditions (Li et al. 2010).

The alterations in growth, development, and stress responses observed in AsA-deficient plants are probably due, at least partially, to modifications in redox-dependent signaling pathways. In this regard, several studies suggest that AsA is part of a complex network that modulates hormone signaling (Pastori et al. 2003; Barth et al. 2004; Kerchev et al. 2011). In addition to chloroplasts, AsA has been found in the cytosol, nuclei, peroxisomes, and mitochondria (Jiménez et al. 1997; Zechmann 2011), and it may therefore contribute to the control of H_2O_2 concentration and redox state in different cellular sites, thereby influencing cell signaling and gene expression.

3 General Introduction to Biological Fixation

Leguminous plants are a major protein source for animal and human nutrition. In addition, crop legumes are able to establish N_2-fixing symbioses with soil bacteria (rhizobia) that contribute fixed nitrogen to the soil (Udvardi and Poole 2013). N_2 fixation occurs within nodules, a unique organ formed mainly on roots as a result of



	Indeterminate	Determinate
Host plant	Alfalfa, pea, *Medicago truncatula*	Bean, soybean, *Lotus*
Geographic origin	Temperate	Tropical and subtropical
Nodule shape	Elongated	Spherical
Initial cell divisions	Inner cortex	Outer cortex
Nodule growth	Cell division. Persistent meristem	Cell expansion
Flavonoids inducing *nod* genes	Isoflavones	Flavones, flavonones
Export of assimilated nitrogen	Amides	Ureides (*Lotus* is an exception)

Fig. 1 Comparison of indeterminate and determinate nodules. The figure shows the major structural and biochemical differences between the two types of nodules. In the photographs, note the red color in the fixation (III) zone of the indeterminate nodule and in the infected zone of the determinate nodule, which is due to the high concentration of Lb. Also note the green color in the senescent (IV) zone of the indeterminate nodule, indicative of Lb degradation to biliverdin-like pigments

a complex molecular dialogue between the plant (macrosymbiont) and the bacteria (microsymbiont) (Oldroyd 2013). In nitrogen-poor soils, legume roots secrete flavonoids that induce the synthesis of lipochitooligosaccharides known as nodulation (Nod) factors. These interact with specific receptors in the plasma membrane of root hair cells triggering a signaling pathway that sequentially involves oscillations in intracellular calcium levels, activation of protein kinases and transcription factors, changes of gene expression, and cell differentiation. The outcome is the formation of a new meristem in the root cortical cells, which are colonized through infection threads. Bacteria are released from the infection threads into single membrane-bound compartments inside the nodule cells, the symbiosomes, where the bacteria differentiate into N_2-fixing bacteroids. According to the growth pattern, two types of nodules can be distinguished (Fig. 1). For terminology of nodule structure, we will follow Vasse et al. (1990) for indeterminate nodules and Minchin et al. (2008) for determinate nodules.

Indeterminate nodules, such as those of pea (*Pisum sativum*), clover (*Trifolium repens*), and the model legume *Medicago truncatula*, have persistent meristems, show a longitudinal gradient of age, and are usually cylindrical. Generally, four zones can be distinguished from the distal to the proximal (closest to the root) region: I (meristem), II (invasion), III (N_2-fixing), and IV (senescent). In old nodules, an additional zone (V) can be observed adjacent to zone IV which contains undifferentiated bacteria. These nodules have, from exterior to interior, an outer

cortex, endodermis, inner cortex (nodule parenchyma) with vascular bundles, and infected zone. Determinate nodules, such as those of bean (*Phaseolus vulgaris*), soybean (*Glycine max*), and the model legume *Lotus japonicus*, do not have persistent meristems or tissues at different developmental stages. They are usually spherical and have a multi-layered cortex and a central infected zone. The cortex of soybean nodules, the best studied, comprises: outer cortex, common endodermis (scleroid layer), mid cortex, and inner cortex (nodule parenchyma). The nodule parenchyma, in turn, is formed by a boundary layer with no apparent intercellular spaces and a distribution zone with small cells and large intercellular spaces. The two types of nodules also differ in biochemical terms (for details on the structure and function of both types of nodules, see Minchin et al. 2008 and Dupont et al. 2012; Fig. 1). For example, indeterminate nodules produce amides (glutamine and asparagine) as nitrogen compounds that are exported to the shoot, whereas determinate nodules produce ureides (allantoin and allantoic acid). There are exceptions to this as *L. japonicus* is an amide exporter.

The plant provides photosynthates to the nodules, mostly sucrose, through the phloem. In the cytoplasm of host cells, sucrose is oxidized to malate and other dicarboxylic acids which are then used up by the bacteroids to sustain N_2 fixation (Udvardi and Poole 2013). This process is catalyzed by a complex of two enzymes, dinitrogenase reductase and dinitrogenase, that fixes N_2 into ammonia. The reaction requires large amounts of ATP and reducing power as well as nearly anaerobic conditions because the enzymes are irreversibly inactivated by O_2. Consequently, mechanisms exist in nodules that strictly control O_2 concentration so as to allow high rates of bacteroid respiration while avoiding oxidation of nitrogenase components. Two major O_2 regulating mechanisms are Lb, a monomeric hemeprotein similar to animal myoglobin that transports O_2 at a steady but low concentration in the cytosol of infected cells (Appleby 1984), and an O_2-diffusion barrier (ODB) localized in the nodule parenchyma which limits the flux of O_2 into the infected zone (Witty et al. 1986). The resulting ammonia is released into the infected cell cytosol, assimilated into carbon compounds by glutamine synthetase and glutamate synthase, and exported to the shoot in the form of amides or ureides. Thus, in simple terms, the legume-rhizobia symbiosis consists of an exchange of carbon and nitrogen between the plant and the bacteria.

4 Production of ROS and RNS in Nodules

In plant cells, respiration, photosynthesis, and other metabolic reactions involving electron transfer may generate ROS such as superoxide radicals and H_2O_2. Two additional, more powerful oxidizing ROS are the hydroxyl radicals and singlet oxygen, which can originate through the Fe-catalyzed Fenton reaction and energy transfer reactions, respectively (Halliwell 2006). Both ROS can readily react with lipids, proteins, and DNA causing extensive damage to membranes and other cellular components. Under physiological conditions, ROS are maintained at low steady-state

concentrations by antioxidant enzymes and metabolites, allowing them to perform signaling functions (Choudhury et al. 2017). In contrast, under adverse conditions and during senescence, the balance between ROS production and antioxidant defenses may be lost, leading to alterations in redox homeostasis, oxidation of cellular components, and, ultimately, plant death. This situation is known as oxidative stress and is manifested by the accumulation of lipid peroxides, carbonylated proteins, and oxidized DNA bases (Pisoschi and Pop 2015). Similarly, RNS such as NO carry out multiple functions in plants, including stress perception and signaling, but the uncontrolled production of NO and NO-derived RNS like peroxynitrite leads to nitrosative stress, characterized by indiscriminate nitrosylation and nitration of proteins and other molecules. Thus, the frontier between oxidative/nitrosative signaling (beneficial situation) and oxidative/nitrosative stress (detrimental situation) seems to be a tenuous one.

The establishment of the legume-rhizobia symbiosis and nodule functioning require finely tuned concentrations of ROS and RNS. Transient increases in both types of reactive molecules have been detected within minutes or hours after perception of Nod factors by the plant at the very early stage of symbiosis (Puppo et al. 2013). Also, superoxide and H_2O_2 have been detected within the infection threads and infected cells of nodules (Santos et al. 2001; Alesandrini et al. 2003; Rubio et al. 2004). Superoxide can be produced by membrane-localized NADPH oxidases (Marino et al. 2011; Arthikala et al. 2014), the autoxidation of oxygenated Lb, and the electron transport chains of bacteroids and mitochondria. Superoxide radicals can then dismutate to H_2O_2 either spontaneously or through a reaction catalyzed by superoxide dismutases.

Likewise, RNS, and especially NO, may arise from different sources. In the nodule host cells, these include a NO synthase-like protein (Cueto et al. 1996) and nitrate reductase activity in combination with the mitochondrial electron transport chain under hypoxic conditions (Horchani et al. 2011). In the bacteroids, NO is formed in the denitrification pathway (Meakin et al. 2007; Sánchez et al. 2010; Horchani et al. 2011) and perhaps also through a bacterial NO synthase-like activity (Meilhoc et al. 2011).

5 AsA and Associated Antioxidant Enzymes in Nodules

Dalton et al. (1986) were the first to report in legume nodules the presence of the AsA-glutathione pathway, as well as high concentrations of AsA (1–2 mM) and APX (0.9% of the total soluble protein). Depending on the legume species, this AsA concentration is 15–40% of that observed in the leaves and slightly greater than that in the roots (Matamoros et al. 2006). However, AsA is not uniformly distributed within the nodule. Antioxidant concentrations are expected to be higher in regions prone to ROS production. In nodules, such conditions are found in the infected cells and in some peripheral cell layers in the cortex. In determinate nodules, the presence of an ODB in the nodule parenchyma physically restricts movement of O_2 into the

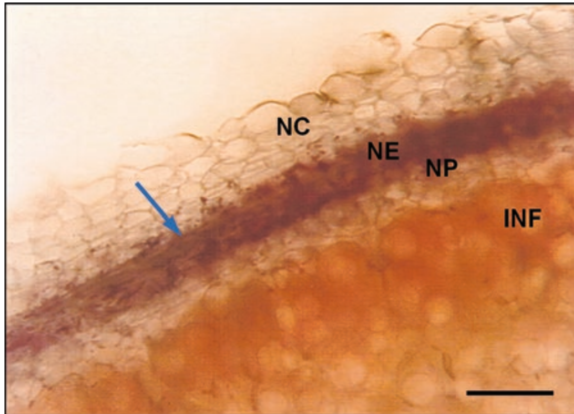


Fig. 2 Localization of respiratory dehydrogenase activity in nodules. The activity, attributable to electrons leaking from the mitochondrial electron transport chain, is localized in the endodermis and parenchyma region *(blue arrow)* of alfalfa nodules. *NC* nodule cortex, *NE* nodule endodermis, *NP* nodule parenchyma, *INF* infected zone. Bar, 100 µm. [Dalton et al. 1998; *printed with permission from American Society of Plant Biologists*]

interior such that the infected zone becomes nearly anaerobic. The anaerobic status is further enforced by elevated rates of respiratory O_2 consumption in the endodermis (indeterminate nodules) or parenchyma (determinate nodules), and indeed we have proposed that this may be an additional mechanism for restricting O_2 access to the central zone (Dalton et al. 1998). Histochemical stains and/or immunolocalization studies revealed that these regions have elevated respiratory dehydrogenase activity (Fig. 2) and high concentrations of AsA (Fig. 3a, b) and APX (Fig. 3c, d). Abundant antioxidants were also located in the infected zone even though the microenvironment here is nearly anaerobic. The central zone of some nodules does not only contain infected cells repleted of bacteroids but also uninfected (interstitial) cells. If the premise that antioxidants are critical to support N_2 fixation is correct, one would expect greater antioxidant levels in infected than in uninfected cells. This appears to be the case at least for APX mRNA for which in situ hybridization showed more intense labeling in the infected cells (Fig. 4). As occurs for AsA and APX, the (homo)glutathione concentration is also enhanced in the nodule parenchyma (Fig. 5), strongly suggesting that the AsA-glutathione pathway is very active in this nodule region and that it may be involved in the operation of the ODB. This would be in keeping with an early proposal that H_2O_2, whose concentration is controlled by the pathway, acts as a signal in the ODB (Minchin 1997).

The evidence from microscopy is supported by careful microdissection of nodules into cortical and infected tissue. This technique does not permit a fine-enough distinction between discrete cell layers such as endodermis and parenchyma, but it does provide a clear separation between peripheral *versus* central infected tissue. Concentrations of AsA and (homo)glutathione were ~44% higher in the peripheral layers of bean nodules (Dalton et al. 1998). The concentration of AsA in the

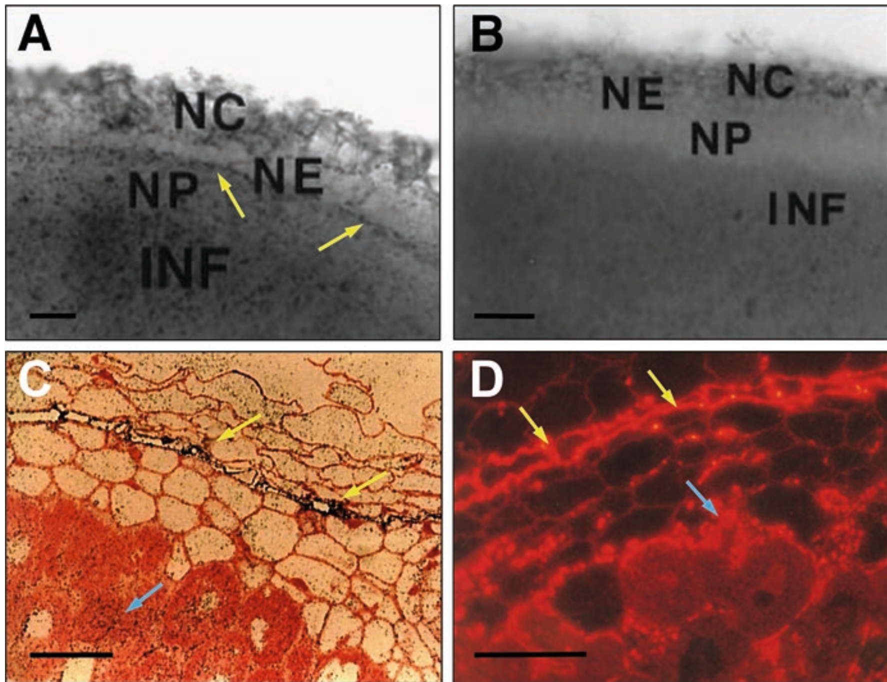


Fig. 3 Localization AsA and APX protein in nodules. (**a**) Histochemical localization of AsA in alfalfa nodules. The presence of high AsA concentrations in the nodule endodermis is marked by deposition of silver particles precipitated fron $AgNO_3$ *(yellow arrows)*. Abbreviations are as in Fig. 2. (**b**) Negative control for (**a**), in which the tissue was preincubated with buffer at pH 9.0 to oxidize the AsA before exposure to $AgNO_3$. (**c**) Immunolocalization of APX in alfalfa nodules. Typical section showing Ag particles (APX protein) concentrated in the infected region *(blue arrow)* and in the nodule endodermis *(yellow arrows)*. Abbreviations are as in Fig. 2. (**d**) Similar to (**c**), except that detection was based on Cy3 fluorescence. Bars, 100 µm. [Dalton et al. 1998; *printed with permission from American Society of Plant Biologists*]

periphery was estimated to be ~1–1.5 mM and that of (homo)glutathione ~0.5 mM. The microdissection studies also allowed a comparison of enzyme-specific activities in different regions of nodules. APX was not significantly different between periphery and infected regions, a misleading fact presumably brought about by the dilution of peripheral extracts with layers in the nodule cortex that are depleted in this enzyme. Even so, extracts from the periphery of bean nodules contained 57%, 77%, and 27% more MDHAR, DHAR, and glutathione reductase activities, respectively, than those present in the infected zone (Dalton et al. 1998). These authors proposed that elevated respiration rates in the endodermis and parenchyma contribute to the restriction of O_2-diffusion into the infected zone, thus protecting nitrogenase from inactivation. High levels of AsA and AsA-glutathione pathway enyzmes at the ODB might contribute to keep respiration-derived ROS at low concentrations. It is also relevant that ascorbate oxidase (AO), an enzyme that catalyzes the oxidation of AsA to DHA using O_2, is induced in nodules and

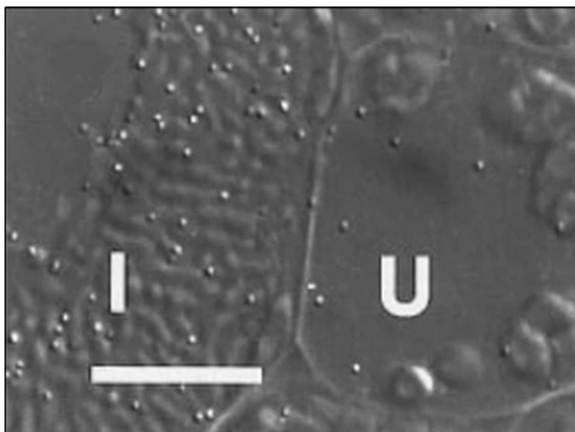


Fig. 4 In situ hybridization of APX mRNA in an alfalfa nodule. The image shows high levels of mRNA in an infected cell (I) and low levels in an adjacent uninfected (interestitial) cell (U). The label is evident as small bright dots. Other features include numerous bacteroids (nearly continuous bumps in I) and starch grains in U. Bars, 10 μm. [Dalton et al. 1998; *printed with permission from American Society of Plant Biologists*]

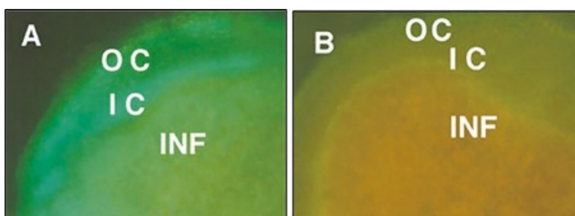


Fig. 5 Histochemical localization of (homo)glutathione in nodules. (**a**) Turquoise blue fluorescence in the parenchyma/inner cortex of a cowpea (*Vigna unguiculata*) nodule. The fluorescence is emitted by the adduct formed between homoglutathione or glutathione and monochlorobimane, a probe that reacts with the thiol groups of both tripeptides. (**b**) Negative control treated similarly except for the omission of monochlorobimane. *OC* outer cortex, *IC* inner cortex, *INF* infected zone. [Dalton et al. 1998; *printed with permission from American Society of Plant Biologists*]

mycorrhizal roots of *L. japonicus* (Balestrini et al. 2012). Notably, the AO mRNA and protein were found to be highly expressed in the intercellular spaces of the nodule parenchyma. These authors proposed that AO could participate in the ODB by directly scavenging O_2 without the production of intermediate ROS. Taken all these observations together, it is clear that the nodule parenchyma is closely linked to AsA and its associated enzymes.

The peripheral location of antioxidants was confirmed in a study that used fusion of the GUS reporter gene with the promoters of the glutathione synthetase and homoglutathione synthetase genes of *M. truncatula* (El Msehli et al. 2011). GUS expression from both reporter constructs was mainly observed in the vascular tissue and the cortex of nodules. Thus, (homo)glutathione appears to be concentrated in

the same region of the nodule as AsA and APX. Interestingly, the expression of γ-glutamylcysteine synthetase (γECS), which is the key regulatory enzyme in the (homo)glutathione biosynthetic pathway, was greater in the meristem area and adjacent developing infection zone.

The hypothesis that symbiotic N_2 fixation depends on antioxidants such as AsA and (homo)glutathione was strengthened by the observation that nodule effectiveness (the rate of N_2 fixation) is correlated with the concentration of antioxidants in nodules (Dalton et al. 1993). In this case, nodules from six effective legume-rhizobia pairings were compared with nodules from six ineffective pairings. All enzymes of the AsA-glutathione pathway were much elevated in the effective pairings, by as much as 5.5-fold in the case of glutathione reductase. In addition, levels of (homo) glutathione were 4.3-fold higher in effective nodules and the ratio of reduced to oxidized (homo)glutathione was 2.9-fold higher in effective nodules. However, no differences in AsA concentration were detected between the pairings.

Varying the concentration of free O_2 surrounding nodules has been reported to have significant effects on antioxidant defenses (Dalton et al. 1991). Higher concentrations of O_2 resulted in increases in the activity of the enzymes of the AsA-(homo) glutathione pathway as well as concentrations of AsA and (homo)glutathione. There was also a correlation with higher concentrations of O_2 leading to increased rates of N_2 fixation (acetylene reduction). These observations support the hypothesis that the supply of O_2 may be limiting to N_2 fixation.

The correlation between antioxidants and N_2 fixation was further confirmed in a study involving γECS (El Msehli et al. 2011). Down-regulation of the γECS gene by RNAi led to decreases in N_2 fixation as well as in levels of Lb, thiols, and thioredoxin (a protein that acts as an antioxidant by facilitating the reduction of other proteins). Overexpression of the γECS gene led to higher contents of (homo)glutathione, Lb, sucrose synthase, and thioredoxin, as well as higher rates of N_2 fixation.

6 Benefits of Exogenous AsA on N_2 Fixation

There are several studies that provide evidence for a direct link between AsA and N_2 fixation in legumes. For instance, foliar application of AsA led to a wide range of beneficial responses in N_2-fixing rhizobia-legume symbioses, including accelerated nodule formation and increases in nodule number (up to tenfold), nodule weight, AsA and Lb, as well as in the nitrogen content of nodules and other plant organs including leaves (Chinoy 1984). Also, the supply of AsA to plants increased yield up to sixfold and delayed nodule senescence. The application of exogenous AsA through root feeding of soybeans led to increases in root and nodule mass, number of nodules, rate of nodule development, and nitrogenase and APX activities (Bashor and Dalton 1999).

In contrast to these favorable responses to exogenous AsA, a similar treatment was found to have no benefit in the response of N_2-fixing pea nodules to water stress

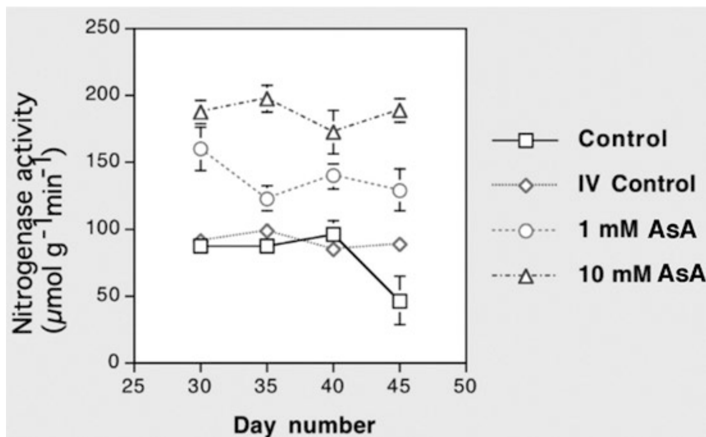


Fig. 6 Nitrogen-fixing activity (acetylene reduction to ethylene) by nodules from soybean plants receiving AsA by stem infusion. Control: no infusion; IV control, infusion with malate/phosphate buffer only; other treatments with concentration of AsA as shown in malate/phosphate buffer. Day number is days after planting. The stem infusion began on day 18 and was continuous through day 45. [Bashor and Dalton 1999; *printed with permission from John Wiley and Sons*]

(Zabalza et al. 2008). The application of exogenous AsA did not produce recovery from water stress as measured by nodule antioxidant enzymes, nodule carbon and nitrogen enzymes, or N_2 fixation. One reason for the lack of response may be that the AsA concentration (5 mM) used in this study may have been below the level necessary to provide benefits. In stem infusion treatments, effects were only observed with 10 mM AsA. Furthermore, the application of AsA to water-stressed plants paradoxically led to much lower foliar concentrations of AsA and glutathione compared to levels in control plants (Zabalza et al. 2008). Consequently, favorable responses to AsA-treatment would not be expected.

Even more profound effects were observed when AsA was provided to plants by stem infusion of a buffered solution of AsA. In this case, AsA led to increases of nitrogenase and APX activities and Lb content (Fig. 6) and to decreases of lipid peroxide levels (Bashor and Dalton 1999). Additional benefits included delayed senescence in which Lb content and nitrogenase activity were maintained through late developmental stages while both factors declined in control plants. This is consistent with the role of AsA in nodule senescence as discussed below.

Because N_2 fixation and other parameters of nodule function are enhanced by treatment with AsA, it is reasonable to suggest that antioxidants may be a limiting factor in N_2 fixation. Such conclusions are linked to the hypothesis that O_2 itself might be the most limiting factor for N_2 fixation (Witty et al. 1986; Hunt and Layzell 1993). O_2 might be limiting because a higher O_2 concentration would require enhanced antioxidants to deal with the increased production of ROS. These complementary hypotheses have implications towards improving N_2 fixation through genetic engineering as discussed later. In particular, enhanced antioxidants may facilitate or enable the entry of more O_2 into the nodule interior to support bacteroid respiration and thus higher rates of N_2 fixation.

AsA has also been shown to be highly beneficial to N_2 fixation in an in vitro reconstitution system (Ross et al. 1999). This system consisted of sealed vials containing the essential components of an intact nodule, namely: N_2-fixing rhizobia, Lb (or myoglobin), purified recombinant APX, and various concentrations of AsA (up to 2 mM) with a gas head space consisting of 3% O_2. The goal was to create a functional N_2-fixing system in which Lb or myoglobin supplies O_2 to meet the respiratory demands of the rhizobia. Just as in nodules, the system would be damaged by H_2O_2 produced by the autoxidation of Lb or myoglobin, hence the need for APX and AsA. Profound benefits were observed when AsA was provided at the highest concentration. These benefits included increased fractional oxygenation of myoglobin indicating that myoglobin remains in the ferrous form and binds O_2. Over the time course of the experiment (9 h), there was a 40% decline in the fractional oxygenation of myoglobin in the absence of AsA. In the presence of AsA, the myoglobin remained fully oxygenated. The inclusion of AsA and APX also resulted in a 4.5-fold increase in nitrogenase activity (acetylene reduction). Nitrogenase activity remained linear for 9 h whereas nitrogenase activity in the absence of AsA and APX was consistently lower and ceased after 6 h.

7 Metabolic Pathways for AsA Biosynthesis in Plants and Nodules

AsA in plants is synthesized primarily by the L-galactose (or Wheeler-Smirnoff) pathway (Wheeler et al. 1998) of which the two main regulatory steps are the conversion of GDP-L-galactose to L-galactose-1-P by GGP and the conversion of GDP-D-mannose to GDP-L-galactose by GDP-mannose 3,5-epimerase (GME). Both enzymes are regulated transcriptionally and GGP also at the translational level (Bulley and Laing 2016). Overexpression of the *GGP* gene leads to an increase of AsA concentrations up to 16-fold in diverse plant species, and co-overexpression of *GGP* and *GME* appears to further increase AsA at least in *A. thaliana* (Bulley and Laing 2016). These and other observations indicate that the route through L-galactose is the major pathway of AsA biosynthesis in plants.

Regarding the rhizobia-legume symbiosis, the high concentration of AsA found in nodules was initially attributed to import from the leaves through the phloem (Groten et al. 2005; Puppo et al. 2005) and proposed to modulate the content of cytosolic peroxiredoxin, a key antioxidant enzyme for H_2O_2 detoxification (Groten et al. 2006). The long-distance transport of AsA from the shoot is a plausible possibility and might account for a fraction of the AsA in nodules. However, many data indicate that nodules possess the necessary biochemical machinery to synthesize AsA from glucose through the L-galactose pathway. A gene coding for GalLDH was cloned and characterized in the model legume *L. japonicus*. The gene codes for a functional protein that is located at the inner membrane of nodule mitochondria and that is expressed in leaves and nodules at similar levels (Matamoros et al. 2006).

Table 1 Experimental evidence for the presence of the galactose pathway of AsA biosynthesis and its regulation in legume nodules

Gene[a]	Evidence in nodules	Regulation[b]	Conditions	References
PMM	Protein			Dam et al. (2014)
GMP/ VTC1	mRNA	T, PT (P)	Cd, aging	Loscos et al. (2008); Marx et al. (2016)
GME	mRNA/protein	T, PT (P, Ac)	Cd, aging	Loscos et al. (2008); Dam et al. (2014); Marx et al. (2016)
GGP/ VTC2	Protein	PT (G)		Matamoros et al. (2018)
GalPP	mRNA	T	Aging	Loscos et al. (2008); Dam et al. (2014)
GalDH	mRNA	T	Cd, aging	Loscos et al. (2008)
GalLDH	mRNA/ activity	T	Cd, H_2O_2, JA, aging	Loscos et al. (2008)

[a]Abbreviations: *PMI* phosphomannose isomerase, *PMM* phosphomannose mutase, *GMP* GDP-mannose pyrophosphorylase, *GME* GDP-mannose 3,5-epimerase, *GGP* GDP-L-galactose phosphorylase, *GalPP* L-galactose-1-P phosphatase, *GalDH* L-galactose dehydrogenase, *GalLDH* L-galactono-1,4-lactone dehydrogenase

[b]*T* transcriptional, *PT* post-transcriptional, (*P*) phosphorylation, (*Ac*) acetylation, (*G*) glycation

Moreover, GalLDH mRNA was localized by in situ hybridization in the infected zone of alfalfa and *L. japonicus* nodules, and high GalLDH activity was detected in the infected zone of alfalfa and bean nodules (Matamoros et al. 2006). Subsequently, genes encoding GMP, GME, L-galactose-1-P phosphatase (GalPP), and L-galactose dehydrogenase (GalDH) were found to be expressed in nodules (Loscos et al. 2008). Although GMP and GME participate in the synthesis of GDP-L-galactose, a common precursor of AsA and cell wall polysaccharides, the enzymes GalPP, GalDH, and GalLDH catalyze the three last steps of the L-galactose pathway (Wheeler et al. 1998). In addition, proteomic studies in *L. japonicus* nodules identified several enzymes involved in AsA metabolism, including phosphomannomutase, GME, and GalPP (Dam et al. 2014). Table 1 shows the AsA biosynthetic pathway highlighting the transcripts and enzymes identified in nodules. Very recently, proteomic studies have detected the key regulatory enzyme, GGP, in bean nodules (Matamoros et al. 2018), further supporting the presence of a complete biosynthetic pathway in these plant organs.

Studies are underway to produce transgenic N_2-fixing plants with elevated AsA content through overexpression of GGP and GME. Preliminary studies in one of our labs (Torres-Jerez et al. 2017) have revealed that GGP-overexpressing plants of *M. truncatula* do indeed have higher rates of N_2 fixation, as do plants that overexpress GME. These results were obtained using the constitutive 35S promoter from cauliflower mosaic virus. Additional studies are planned using the strong nodule-specific promoter for Lb. The practical implications of this work could be striking since even a small increase in nitrogen fixed or yield could translate to huge agronomic benefits.

Likewise, given the profound, positive responses to stem infusion of AsA, specifically the fourfold to fivefold increase in nitrogenase activity, it is reasonable to expect that increased production of endogenous AsA should lead to increased nitrogenase activity and delayed nodule senescence. Elevated levels of AsA should also confer increased tolerance of stresses such as drought or salinity, probably because of the exacerbated production of ROS under stressful conditions (Miller et al. 2010). Exogenous AsA has been reported to increase tolerance of salinity for tomato seedlings (Shalata and Neumann 2001). Similarly, maize seeds soaked in AsA resulted in seedlings with greater tolerance to salinity (Rady and Hemida 2016). On the contrary, a lower AsA level such as that found in the *vtc1* mutants of *A. thaliana* caused accumulation of H_2O_2 and decreases in activities of the AsA-glutathione enzymes, chlorophyll content, CO_2 assimilation, and photosystem II activity (Huang et al. 2005).

Alternative pathways for AsA biosynthesis through *myo*-inositol (Lorence et al. 2004) or D-galacturonic acid (Valpuesta and Botella 2004) have been proposed in several species. For example, in strawberry fruits, AsA biosynthesis occurs through D-galacturonic acid, a component of cell wall pectins, and overexpression of D-galacturonic acid reductase increases AsA concentration (Agius et al. 2003). However, the contribution of these pathways to AsA biosynthesis in nodules has not been investigated so far.

8 Regulation of AsA Biosynthesis in Nodules During Senescence and Stress

Natural senescence or aging is a complex and tightly regulated process entailing the loss of redox homeostasis and the degradation of organelles and macromolecules (Lim et al. 2007). Environmental constraints affect regulation of AsA biosynthesis that may occur through transcriptional and translational control of the L-galactose pathway (Bulley and Laing 2016). In nodules, aging is associated with an almost complete transcriptional inhibition of some genes involved in AsA biosynthesis, a strong decline of GalLDH activity, and consistent decreases in AsA and glutathione (Groten et al. 2005; Loscos et al. 2008). These observations indicate a progressive switch-off of the AsA biosynthetic pathway during nodule aging. Moreover, the capacity for AsA regeneration from MDHA and DHA is also negatively affected. In senescent bean nodules, that capacity declines faster in the mitochondria than in the cytosol, which could lead to alterations in the mitochondrial redox state (Matamoros et al. 2013).

In nodules, senescence induced by drought, nitrate, or prolonged darkness shares some features with aging, including inhibition of nitrogenase, degradation of Lb, and decrease of antioxidant redox buffering capacity (Puppo et al. 2005; Becana et al. 2010 and references therein). The concentration of AsA declined by 58–67% with drought stress in pea and alfalfa nodules (Gogorcena et al. 1995; Naya et al. 2007).

However, AsA decreased by 41% in pea nodules after supply with excess nitrate (Escuredo et al. 1996) but remained constant in common bean nodules under similar conditions (Matamoros et al. 1999). Likewise, drastic declines of AsA were observed with advancing age of pea nodules (Groten et al. 2005, 2006) but not of soybean nodules (Dalton et al. 1986). Therefore, there are species-specific differences in AsA metabolism and/or transport in response to, at least, natural and nitrate-induced nodule senescence. The decline in AsA concentration was accompanied in many cases by corresponding decreases in APX, MDHAR, and DHAR activities in nodules and by increases in oxidative damage of lipids and proteins (Escuredo et al. 1996; Matamoros et al. 1999; Hernández-Jiménez et al. 2002). This suggests that the function of the AsA-glutathione pathway becomes somewhat limited during senescence and that the resulting uncontrolled ROS production may contribute to the inhibition of N_2 fixation. The mechanisms for this inhibition, however, probably vary with the symbioses and with the type, intensity, and duration of stress.

The regulation of AsA biosynthesis in nodules is poorly known (Table 1). GalLDH activity is post-transcriptionally regulated but is not a major determinant for AsA content in nodules under stress conditions (Loscos et al. 2008). Nodules exposed to cadmium, a toxic heavy metal, showed reduced expression of the genes for GMP, GME, GalDH, and GalLDH, whereas high salinity had no effect on their expression. This indicates specific transcriptional regulation of AsA biosynthesis in response to various stresses (Table 1). Additional regulatory mechanisms might exist. For example, a glycated form of GMP has been very recently identified in bean nodules (Matamoros et al. 2018). Protein glycation is a post-translational modification originated by the reaction of arginine and lysine residues with reducing sugars or α-carbonyls derived from them (Bilova et al. 2017). However, information on this modification in plants is virtually nonexistent. There are also examples of other post-translational modifications (Table 1). Thus, phosphorylated and acetylated peptides from enzymes of AsA biosynthesis have been recently detected as a result of an in-depth proteome analysis of several *M. truncatula* organs (Marx et al. 2016). These data revealed that GME can be acetylated and phosphorylated and that GMP can be phosphorylated, suggesting that both types of modifications may play regulatory roles of the enzyme activities.

The regulation of AsA metabolism is also influenced through hormonal cross-talk. Jasmonates (JA) are important regulators of plant development and stress responses (Wasternack and Hause 2013) and induce genes of biosynthesis of glutathione (Xiang and Oliver 1998) and AsA (Wolucka et al. 2005; Loscos et al. 2008). In bean nodules, JA induced GalLDH expression, inhibited DHAR activity at the post-translational level, and increased AO mRNA and activity (Loscos et al. 2008). The two last effects combined may trigger a rapid change in the apoplastic redox state due to AsA oxidation, which in turn might influence the capacity of the apoplast to generate and propagate redox signals.

9 Conclusions

AsA is abundant in nodules and plays a critical role in supporting N_2 fixation by minimizing damage from ROS. AsA concentration in nodules and other plant organs depends on the rates of biosynthesis, degradation, and transport. Moreover, the ratio between the reduced (AsA) and oxidized (MDHA and DHA) forms may influence signaling networks. Research from different laboratories is starting to elucidate the numerous factors and complex regulatory mechanisms that determine AsA homeostasis in plant cells. However, the information on AsA metabolism and regulation in legume nodules is scant and further research is necessary to fill in this gap. In this regard, the generation of legumes with altered AsA concentrations constitutes an excellent tool to investigate the role of this important antioxidant, redox buffer, and signaling metabolite. These plants may help shed light on the mechanisms by which AsA increases N_2 fixation. Thus, from an agronomical viewpoint, enhanced AsA levels might improve the stress tolerance and yield of crop legumes. Another major feature of AsA in nodules is its potential contribution to the operation of the ODB. The enhanced levels of AsA, APX, and (homo)glutathione, along with a high respiratory activity in the nodule parenchyma, provide strong support for the involvement of the mitochondrial respiration and the AsA-glutathione pathway in the ODB, probably entailing rapid fluctuations in H_2O_2 concentration. Extensive physiological, biochemical, and cellular studies with transgenic plants are anticipated to be invaluable to test this appealing hypothesis.

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Importance of Vitamin C in Human Health and Disease

Matthew Chisnall and Richard Macknight

Abstract Ascorbic acid (AsA or vitamin C) is an essential human micronutrient predominantly obtained from plants. Our primate ancestor lost its ability to synthesize AsA at a time when its diet was rich in AsA. Today, eating sufficient fruits and vegetables to obtain more than the minimum level of AsA consistently is a challenge for many people. Research is revealing the importance of AsA in human health well beyond merely preventing scurvy. AsA acts as a cofactor for enzymes involved in epigenetic programming. The link between AsA and epigenetics has profound implications on how dietary AsA might impact on human health. Epigenetic programming plays a crucial role in embryonic development, the progression of cancer, and age-related diseases and there is evidence AsA influences all of these processes. AsA also plays a key role in regulating iron uptake. Iron deficiency anaemia is the most common and widespread micronutrient deficiency, affecting around two billion people worldwide, especially women and children. While it has been long known that AsA enhances uptake of non-haem iron from food, recent studies have found that rather than simply acting in the gut to convert iron into a form that is more readily absorbed, AsA is likely a key regulator of cellular iron uptake. Increased understanding of the various cellular roles of AsA is revealing that regularly obtaining sufficient dietary AsA is important for long-term human health. Enhancing the AsA contents of crop plants has the potential to improve the uptake of dietary AsA and improve human health.

Keywords Vitamin C · Epigenetics · Iron absorption · Malnutrition

M. Chisnall
Department of Biochemistry, University of Otago, Dunedin, New Zealand

R. Macknight (✉)
Department of Biochemistry, University of Otago, Dunedin, New Zealand

New Zealand Institute for Plant and Food Research Ltd, Dunedin, New Zealand
e-mail: richard.macknight@otago.ac.nz

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1 Introduction

Unlike most animals, humans cannot synthesize ascorbic acid (AsA) due to mutations within the L-*gulono-γ-lactone oxidase* (*GLO*) gene, which in other animals encodes the enzyme catalysing the last step in the AsA biosynthetic pathway (Drouin et al. 2011). Humans must obtain AsA on a regular basis through their diet (Nishikimi et al. 1994) and the main source is fresh fruits and vegetables, which can vary greatly in their AsA contents.

Severe AsA deficiency results in scurvy, which was a major problem for sailors who lacked fresh fruits and vegetables in their diet. It has been estimated that two million sailors died of scurvy between 1500 and 1800 (Drymon 2008). In 1747, James Lind conducted the first clinical trial where he divided sailors into different trial groups and showed that citrus juice prevents scurvy (Lind 1757). However, Lind's discovery was largely ignored, and it took another 40 years before the British Navy began giving out lemon juice routinely. In 1907, Norwegian researchers Holst and Frölich were able to reproduce scurvy in guinea pigs (which fortuitously also have a non-functional *GLO* gene) by feeding them a diet of grain and flour (Holst and Frölich 1907) and 20 years later it was shown that scurvy is specifically caused by lack of AsA. Since then much research has been devoted to understanding AsA biosynthesis and catabolism, and its biological roles in plants and animals. We now appreciate that AsA is an essential molecule with diverse and critical biological functions.

2 Ascorbic Acid and Human Health

Why do we need dietary AsA? The physiological and biochemical functions of AsA are due to its ability to donate electrons (Du et al. 2012). AsA has two broad roles; it functions as a general antioxidant, and it is cofactor for mono- and di-oxygenase enzymes.

2.1 Ascorbic Acid as a General Antioxidant

Reactive oxygen species (ROS) are the by-products of normal metabolism, and the cell uses a range of antioxidants, including AsA, to avoid ROS damage. Cellular damage caused by ROS plays a role in ageing and many degenerative diseases, such as Alzheimer's disease (Hensley et al. 2000). Given the potent antioxidant properties of AsA, would increasing AsA consumption, e.g. by taking AsA supplements, be beneficial? While the supplement industry has promoted the health benefits of taking extra AsA and other antioxidants, there is no clear evidence that taking high doses of AsA in an attempt to increase the normal physiological levels of AsA

reduces oxidative stress or slows ageing or the progression of age-related diseases. All organisms already have sufficient antioxidants to keep ROS in balance, and there is no reason to believe taking large doses would be beneficial. However, it is important to ingest sufficient AsA to avoid deficiency. The balance between oxidation and antioxidation (redox balance) is critical in maintaining a health biological system (Bouayed and Bohn 2010). The antioxidant properties of AsA are needed throughout the body and play specific roles in some tissues. For example, AsA is found in eye tissue at high, millimolar levels, and this is proposed to protect the eyes from potential oxidative damage caused by solar radiation (Brubaker et al. 2000; Kern and Zolot 1987). AsA has an important role in the regeneration of other physiological antioxidants, in particular, vitamin E (Golumbic and Mattill 1941). Vitamin E functions to prevent lipid membrane oxidation and becomes oxidized in the process. AsA can reduce the oxidized vitamin E to maintain protection of membranes (Csallany et al. 1962). ROS also play important roles in the body and are involved in cellular signalling, regulation of immune responses, and fostering antioxidant defence responses (Bouayed and Bohn 2010). This balance means that high doses of antioxidants could be detrimental. For example, the immune system uses ROS to kill cancer cells before they metastasize and spread throughout the body and one study showed that mice given exogenous antioxidants had increased rates of metastasizing melanomas (Piskounova et al. 2015).

2.2 Ascorbic Acid as a Cofactor

Ascorbic acid serves as a cofactor for di-oxygenase (the Fe^{2+}/2-oxoglutarate-dependent di-oxygenases; 2-OGDO) and monooxygenase enzymes. The activity of the 2-OGDO enzymes require that core reactive iron atoms are reduced to Fe^{2+}, a step requiring AsA. Scurvy causes swollen, bleeding gums—sometimes teeth can fall out, severe joint or leg pain, skin that bruises easily. These symptoms arise from the reduced activity of the 2-OGDO enzyme, collagen prolyl 4-hydroxylase (CP4H), due to a lack of AsA. CP4H is required to hydroxylate the proline residues of collagen that form stable collagen fibres. Collagen is an essential component of connective tissue and as such has an essential role in wound healing. While scurvy is rare, poor vitamin C status is more common and is often undiagnosed since the early symptoms—fatigue, malaise, depression, and irritability are non-specific and unremarkable (Ben-Zvi and Tidman 2012). The fatigue and weakness of early scurvy is likely due to AsA role in the biosynthesis of L-carnitine (Du et al. 2012). L-Carnitine functions in energy metabolism, specifically the transport and oxidation of fatty acids and therefore is most important in tissues which derive their energy from fatty acids (Flanagan et al. 2010). The depression and mood swings characteristic of early scurvy are due to reduced neurotransmitters, as AsA is a cofactor for dopamine-β-monooxygenase, which converts dopamine to norepinephrine (Wimalasena and Wimalasena 1995).

Most people treated for scurvy feel better within 2 days and recover within 2 weeks. However, there may be more long-lasting effects of not consuming sufficient dietary AsA. Two classes of 2-OGDO enzymes are involved in epigenetic programming; the methylcytosine di-oxygenases (known as ten-eleven translocation (TET) di-oxygenases) that are responsible for DNA demethylation (Camarena and Wang 2016; Blaschke et al. 2013; Chen et al. 2013) and the Jumonji C domain-containing histone demethylases (Wang et al. 2011). The link between ascorbate and epigenetics has profound implications on how dietary ascorbate might impact on human health (Macknight et al. 2017). Epigenetic programming plays a key role in regulating development programmes and a link between AsA, epigenetics, and cancer has recently been established. Two recent high-profile papers have provided a link between AsA and leukaemia (Agathocleous et al. 2017; Cimmino et al. 2017). AsA was shown to play a role in the differentiation of blood-forming haematopoietic stem cells. A key step in this process of differentiation is the demethylation of DNA which alters gene expression. The enzyme TET2 carries out the first step in removing DNA methylation (the conversion of 5-methylcytosine to 5-hydroxy methylcytosine) and to achieve this the haematopoietic stem cells import 2–20 times more AsA via the upregulation of a specific ascorbate transporter (Agathocleous et al. 2017). AsA is thought to maintain TET activity by reducing Fe^{3+} to Fe^{2+} in the active site of the enzyme. Consistent with having an important role in stem cell differentiation, mutations in the TET2 gene are associated with various forms of leukaemia in humans. Experimentally reducing TET2 activity in mice also results in increased rates of leukaemia. In these mice, TET2 activity could be increased by administering AsA and this increased stem-cell differentiation and reduced cancer rates (Agathocleous et al. 2017). Similarly, when human acute myeloid leukaemia cancer cells were transplanted into mice, AsA supplementation induced differentiation and the death of the leukaemia cells (Cimmino et al. 2017). These experiments are consistent with epidemiological evidence indicating that a higher consumption of fruits and vegetables correlates with a lower risk of most types of cancer (Carr and Frei 1999; Li and Schellhorn 2007). However, randomized clinical trials have suggested that AsA supplementation does not reduce the risk of cancer (Galan et al. 2005; Gaziano et al. 2009; Hercberg et al. 2004; Lin et al. 2009; Qiao et al. 2009; Taylor et al. 1994). This apparent discrepancy is likely due to both, the AsA status of the patients in the clinical trials, and the longer term effects of low AsA consumption. People who are AsA deficient or had been AsA deficient for periods of their life might have higher rates of cancer. However, simply giving people who have had sufficient dietary AsA, extra AsA would likely have no effect on cancer rates.

There is a large body of evidence that maintaining normal AsA levels provides protection against other human diseases, such as cardiovascular and neurodegenerative disease (Chambial et al. 2013; Harrison 2012). Given the fundamental roles epigenetics has in controlling gene expression throughout our life, any alteration in this process could result in disease, especially those that might involve slow cumulative changes typical of age-related diseases (Camarena and Wang 2016). Again, there is no evidence that taking AsA supplements on top of a normal, healthy diet provides any protective effects (Chambial et al. 2013; Harrison 2012).

3 Role of AsA in Iron Absorption

AsA also plays an important role in the uptake of dietary iron. Iron deficiency is the most common and widespread nutritional disorder in the world, predominantly affecting developing countries but is also the only nutrient deficiency significantly prevalent in industrialized countries (World Health Organization 2015). The predominant dietary source of iron is from non-haem iron, sourced from fruits and vegetables. Absorption of iron from non-haem sources is lower than that of haem iron from animal sources and several factors influence this (Lynch and Cook 1980). AsA was initially thought to have roles in non-haem iron absorption as a reductant and a chelator (Conrad and Schade 1968). When iron is consumed, it is oxidized to the Fe^{3+} state, and for absorption to occur, iron must be reduced to the Fe^{2+} state. AsA was proposed to be a reducing agent responsible for the reduction of iron (Gunshin et al. 1997), but this has now been shown to be performed by the action of ferrireductase (McKie et al. 2001). However, this does not exclude AsA from being involved in the reducing process. The main role for AsA in iron absorption is therefore proposed to be promoting iron stability (Teucher et al. 2004). AsA could act to improve iron stability through its action as a chelator, forming an AsA-iron complex (Conrad and Umbreit 1993). This complex would maintain the solubility of iron with the increasing pH of the small intestine (duodenum) allowing for its absorption via the intestinal mucosal lining cells (Teucher et al. 2004). AsA also influences iron absorption by acting to negate the effect of inhibitors such as phytates and polyphenols (Hurrell and Egli 2010).

In addition to enhancing the iron absorption in the gut, AsA aids cellular iron uptake. Iron is a cofactor for a number of enzymes and is also needed for the haemoglobin and myoglobin to bind oxygen. Iron is highly toxic to cells and virtually all iron transported in the plasma is bound to the iron-binding protein, transferrin. Recent work indicates that AsA enhances transferrin-dependent iron uptake and also stimulates the synthesis of the cellular iron-binding protein, ferritin (Lane et al. 2013; Lane and Richardson 2014). Thus, AsA appears to play a central role in controlling iron levels and this might explain why vitamin C deficiency can result in anaemia.

Early feeding studies involving a single meal eaten with and without orange juice showed significant differences in iron absorption; iron absorption increased from 3.7 to 10.4% in one study (Callender et al. 1970) and 2.5-fold in a second study (Rossander et al. 1979). Direct supplementation of AsA in a single meal has resulted in a 2.9-fold increase in non-haem iron absorption (Fidler et al. 2004). Variation in single meal studies has been attributed to differences in meal compositions, in particular, the levels of iron absorption inhibitors and enhancers other than AsA. The molar ratios of AsA to non-haem iron and inhibitors has been shown to affect iron absorption (Gillooly et al. 1983, 1984; Hallberg et al. 1986, 1989; Siegenberg et al. 1991).

Studies which have looked at the effect of AsA on non-haem iron in a complete diet have shown either no or only a small effect compared to single meal studies

(Cook and Reddy 2001). A significant increase in iron absorption was observed in Mexican women, with a two-fold increase in dietary AsA resulting in a 3.3-fold increase in iron absorption (Diaz et al. 2003). The women in this study were iron deficient and normally had a diet low AsA (Diaz et al. 2003). It is likely that if individuals with normal levels of iron and AsA, consume more AsA it will have little effect on iron uptake. However, for individuals deficient in iron and AsA, increasing dietary AsA will have the added benefit of increasing iron uptake. Thus, increasing AsA levels in food crops might be an effective strategy to reduce iron deficiency.

4 Ascorbic Acid and the Common Cold

AsA supplementation is commonly marketed for the maintenance of good health and the prevention of the common cold. However, there has been significant controversy in the scientific literature for at least 70 years about whether AsA prevents or treats the common cold. Reviews of the literature have shown that AsA does not reduce the incidence of colds in the general population, showing AsA supplementation for this purpose to be unjustified (Hemilä and Chalker 2013). However, individuals exposed to brief periods of severe physical exercise, cold environments, or those with marginal AsA status could benefit from AsA supplementation to reduce the incidence of colds (Douglas et al. 2007; Wintergerst et al. 2006). While there is some evidence that AsA reduces duration and severity of colds when AsA is taken on regular basis, supplementation once a cold has been contracted was ineffective (Hemilä and Chalker 2013). As with AsA other roles, it is likely that the major benefit is when individuals who are not getting sufficient dietary AsA are provided with supplementary AsA (Johnston et al. 2014).

5 Recommended Daily Intake of AsA

How much dietary AsA do we need? AsA is water soluble and must be regularly ingested, as excess cannot be stored. The recommended daily intake (RDI) or recommended daily allowance (RDA) for nutrients is the amount needed to meet the requirements of 97–98% of healthy individuals and are set by representative agencies for each country. The intake of a particular nutrient required each day is dependent on the body's needs, and the efficiency of uptake and excretion. RDA for AsA varies between countries (Table 1), and it has been suggested a higher recommended intake might be required for optimum health (Carr and Frei 1999; Levine et al. 1996). The amount of dietary AsA needed also depends on an individual's lifestyle. The USA RDA states a requirement for smokers to have an AsA intake 35 mg/day higher than their non-smoking counterparts (Institute of Medicine 2000), due to smoking significantly reducing AsA levels (Schectman et al. 1989). Associated with RDI and RDA is the upper level of intake or maximum daily allowance. This is the

Table 1 AsA recommended daily amounts in mg

Individuals	Australia/NZ[a]	USA[b]
0–6 months	35	40
7–12 months	35	50
1–3 years	35	15
4–8 years	35	25
9–13	40	45
14–18	40	75(M), 65(F)
19+	45	90(M), 75(F)
Pregnancy 18 and under	55	80
Pregnancy 19 and older	60	85
Breastfeeding 18 and under	80	115
Breastfeeding 19 and over	85	120

[a]Capra (2006)
[b]Institute of Medicine (2000)

intake at which a mineral or nutrient becomes toxic due to excess. The selective uptake of AsA by humans means there is a very low risk of toxicity from AsA. The Australian and New Zealand guidelines have set a prudent limit of 1000 mg/day (Capra 2006).

AsA deficiency is prevalent in developing nations and is also present in developed nations. Older studies revealed 20–30% of adults in the USA consume less than 60 mg AsA daily and about 15% were deficient (Hampl et al. 2004). More recent studies have revealed insufficient levels in Canadians with 14% AsA deficient and 33% consuming suboptimal amounts (Cahill et al. 2009) and 40% of Mexican women AsA deficient (Garcia et al. 2003). Given our new understanding of the importance of consuming sufficient AsA, this could be a significant factor in reduced life-long good health.

6 Conclusion and Future Perspectives

When our primate ancestors lost the ability to synthesize AsA, their diet of vegetation and fruits would have provided 25–100 times more AsA than the current RDA (Macknight et al. 2017). In this situation, there would have been no evolutionary disadvantage in not being able to make AsA. However, today consuming sufficient fruits and vegetables to provide the optimal intake of dietary AsA is a challenge for many people, with the majority of staple crops having very low levels of AsA.

As we begin to understand the fundamental importance of AsA in health and disease, it is clear that consuming sufficient AsA is not just important to avoid scurvy. AsA plays a crucial role in many essential cellular processes and while consuming excess AsA is likely to be of little benefit, consuming insufficient AsA will likely contribute to poor health. As our knowledge of how plants regulate the

synthesis of AsA increases, there will be opportunities to increase the AsA content of crops to help alleviate AsA deficiency (Macknight et al. 2017). Since AsA also regulates iron uptake, increasing dietary AsA should also help reduce iron deficiency.

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