

Chapter 18

Transgenic Plants Over-expressing Glutathione Biosynthetic Genes and Abiotic Stress Tolerance

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Abstract Plants cannot survive without glutathione, or a functionally homologous thiol, as glutathione has diverse functions in plant growth and development, many of which cannot be performed by other thiols or antioxidants. The roles of glutathione in plants include the regulation of redox homeostasis, cell signaling and gene expression, and essential roles in key physiological and metabolic processes such as photosynthesis and sulfur assimilation. The cellular pool of reduced glutathione (GSH) can be depleted by oxidation of GSH to glutathione disulfide (GSSG), by reactive oxygen species (ROS), or by reacting with methylglyoxal (MG). The generation of ROS and MG increases in plant cells under abiotic stress, e.g., in plants exposed to heavy metals, salinity, drought, high or low temperatures, herbicides, or air pollutants. There is considerable evidence to suggest that enhanced activities of GSH utilizing and regenerating enzymes are crucial for abiotic stress tolerance in both model and cultivated plant species. Recently, the use of transgenic plants has clearly demonstrated the importance of GSH for stress tolerance, with plants over-expressing GSH biosynthetic genes and genes associated with maintaining GSH levels having increased GSH levels and showing improved tolerance to individual stressors. In addition, modulating the activities of GSH-related enzymes has also been shown to be important for multiple stress tolerance; however, many of the details of the roles GSH plays in multiple stress tolerance are still unresolved.

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The aim of this chapter is to provide a comprehensive overview of the diverse roles of GSH biosynthetic genes in improving abiotic stress tolerance by critically evaluating the research conducted using transgenic plants, expressing GSH-associated genes, grown under abiotic stress.

Keywords Glutathione • Abiotic stress • Transgenic plants • Antioxidant • Glutathione biosynthetic genes

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1 Introduction

All aerobic organisms including plants require molecular oxygen for their survival. As a consequence of cellular respiration, molecular oxygen is reduced to H₂O, and during this process, reactive oxygen species (ROS) including superoxide anion radical (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical (•OH) are often produced (Asada 1999; Halliwell and Gutteridge 2007; Krumova and Cosa 2016; Sewelam et al. 2016). In addition, plants grown in the field are constantly exposed to a range of abiotic stresses including heavy metals, salinity, drought, high or low temperature, herbicides, and air pollutants, and the generation of ROS is a common consequence of such abiotic stresses (Shimazaki and Sugahara 1980; Foyer et al. 1994; Prasad 1996; Gaber et al. 2012; Ahmad et al. 2016; Hussain et al. 2016; Akram et al. 2017). As ROS are extremely reactive at high concentration, they can cause severe damage to cell components, e.g., by oxidizing proteins and inactivating enzymes, oxidizing DNA, and initiating the peroxidation of unsaturated fatty acids in cell membranes (Foyer and Harbinson 1994). However, ROS at lower levels perform important roles in the activation of defense gene expression, as part of the protective mechanisms plants use to cope with biotic and abiotic stressors, and so careful regulation of cellular ROS levels is important for plant survival under field conditions (Karpinski et al. 1999; Grant and Loake 2000; Fryer et al. 2003; op den Camp et al. 2003; Cheng et al. 2015; Avery 2011; Krumova and Cosa 2016).

Plant cells have various mechanisms to regulate cellular ROS levels like enzymatic defense systems, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), glutathione

S-transferases (GSTs), glutathione peroxidase (GPX), peroxiredoxins, and thioredoxins and nonenzymatic antioxidants including reduced glutathione (GSH), ascorbate (AsA), vitamin E, lipoic acid, beta-carotene, and flavonoids (Foyer et al. 1994; Gaber et al. 2004, 2006; Halliwell and Gutteridge 2007; Hossain et al. 2014, 2015; Mostofa et al. 2017). Noctor and Foyer (1998), when reviewing the roles of GSH and AsA in plants, concluded that one of the main functions of these molecules is to “keeping reactive oxygen under control.” However, more recently studies have shown that GSH and AsA also play very important roles in the regulation of the cellular redox status and in mediating redox and ROS relating signaling in plant cells (Foyer and Noctor 2005a, b, 2009).

Reduced glutathione is the predominant low-molecular-weight thiol found in plant cells and plays an important role in the protection of cells against free radical-mediated damage (Chen et al. 2012, 2015; Noctor et al. 2012; Munné-Bosch et al. 2013). The majority of the cellular GSH is present in the cytosol, with the remaining found in mitochondria, chloroplasts, the nuclear matrix, and peroxisomes (Zechmann 2014). Due to the presence of the cysteine residue, GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances, e.g., free radicals and reactive oxygen/nitrogen species. Cellular GSH concentrations often decline in response to biotic or abiotic stressors as a result of increased oxidative and/or MG-induced stress (Lu 2000; Hossain et al. 2011; Hoque et al. 2016). The GSH/GSSG ratio, which is frequently used as an indicator of the cellular redox state, is often >10 under normal physiological conditions but declines rapidly in plants under stress (Lu 2000). In both animals and plants, it is well-known that shifting the GSH/GSSG ratio toward a more oxidizing state can activate several signaling pathways/factors including protein kinase B, calcineurin (calcineurin B-like proteins in plants), nuclear factor- κ B, c-Jun N-terminal kinase, apoptosis signal-regulated kinase 1, and mitogen-activated protein kinases, thereby reducing cell proliferation and increasing apoptosis (Jones 2000). Recent studies on plants have shown the importance of GSH for abiotic stress tolerance; therefore, the aim of this chapter is to provide an overview of the use GSH biosynthetic gene expression in transgenic plants as a means for improving plant abiotic stress tolerance and possibly crop yields.

2 The Biosynthesis of Glutathione and Relationship of Glutathione to Plant Stress Tolerance

Glutathione is synthesized in two ATP-dependent steps catalyzed by two enzymes, a plastidial γ -glutamylcysteine synthetase (γ -ECS, GSH1; EC 6.3.2.2) and a cytosolic glutathione synthetase (GS, GSH2; EC 6.3.2.3) (May and Leaver 1993; Rawlins et al. 1995; Cobbett et al. 1998; Noctor et al. 2002; Galant et al. 2011). γ -ECS catalyzes the rate-limiting step in GSH biosynthesis in mammals, in yeasts and in plants, and levels of this enzyme can be regulated at the transcriptional and/or translational levels (Xiang and Oliver 1998; Noctor et al. 1996, 2002, 2012; Liedschulte et al. 2010). Studies have shown that increases in GSH levels observed in response to stress are correlated with increased γ -ECS activity (Chen and

Goldsborough 1994; Kocsy et al. 2001). In addition, the over-expression or inhibition of *GSH1* and *GSH2* causes increased or decreased levels of GSH, respectively, in plants and was found to modulate or inhibit stress-responsive pathways required for plant growth, development, and stress tolerance (Cobbett et al. 1998; Xiang and Oliver 1998; Szalai et al. 2009; Ghanta and Chattopadhyay 2011; Noctor et al. 2011, 2012; Cheng et al. 2015; Liu et al. 2015). Increased expression of γ -ECS in transgenic plants and enzymes associated with sulfur assimilation pathway or GR has been shown to cause substantial increases in leaf GSH levels (Harms et al. 2000; Foyer et al. 1995; Noctor et al. 1996). Also, it has been reported that an increased capacity to maintain GSH levels or an increase in the cellular pool of GSH can lead to modified amino acid metabolism and enhanced stress tolerance (Noctor et al. 1998a, b). In addition to the transgenic approach, analysis of mutants deficient in GSH and the treatment of plants with GSH, combined with transcript profiling, has provided valuable information on how GSH and GSSG regulate cell signaling and plant development and ability to tolerate stress. For example, transcript profiling studies have identified the relationships that exist between the regulation of stress-related defensive networks and antioxidant metabolism in plants (Willekens et al. 1997; Rossel et al. 2002; Pneuili et al. 2003). Studies have also shown that GSH can influence cellular levels of the regulatory proteins NPR1 and protein phosphatase 2C (ABI2), which are important in salicylic acid (SA) and abscisic acid (ABA) signaling, respectively (Meinhard et al. 2002; Mou et al. 2003). In a more recent study, using transcriptomic analyses of steady-state and polysome-bound mRNAs in GSH-treated plants, Cheng et al. (2015) reported that GSH had an even greater potential impact on plant growth, development, and stress tolerance than what was apparent from previous total mRNA profiling studies. They demonstrated that the translational changes induced by GSH treatment were associated with changes in numerous hormone and stress signaling pathways and suggested that these changes might contribute to enhance stress tolerance in GSH-treated plants (Cheng et al. 2015). Recently, studies on a range of plant species that used a priming approach, thermal (heat or cold treatments) or chemical (proline, betaine, nitric oxide, selenium, salicylic acid, polyamines, etc.), have shown that priming can increase GSH biosynthesis, elevate cellular GSH pools, and increase the activities of glutathione-utilizing and glutathione-regenerating enzymes, with the end result being plants that have improved abiotic stress tolerance (Hossain et al. 2010, 2011, 2012, 2013a, b and references therein).

3 Transgenic Plants Over-expressing GSH Biosynthetic Genes: Heavy Metal Tolerance

Several studies have shown that cellular GSH levels can regulate the expression of a range of defense genes that confer stress tolerance to plants (Wingsle and Karpinski 1996; Karpinski et al. 1997; Wingate et al. 1988; Loyall et al. 2000; Cheng et al. 2015). A significant role for GSH was found in plants responding to excessive levels

of cadmium (Cd) and other heavy metals, as GSH is the precursor of phytochelatins (PCs) ($[\gamma\text{-Glu-Cys}]_n\text{-Gly}$) that are synthesized by the enzyme phytochelatin synthase (PCS) (Grill et al. 1987, 1989; Cobbett and Goldsbrough 2002; Rea et al. 2004; Hossain et al. 2012). Phytochelatins form complexes with potentially toxic metals, which are then sequestered into the vacuole, reducing their cytotoxicity (Grill et al. 1987, 1989; Cobbett and Goldsbrough 2002; Rea et al. 2004). Transgenic plants over-expressing GSH biosynthetic genes have been generated for various plants species and all showed improved tolerance to metal toxicity as compared to wild-type (WT) plants (Table 18.1).

The above studies clearly indicate that the up-regulation of GSH biosynthesis can improve the tolerance of plants to heavy metals and can, in some cases, enhance metal uptake, utilization, and detoxification. Hence, the development of transgenic plants over-expressing GSH biosynthetic genes and displaying increased heavy metal tolerance could not only be used to allow crop plants to be grown in soils high in heavy metals but could also be used for phytoremediation purposes.

4 Transgenic Plants Over-expressing GSH Biosynthetic Genes: Salt and Osmotic Stress Tolerance

The role of GSH and GSH metabolism in tolerance to salt stress has been studied using salt-tolerant and salt-susceptible genotypes in several plant species (Mittova et al. 2003a, b; El-Shabrawi et al. 2010). In general, salt-tolerant species show greater GSH biosynthesis and cellular GSH levels; higher GSH/GSSG ratio; higher GPX, GST, GR, Gly I, and Gly II activities; and lower levels of oxidative damage when exposed to salt stress (Mittova et al. 2003a, b; El-Shabrawi et al. 2010). Application of GSH to plants has also been shown to improve salt tolerance and yields in crop plants (Hussain et al. 2016; Akram et al. 2017). Several studies using transgenic plants have shown that over-expression of GSH biosynthetic genes improves salt tolerance in a wide range of plant species (Bae et al. 2013; Choe et al. 2013; Li et al. 2015; Park et al. 2017). For example, Choe et al. (2013) showed that transgenic rice (*Oryza sativa*) plants over-expressing *OsECS* had improved salinity stress tolerance, as indicated by a bright green phenotype, and maintained a higher GSH/GSSG ratio as compared to WT plants. These transgenic plants also showed lower ion leakage and higher chlorophyll fluorescence when exposed to MV-induced oxidative stress, and the seeds of these plants showed higher germination rates under saline conditions. In addition, *OsECS* over-expressing rice plants accumulated more biomass and had higher yields when grown in paddy fields in the absence of any stress. Other studies on rice plants in which GSH levels have also been manipulated have also shown improved tolerance to salinity. Bae et al. (2013) reported that transgenic rice plants over-expressing a *Brassica juncea* L. *ECS* (*BrECS*) gene showed improved salt tolerance and higher yields and biomass when grown in the paddy fields. Li et al. (2015) showed that transgenic plants over-expressing a *Pyrus*

Table 18.1 Transgenic plants over-expressing GSH biosynthetic genes and heavy metal stress tolerance

Gene name	Transgenic plant species	Tolerance to	Phenotypic response and factors determining tolerance	References
<i>γ-ECS</i>	<i>Agrostis palustris</i>	Cd	Transgenic plants exhibited more effective growth as compared to WT plants under Cd stress due to increased sequestering of Cd ²⁺ and PC synthesis	Zhao et al. (2010)
<i>γ-ECS</i> + <i>ArsC</i> (arsenate reductase)	<i>Arabidopsis thaliana</i>	Arsenic (As)	Transgenic plants over-expressing both genes showed 4- to 17-fold higher shoot fresh weights and accumulated two- to three-fold higher As per gram tissue as compared to WT or plant over-expressing <i>γ-ECS</i> or <i>ArsC</i>	Dhankher et al. (2002)
<i>γ-ECS</i>	<i>cad2-1</i> mutant of <i>Arabidopsis thaliana</i>	As	Transgenic plants showed 6- to 100-fold higher <i>γ</i> -glutamylcysteine (EC), PC2, and PC3 peptide levels in root tissues over mutants that were equivalent to WT plants. The shoot and root levels of GSH were two- to five-fold above those in WT plants, with or without treatment with As	Li et al. (2006a)
<i>γ-ECS</i> or <i>GS</i>	<i>Arabidopsis thaliana</i>	Mercury (Hg) and As	Over-expression of <i>γ-ECS</i> or <i>GS</i> caused a significant increase in GSH and/or PCs, and plants showed resistance to As and Hg, as compared to WT plants. Co-expression of both <i>ECS</i> and <i>GS</i> resulted in tolerance to Hg, and plants accumulated 35-fold more biomass and three-fold more Hg aboveground than the WT when grown on Hg(II), due to enhanced synthesis of PCs as compared to the WT plants or lines expressing <i>ECS</i> or <i>GS</i> alone	Li et al. (2006b)
<i>γ-ECS</i> + <i>PCS</i> + <i>serine acetyltransferase</i>	<i>Nicotiana tabacum</i>	Cd	Plants over-expressing transgenes (either separately or in combination) showed increased Cd sequestering in roots but not in shoots compared with WT plants. Importantly, transgenic plants over-expressing all three genes showed the greatest effects (about eight-fold elevation of thiols) as compared to single gene transgenic or to WT plants	Wawrzynski et al. (2006)
<i>γ-ECS</i>	<i>Arabidopsis thaliana</i>	As, Hg, and Cd	The level of GSH, PCs were increased 3- to 20-fold in response to As, Hg, and Cd exposure as compared to WT plants. Transgenic plants were highly resistant to As and weakly resistant to Hg but sensitive to Cd indicating different tolerance mechanisms for the metals	Li et al. (2005)

γ -ECS or GS	<i>Brassica juncea</i>	Heavy metal and metalloids	Transgenic plants over-expressing γ -ECS or GS showed significantly higher capacities to tolerate and accumulate a variety of metals (particularly As, Cd, and Cr) as well as mixed-metal combinations (As, Cd, Zn/As, Pb, and Zn) due to greater availability of GSH and higher PC synthesis as compared to WT plants	Reisinger et al. (2008)
GS	<i>Brassica juncea</i>	Cd	Transgenic plants showed enhanced Cd tolerance at various stages of plant development and accumulated significantly (25%) higher Cd levels than the WT plants. Cd accumulation and tolerance were correlated with the <i>gshI</i> expression levels. Transgenic plants also showed higher GSH, thiol, S, Ca, and PC contents as compared to WT plants	Zhu et al. (1999a)
γ -ECS	<i>Brassica juncea</i>	Cd	The transgenic plants showed increased tolerance to Cd and had higher concentrations of PCs, GSH, and total nonprotein thiols compared to WT plants. The transgenic plants accumulated more Cd than WT plants: shoot Cd concentrations were 40–90% higher. Over-expression of γ -ECS increases biosynthesis of GSH and PCs, which in turn enhances Cd tolerance and accumulation	Zhu et al. (1999b)
γ -ECS	Cottonwood (<i>Populus deltoides</i>)	As	Transgenic plants had higher ECS activity and thiol levels and showed enhanced growth in the presence of As as compared to WT plants. Furthermore, roots of transgenic plants accumulated significantly more (two-fold in root tissues and two- to three-fold in shoot tissues) As compared to non-transgenic plants	LeBlanc et al. (2011)
ECS + PCS	<i>Arabidopsis thaliana</i>	Cd and As	Transgenic plants over-expressing either ECS or PCS showed higher tolerance to Cd and As as compared to WT plants. Importantly, transgenic plants over-expressing both of the genes showed 2-fold Cd accumulation and higher PCs synthesis as compared to single-gene transgenic lines	Guo et al. (2008)

(continued)

Table 18.1 (continued)

Gene name	Transgenic plant species	Tolerance to	Phenotypic response and factors determining tolerance	References
<i>γ-ECS</i>	<i>Populus nigra</i> , <i>Populus × canescens</i>	Zinc (Zn)	Transgenic plants over-expressing <i>γ-ECS</i> either in the cytosol or chloroplasts showed elevated levels of GSH under Zn stress. Transgenic plants showed higher HM uptake and GST activity as compared to WT plants	Bittsánszky (2005)
<i>γ-ECS</i>	<i>Populus tremula</i> × <i>P. alba</i>	Heavy metals and metalloids	Transgenic plants over-expressing <i>γ-ECS</i> in the cytosol showed a two-fold increase in foliar GSH concentrations. Biomass accumulation of WT plants decreased in contaminated soil by more than 30-fold, whereas <i>γ-ECS</i> transgenic plants showed only a two-fold decrease compared to control plants grown in uncontaminated soils	Ivanova et al. (2011)

calleryana γ -ECS (*Pc γ ECS*) gene showed higher GSH biosynthesis and salt tolerance. Recently, Park et al. (2017) reported that transgenic rice plants over-expressing a GS gene (*OsGS*) showed improved growth and oxidative stress tolerance when planted in paddy fields. The transgenic plants showed improved oxidative stress (induced by MV) tolerance as indicated by lower MDA and H₂O₂ accumulation. Importantly, the transgenic plants showed improved grain yields and increased biomass under variable climatic conditions. The above evidence demonstrates that genetically engineered plants over-expressing γ -ECS or GS genes show improved salt tolerance as well as increased biomass and yields under salt stress and in the absence of stress.

5 Transgenic Plants Over-expressing GSH Biosynthetic: Drought Stress Tolerance

Drought is one of the most important stressors that impacts crop productivity worldwide (George and Parida 2010; Prabu et al. 2011; Su et al. 2011). It is well known that roots are the first plant organs to respond to drought (Davies and Zhang 1991; Sengupta and Reddy 2011; Sengupta et al. 2011). Drought stress is associated with reduced CO₂ fixation and higher ROS accumulation that can cause oxidative damage (Baena-Gonzalez et al. 2007; Cruz de Carvalho 2008; Miller et al. 2010; Gechev et al. 2012). Therefore, for the survival of plants under drought stress, fully functional ROS-detoxifying systems are essential for normal plant growth and development (Kranter et al., 2002; Hossain et al. 2013a, b). Ahmed et al. (2013) showed that drought-tolerant wild barley showed greater GSH biosynthesis under drought or salt stress alone or in combination and higher levels of antioxidant enzymes. Greater synthesis of GSH under drought stress was also found in drought-tolerant wheat genotypes, as compared to susceptible cultivars (Islam et al. 2015). Exogenous application of GSH improved drought stress tolerance through the up-regulation of ROS and MG detoxification pathways (Nahar et al. 2015). Compartment specific studies of ROS and antioxidant metabolism in GSH-deficient *pad2-1* mutants demonstrated diverse roles for GSH in regulating drought tolerance. Koffler et al. (2014) showed that GSH-deficient *pad2-1* mutant plants exposed to drought stress had significantly lower GSH levels in most cell compartments (51% in mitochondria, 31% in chloroplasts, 34% in nuclei, and 28% in the cytosol), whereas increased GSH levels were found in WT plants under drought. The levels of GSH and AsA decreased significantly in chloroplasts and peroxisomes with a large increase in cellular H₂O₂ levels. Sengupta et al. (2012) investigated the importance of *Vigna radiata* (L.) γ -ECS (*Vr γ ECS*) under progressive drought stress. Analysis of H₂O₂ levels, lipid peroxidation and *Vr γ ECS* enzyme activity was linked during drought stress and recovery. Additionally, the delicate inter-relationships, putative regulatory mechanism and functioning in the root system under adverse drought conditions, was associated with these factors (Sengupta et al. 2012). In another study, transgenic tobacco plants over-expressing γ -ECS

showed greater drought stress tolerance as indicated by higher germination rate, water retention, water recovery, chlorophyll, and proline content as compared with WT plants. The transgenic tobacco plants also showed higher levels of expression of stress-related genes including *heat shock protein 70(HSP70)*, *GPX*, *thioredoxin peroxidase*, *chalcone synthase*, *1-aminocyclopropane-1-carboxylic acid (ACC) oxidase*, and *heme oxygenase 1* (Kumar et al. 2014).

6 Transgenic Plants Over-expressing GSH Biosynthetic Genes: Herbicide Tolerance

In modern agriculture, herbicides are frequently applied to eradicate weeds as they are more labor- and energy-effective than manual or mechanical weed control (De Block et al. 1987). Recent studies have investigated the role of GSH and its related enzymes and herbicide tolerance (Katerova and Miteva 2010; Burns et al. 2017). GSH can directly detoxify herbicides by forming conjugates, a process that can also be catalyzed by the enzyme GST. Once formed, conjugates can be metabolized and excreted or can be stored in vacuoles or dead cells (Katerova and Miteva 2010). Proteomic and genomic studies of GSH-related proteins in *Avena fatua* L. have shown higher DHAR activity in herbicide-resistant genotypes as compared to susceptible genotypes (Burns et al. 2017). Tseng et al. (2013) also showed that a paraquat-tolerant rice mutant showed higher GSH biosynthesis (3.5-fold) as compared to susceptible one. Transgenic poplar hybrids (*Populus tremula* × *Populus alba*) over-expressing a bacterial γ -ECS gene in the cytosol or in chloroplasts displayed increased resistance to chloroacetanilide herbicides and had higher cellular levels of γ -ECS and GSH as well as higher GST activity as compared to WT plants (Gullner et al. 2001). In *Brassica juncea*, over-expressing γ -ECS or GS gene showed a twofold increase in nonprotein thiol levels and enhanced plant resistance to herbicide atrazine (Flocco et al. 2004). These studies demonstrate the potential for that over-expressing genes associated with GSH metabolism has for the production of transgenic herbicide-tolerant crops plants.

7 Conclusions and Future Perspectives

Glutathione has a broad range of functions in plant growth, development, and stress tolerance, and glutathione metabolism is now considered as a prime candidate for the deliberate manipulation of plants to enhance stress tolerance and to improve yields and quality and also for phytoremediation purposes. Transgenic plants over-expressing GSH biosynthetic genes have showed higher stress tolerance, due to greater GSH accumulation, modulation of redox homeostasis, and increased expression of genes associated with stress tolerance in plants. However, there are still numerous questions to be answered with respect to the roles glutathione plays in plant stress tolerance. For instance, the regulation of GSH biosynthesis and its mechanistic interaction with other redox active molecules and the interconnections

between GSH and various biochemical pathways concern with abiotic stress tolerance. A complete understanding of the regulatory factors associated with GSH biosynthesis in plants and how this important molecule interacts with other plant metabolic processes will open up a new horizon for stress tolerance and crop improvement through the genetic engineering of GSH biosynthetic genes into plant cells.

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