Shamsul Hayat · Mohammad Yusuf
Renu Bhardwaj · Andrzej Bajguz *Editors*

Brassinosteroids: Plant Growth and Development

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Preface

Brassinosteroids are endogenous plant growth-promoting hormones found throughout the plant kingdom that influence cellular expansion and proliferation, and the phenotype of mutant affected in brassinosteroid biosynthesis and signaling clearly shows that these plant steroids are essential regulators of physiological processes including organ elongation, vascular differentiation, male fertility, timing of senescence, and leaf development. Several books covering various aspects of brassinosteroid biology and chemistry appeared in 1991, 1999, 2003, and 2011. However, in the past 7 years, a great deal of progress has been made in understanding specific components of brassinosteroid signal transduction and in clarifying mechanism by which brassinosteroid perception ultimately results in changes in the expression of specific genes associated with different developmental programs. The number of physiological processes known to involve brassinosteroid action has also expanded, and significant experiments quantifying the utility of brassinosteroid application in practical agriculture have been documented. Therefore, it is a need of the hour to gather the information in a book form.

The book is comprised of 16 chapters. Chapter 1 of this book gives a survey of diversity of brassinosteroids in plants. Chapter 2 deals with the currently available data of brassinosteroids in microalgae, which has not been covered in any earlier volume of brassinosteroids. The recent progress in brassinosteroids in cereals is covered in Chap. 3. Chapter 4 summarizes the importance of fluoroxyl and hydroxyl substitutions in brassinosteroids for shooting control and the use of in vitro-grown shoots as test systems. Chapter 5 deals with the role of brassinosteroids in plant response to stress. Physiological action of brassinosteroids which depends on their concentration discussed in a Chap. 6 is solely for the role of brassinosteroids during senescence. Regulation of photosynthesis is discussed in Chap. 7. Chapter 8 deals with the genetic and molecular bases of brassinosteroid metabolism and interactions with other phytohormones. In Chap. 9, transformation of matter and energy in crops under the influence of brassinosteroids is briefly described. Chapter 10 covers the use of transcriptomics and proteomics techniques to study the regulation of brassinosteroids in plants. In Chap. 11, the interplay between antioxidant enzymes and brassinosteroids in the control of plant development and stress tolerance is discussed.

Chapter 12 possesses the information of brassinosteroids in relation to horticultural crops. A current scenario on the role of brassinosteroids in plant defense triggered in response to biotic challenges has been discussed in Chap. 13. Anticancer potential of brassinosteroids is described in Chap. 14. Chapter 15 covers the potential of brassinosteroids in abiotic stress tolerance. Finally, a cross talk of brassinosteroid with other phytohormones is summarized in Chap. 16.

This book is not an encyclopedia of review but includes a selected collection of newly written, integrated, and illustrated chapters describing our knowledge of brassinosteroids. The aim of this book is to tell all about brassinosteroids by the present time. The various chapters incorporate both theoretical and practical aspects and may serve as a baseline information for future researches through which significant developments are possible. It is intended that this book will be useful to the students, teachers, and researchers, both in universities and research institutes especially in relation to biological and agricultural sciences.

With great pleasure, we extend our sincere thanks to all the contributors for their timely response, their excellent and up-to-date contributions, and their consistent support and cooperation. We are thankful to all who has helped us in any way during the preparation of this volume. We are extremely thankful to Springer Nature for the expeditious acceptance of our proposal and completion of the review process. Subsequent cooperation and understanding of their staff are also gratefully acknowledged. We express our sincere thanks to the members of our family for all the support they provided and the neglect and loss they suffered during the preparation of this book.

Finally, we are thankful to the Almighty who provided and guided all the channels to work in cohesion of the idea to the development of the final version of this treatise *Brassinosteroids: Plant Growth and Development* until the successful completion of the job.

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Al Ain, UAE — Mohammad Yusuf
Amritsar, India — Renu Bhardwaj
Bialystok, Poland — Andrzej Bajguz

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Editors

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Chapter 1
The Brassinosteroids Family – Structural Diversity of Natural Compounds and Their Precursors

Marco Antonio Teixeira Zullo and Andrzej Bajguz

Abstract The members of the brassinosteroids family, defined as the 3-oxygenated (20β)-5α-cholestane-22α,23α-diols or their derived compounds isolated from plants, bearing additional alkyl or oxy substituents, are presented. Further, brassinosteroids are grouped into C_{27}, C_{28}, and C_{29} depending upon the number of carbons in their skeletons. Their structural variations occur due to the substitution in A and B-rings as well in the side chain. They occur in both free and conjugated forms to sugars, fatty and inorganic acids. Their presence in Algae, Bryophyta, Pteridophyta and Angiosperms indicates a ubiquitous distribution in the plant kingdom. The related brassinosteroids precursors, as well as their occurrence, are also presented. Brassinosteroids are considered as the 6th class of plant hormones which have been established after the discovery of brassinolide and other related compounds.

Keywords Natural brassinosteroids · Brassinosteroids precursors · Brassinosteroids occurrence

1 Introduction

Intrigued with previous reports of growth regulating properties of pollen extracts, Mitchell and Whitehead (1941) examined the growth responses and histological changes that resulted from the application of ethereal extracts of corn pollen on intact bean plants or on the cut surfaces of decapitated stems. They observed that the first internode of the plants where these extracts were applied grew significantly more and faster than the untreated ones or treated with some known auxins, as well as gained more fresh and dry weights than the controls. They demonstrated that

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these were light dependent phenomena, and due to cell elongation rather than cell division. When applied to tap roots, these extracts inhibited root elongation and provoked the appearance of small tumors distal to the application point. When these pollen extracts where applied to the cut surfaces of decapitated stems they caused pronounced radial elongation of epidermal, cortical parenchyma, and endothelial cells. Later Mitchell et al., reported that immature bean seeds also contained plant growth-stimulating hormones (Mitchell et al. 1951) and that *Brassica napus* pollen contained new, yet unknown, hormones they called *brassins* (Mitchell et al. 1970), all of them with properties similar to those reported earlier (Mitchell and Whitehead 1941).

About 60 kinds of pollen were then screened for plant growth activity in the bean second internode assay, and "a few samples, notably the pollen from rape plant (*Brassica napus* L.) and alder tree (*Alnus glutinosa* L.), produced an unusual response that combined elongation (the typical gibberellin response) with swelling and curvature" (Mandava 1988). At the same time, some experiments showed that application of brassins to young bean and Siberian elm tree plants promoted overall plant growth (Mitchell and Gregory 1972), what led United States Department of Agriculture (USDA) to initiate an effort aimed to explore the agricultural perspectives of brassins and to isolate their component(s). After processing 500 libers of rape pollen, finally, the USDA team announced the isolation and structure elucidation of the active principle, brassinolide (**1**) (Grove et al. 1979), the first plant hormone of steroidal nature, presenting, unlike animal steroidal hormones, (*i*) a 22α,23α-dihydroxylated campestane side chain, (*ii*) a B-ring lactone, and, (*iii*) a 2α,3α-dihydroxylated ring A. Bean second internodes exhibited elongation, curvature, swelling and even splitting when treated with increasing amounts of brassinolide (**1**) (Grove et al. 1979) (Fig. 1.1), a very distinct effect never observed with any other known plant hormone. Its isolation was followed by its partial synthesis (Fung and Siddall 1980; Ishiguro et al. 1980) and of its analogues (Thompson et al. 1979, 1981, 1982; Mori 1980; Takatsuto et al. 1981; Sakakibara and Mori 1982; Sakakibara et al. 1982; Mori et al. 1982), some later recognized as plant hormones themselves.

The early synthetic work furnished many compounds with similar or weaker brassin activity, what prompted natural products chemists to search for brassinolide related compounds in plant species other than rape. To the first of them, the 6-ketosteroid castasterone (**2**) (Yokota et al. 1982a), the putative biosynthetic precursor of brassinolide (**1**), followed that of dolicholide (**3**) (Yokota et al. 1982b), dolichosterone (**4**) (Baba et al. 1983), both with a 24-methylene-5α-cholestane structure, and 28-homodolichosterone (**11**) (Baba et al. 1983), with a 24(*E*)-ethylidene-5α-cholestane skeleton instead of a 5α-campestane basis as in brassinolide (**1**) and castasterone (**2**), and then a multitude of brassinosteroids (BRs) of different side chain structures and oxygenation patterns were isolated, giving rise to the class of brassinosteroids phytohormones, the components of which will be described ahead.

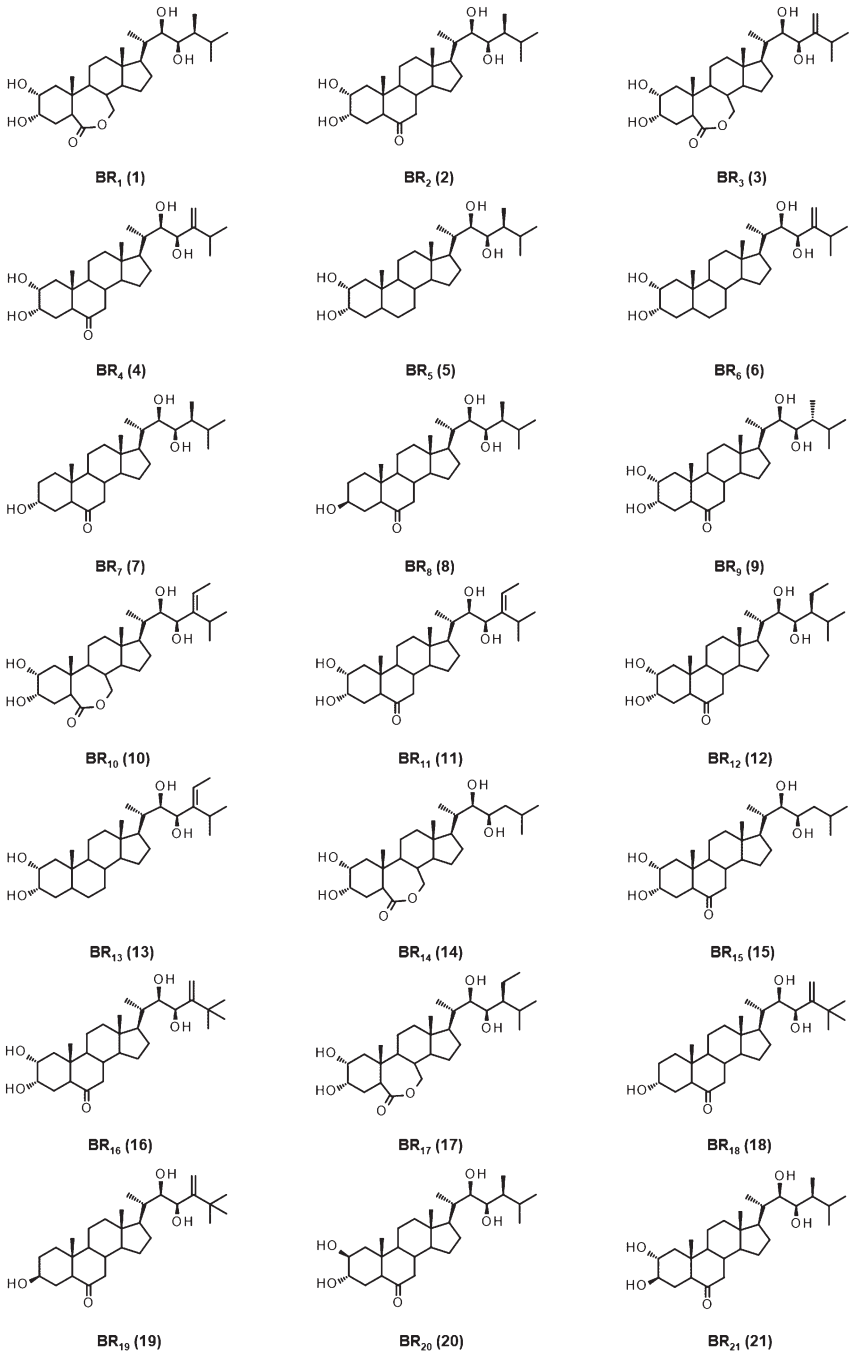

Fig. 1.1 Natural brassinosteroids

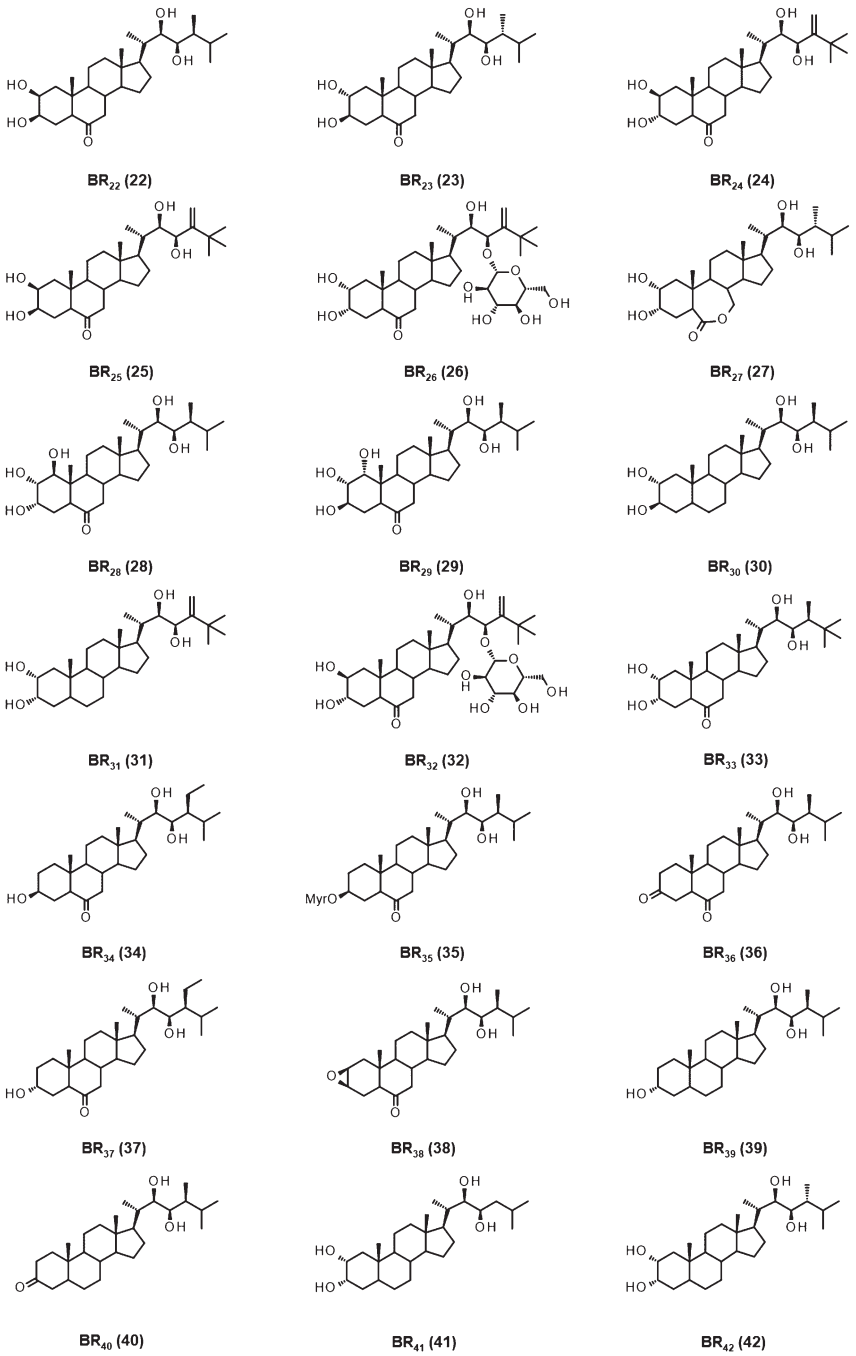

Fig. 1.1 (continued)

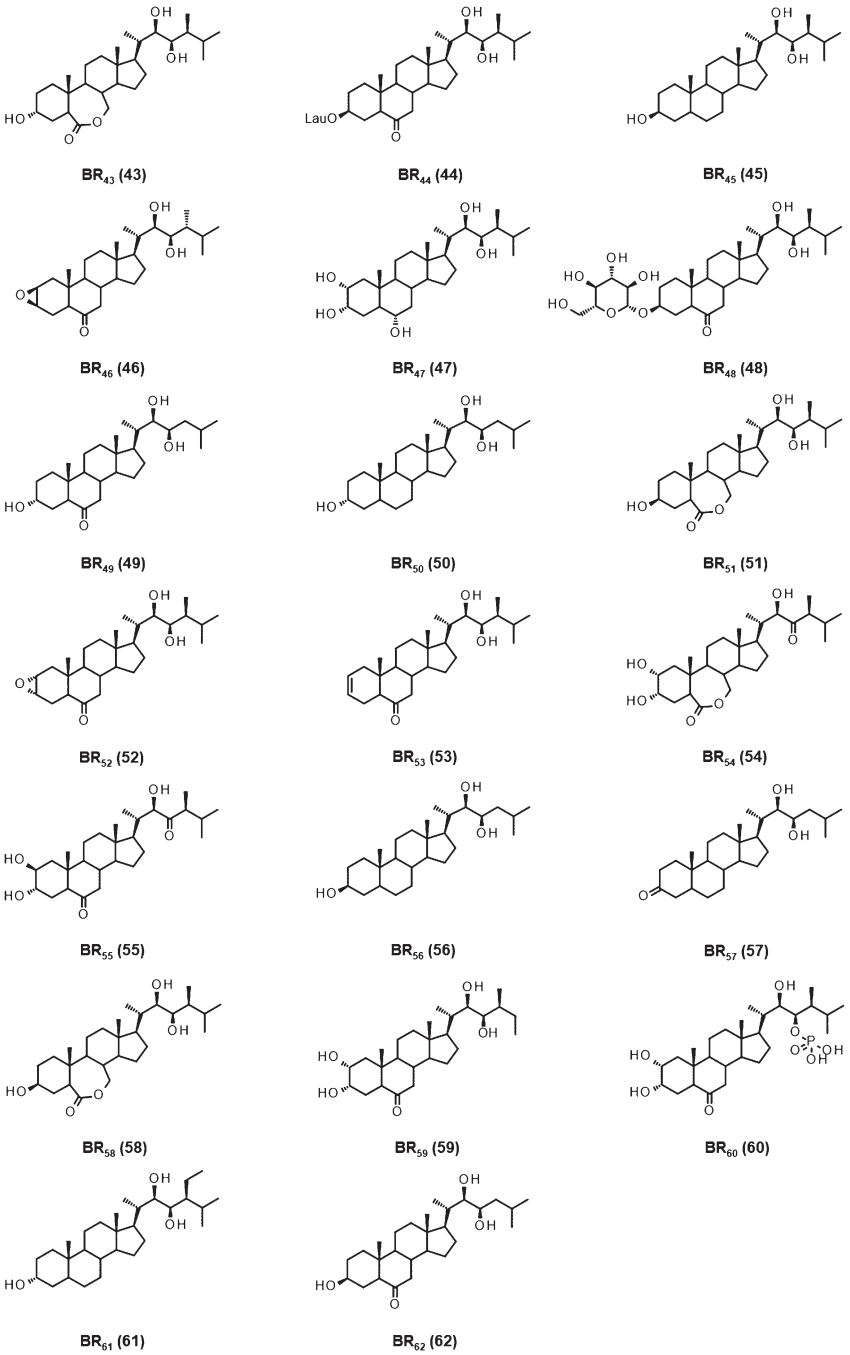

Fig. 1.1 (continued)

2 Natural Brassinosteroids

About sixty compounds with structures related to that of brassinolide (**1**) were isolated from or detected in plant materials in the last forty years (see Table 1.1 and Fig. 1.1). They were found in 26 species of 6 families of Algae, in 2 species of 2 families of Bryophyte, in 15 species of 8 families of Pteridophyte, in 6 species of 4 families of Gymnospermae, in 74 species of 35 families of Angiospermae (in 18 species of 6 families of Monocotyledoneae and 56 species of Dicotyledoneae), and in some plant derived products. About 15 biosynthetic precursors of brassinosteroids, some presenting brassinosteroid activity themselves, were found in many plant species.

Table 1.1 First report of a natural brassinosteroid

BR_n	Trivial name	References
1	Brassinolide	Grove et al. (1979)
2	Castasterone	Yokota et al. (1982a)
3	Dolicholide	Yokota et al. (1982b)
4	Dolichosterone	Baba et al. (1983)
5	6-Deoxocastasterone	Yokota et al. (1983c)
6	6-Deoxodolichosterone	Yokota et al. (1983c)
7	Typhasterol	Schneider et al. (1983)
8	Teasterone	Abe et al. (1984a)
9	24-Epicastasterone	Yokota et al. (1987b)
10	28-Homodolicholide	Yokota et al. (1983b)
11	28-Homodolichosterone	Baba et al. (1983)
12	28-Homocastasterone	Abe et al. (1983)
13	6-Deoxo-28-homodolichosterone	Yokota et al. (1987c)
14	28-Norbrassinolide	Abe et al. (1983)
15	28-Norcastasterone	Abe et al. (1983)
16	25-Methyldolichosterone	Kim et al. (1987)
17	28-Homobrassinolide	Ikekawa et al. (1984)
18	2-Deoxy-25-methyldolichosterone	Takahashi et al. (1988)
19	3-Epi-2-deoxy-25-methyldolichosterone	Yokota and Takahashi (1988)
20	2-Epicastasterone	Takahashi et al. (1988)
21	3-Epicastasterone	Takahashi et al. (1988)
22	2,3-Diepicastasterone	Takahashi et al. (1988)
23	3,24-Diepicastasterone	Takahashi et al. (1988)
24	2-Epi-25-methyldolichosterone	Takahashi et al. (1988)
25	2,3-Diepi-25-methyldolichosterone	Takahashi et al. (1988)
26	23-*O*-β-*D*-Glucopyranosyl-25-methyldolichosterone	Yokota et al. (1987a)
27	24-Epibrassinolide	Ikekawa et al. (1988)
28	1β-Hydroxycastasterone	Takahashi et al. (1988)

(continued)

Table 1.1 (continued)

BR_n	Trivial name	References
29	1α-Hydroxy-3-epicastasterone	Kim (1991)
30	3-Epi-6-deoxocastasterone	Kim (1991)
31	6-Deoxo-25-methyldolichosterone	Kim (1991)
32	23-*O*-β-*D*-Glucopyranosyl-2-epi-25-methyldolichosterone	Kim (1991)
33	25-Methylcastasterone	Taylor et al. (1993)
34	28-Homoteasterone	Schmidt et al. (1993b)
35	Teasterone-3-myristate	Asakawa et al. (1994)
36	3-Dehydroteasterone	Abe et al. (1994)
37	28-Homotyphasterol	Abe et al. (1995a)
38	Secasterone	Schmidt et al. (1995b)
39	6-Deoxotyphasterol	Griffiths et al. (1995)
40	3-Dehydro-6-deoxoteasterone	Griffiths et al. (1995)
41	6-Deoxo-28-norcastasterone	Spengler et al. (1995)
42	6-Deoxo-24-epicastasterone	Spengler et al. (1995)
43	2-Deoxybrassinolide	Schmidt et al. (1995c)
44	Teasterone-3-laurate	Asakawa et al. (1996)
45	6-Deoxoteasterone	Fujioka et al. (1998b)
46	24-Episecasterone	Friebe et al. (1999)
47	6α-Hydroxycastasterone	Fujioka et al. (2000b)
48	3-*O*-β-*D*-Glucopyranosylteasterone	Soeno et al. (2000b)
49	28-Nortyphasterol	Fujioka et al. (2000a)
50	6-Deoxo-28-nortyphasterol	Yokota et al. (2001)
51	3-Epibrassinolide	Konstantinova et al. (2001)
52	2,3-Diepisecasterone	Antonchick et al. (2003)
53	Secasterol	Antonchick et al. (2003)
54	Cryptolide	Watanabe et al. (2000)
55	23-Dehydro-2-epicastasterone	Hwang et al. (2006)
56	6-Deoxo-28-norteasterone	Bhardwaj et al. (2007)
57	3-Dehydro-6-deoxo-28-norteasterone	Bhardwaj et al. (2007)
58	3-Epi-2-deoxybrassinolide	Katsumata et al. (2008)
59	26-Norcastasterone	Son et al. (2013)
60	Castasterone 23-phosphate	Kim et al. (2015)
61	6-Deoxo-28-homotyphasterol	Xin et al. (2016)
62	28-Norteasterone	Oklestkova et al. (2017)

The finding that the rice lamina inclination assay, developed by Maeda (1965), to test for auxin activity could be used to detect the activity of brassinosteroids at even nanomolar or subnanomolar concentrations (Wada et al. 1981) and the development of a microanalytical method for the quantification of 22α, 23α-dihydroxybrassinosteroids (Takatsuto et al. 1982), allowed a rapid expansion of the number of known brassinosteroids. The first brassinosteroids isolated presented, as common features, (*i*) a 5α-cholestane or a 6,7-seco-5α-cholestane

derived skeleton, (*ii*) ring A with one to three oxygen functions (one always at carbon 3), (*iii*) ring B fully saturated or with varying degree of oxidation at carbon 6, (*iv*) all-*trans* ring junctions and (*v*) 22α,23α-dihydroxylation. In this sense, 3-oxygenated (20β)-5α-cholestane-22α,23α-diols of plant origin, bearing additional alkyl or oxy substituents, were considered as natural brassinosteroids (Zullo and Adam 2002). A more restricted definition states that, in the biosynthetic route to a brassinosteroid lactone, "one would consider as brassinosteroids only those compounds originated after the 22α,23α-dihydroxylation (i.e., those between teasterone or 6-deoxoteasterone and brassinolide), and hence as *brassinosteroid precursors* those before dihydroxylation occurs (i.e., those compounds up to cathasterone and 6-deoxocathasterone)" (Zullo et al. 2003; Zullo and Kohout 2004). After then some other brassinosteroids presenting 2,3-epoxy, 23-dehydro, 23-glycosidic, 23-ester functions, or 26-nor side chain or even 2,3-unsaturation were isolated, "allowing to consider as *natural brassinosteroids* the 3-oxygenated (20β)-5α-cholestane-22α,23α-diols or their derived compounds isolated from plants, bearing additional alkyl or oxy substituents" (Zullo 2018).

The unconjugated brassinosteroids so far isolated present 27 (C_{27}), 28 (C_{28}) or 29 (C_{29}) carbons, with 5α-cholestane or 26-nor-5α-campestanes (= 26-nor-24α-methyl-5α-cholestane) structures for the C_{27} series, 5α-campestane (= 24α-methyl-5α-cholestane), 5α-ergostane (= 24β-methyl-5α-cholestane) or 24-methylene-5α-cholestane skeletons for the C_{28} series, and 5α-sitostane (= 24α-ethyl-5α-cholestane), 24(*Z*)-ethylidene-5α-cholestane, 25-methyl-5α-campestane and 24-methylene-25-methyl-5α-cholestane structures for the C_{29} series (Fig. 1.2). Only one of the side chains of isolated brassinosteroids is of a 26-nor sterol, although C_{26}-demethylation of brassinosteroids have been demonstrated in metabolic studies with some species (Joo et al. 2012, 2015; Kim et al. 2000a, b). From the 12 different side chains of natural brassinosteroids, 9 of them present 22α,23α-dihydroxylation, while one presents a 22α-hydroxy-23-oxo group, another one presents conjugation of one glucose unit at the 23α-hydroxyl, and a last different side chain shows phosphorylation at the 23α-hydroxyl. Feeding studies shows that side chain glucosylation can occur at either C-23 (Poppenberger et al. 2005) or C-22 (Soeno et al. 2006), and also at C-25 or C-26 after hydroxylation at these carbons (Hai et al. 1996). Phosphorylation (Kim et al. 2015) and sulfonation (Rouleau et al. 1999) have been demonstrated to occur at the side chain of brassinosteroids, but while the first occurs at C-23, the second occurs at C-22, at least with the actual experimental data available.

It is known that the bioactivity of brassinosteroids is dependent on the structure of the side chain and of the A/B rings (Takatsuto et al. 1983b; Takatsuto et al. 1983a; Brosa et al. 1996; Takatsuto et al. 1987; Mandava 1988; Liu et al. 2017; Zullo and Adam 2002). Regarding to the side chain, as general rules, employing the rice lamina inclination assay on any of its versions (Maeda 1965; Wada et al. 1981; Fujioka et al. 1998a), for the same A/B ring structures, 22α,23α-dihidroxybrassinosteroids of the brassinolide series are so active as of the 28-homobrassinosteroids series (Takatsuto et al. 1983a), and more active than those of 24-epi- or 28-norbrassinosteroids (Takatsuto et al. 1983a; Wada et al. 1983),

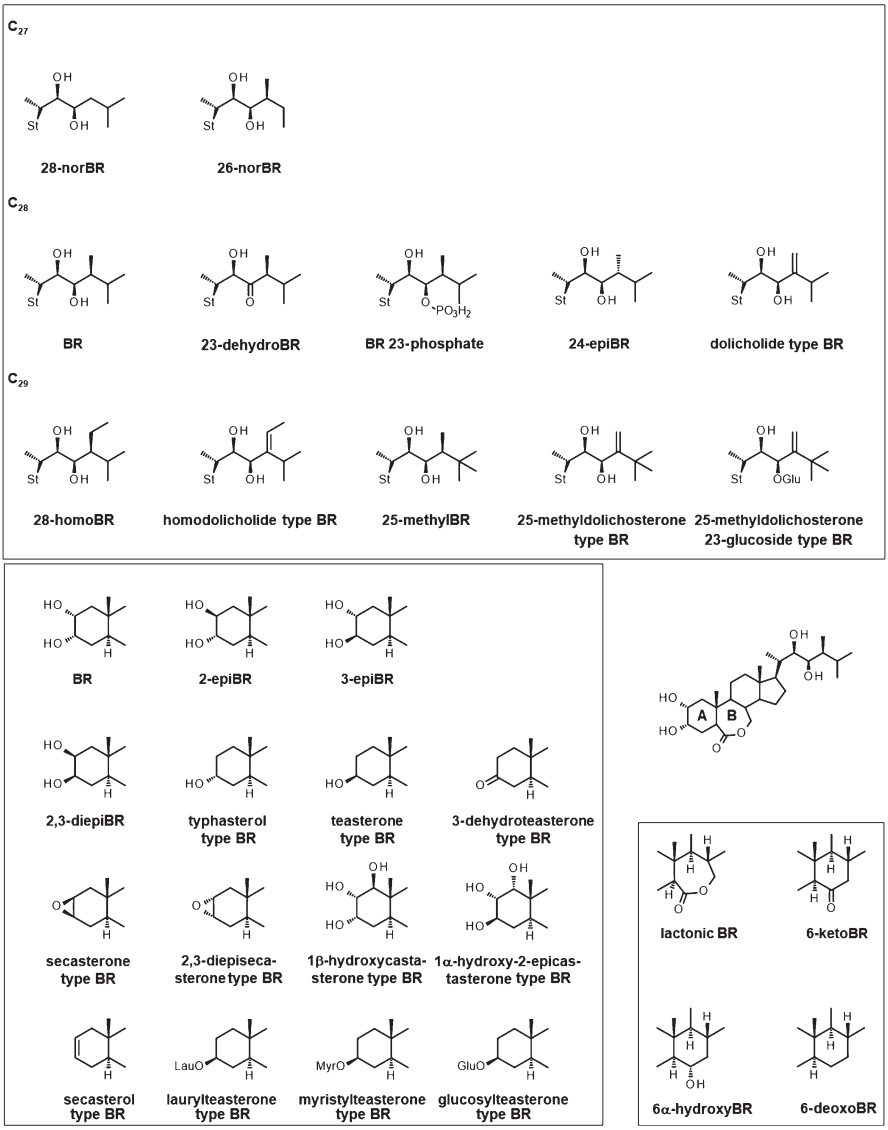

Fig. 1.2 Brassinolide and structural variations of brassinosteroids

which are more active than 26-norbrasssinosteroids (Kim et al. 2000a; Watanabe et al. 2001). 23-Dehydrogenation (Watanabe et al. 2001), or conjugation at one of the side chain hydroxyls (Suzuki et al. 1993b; Kim et al. 2015; Rouleau et al. 1999), diminishes (Yokota et al. 1998; Suzuki et al. 1993b) or abolishes the biological activity (Kim et al. 2015; Rouleau et al. 1999), an effect contrary to that observed with 25-methylation (Mori and Takeuchi 1988). It is to note that the relative

biological activity of brassinosteroids vary according to the biological assay performed for their evaluation, not only in relation to the side chain but also to the other active sites of their molecules (Takatsuto et al. 1983b; Watanabe et al. 2001; Zullo and Adam 2002; Liu et al. 2017).

A greater structural variation is observed in ring A, with 15 different structures reported, ranging from $\Delta^{2,3}$-unsaturated to trioxygenated and conjugated brassinosteroids: even so, this variation still does not reflect all the possible substructures at this ring, presumed either by efforts of large scale isolation of brassinosteroids (Kim 1991; Fujioka 1999), or by the study of the metabolism of brassinosteroids (Zullo 2018). The biological activity for brassinosteroids with A/B *trans* ring junctions increases as substitution in ring A changes in the order 3β-hydroxy ≤ 3-oxo ≤ 3α-hydroxy < 2α,3α-dihydroxy, and diminishes as deviates from these patterns (Mandava 1988; Zullo and Adam 2002; Liu et al. 2017; Takatsuto et al. 1987; Fujioka et al. 1995a).

The structural variations in ring B reflect the main steps in the biosynthesis of brassinosteroids (Vriet et al. 2013), being more active as its oxidation state increases (Mandava 1988) sequentially from the 6-deoxo to the 6α-hydroxy to the 6-oxo and to the 7-oxalactone types. Therefore, brassinosteroids can be classified, according to the B ring structure, as: (a) **6-oxo-7-oxalactonic brassinosteroids:** (*i*) 2α,3α-dihydroxylated: brassinolide (**1**), dolicholide (**3**), 28-homodolicholide (**10**), 28-norbrassinolide (**14**), 28-homobrassinolide (**17**), 24-epibrassinolide (**27**), cryptolide (**54**); (*ii*) 2α, 3β-dihydroxylated: 3-epibrassinolide (**51**); (*iii*) 3α-hydroxylated: 2-deoxybrassinolide (7-oxatyphasterol, **43**); (*iv*) 3β-hydroxylated: 3-epi-2-deoxybrassinolide (7-oxateasterone, **58**); (b) **6-oxo (or 6-keto) brassinosteroids:** (*i*) 2α,3α-dihydroxylated: castasterone (**2**), dolichosterone (**4**), 24-epicastasterone (**9**), 28-homodolichosterone (**11**), 28-homocastasterone (**12**), 28-norcastasterone (**15**), 25-methyldolichosterone (**16**), 25-methylcastasterone (**33**), 26-norcastasterone (**59**); (*ii*) 2β,3α-dihydroxylated: 2-epicastasterone (**20**), 2-epi-25-methyldolichosterone (**24**), 23-dehydro-2-epicastasterone (**55**); (*iii*) 2α,3β-dihydroxylated: 3-epicastasterone (**21**), 3,24-diepicastasterone (**23**); (*iv*) 2β,3β-dihydroxylated: 2,3-diepicastasterone (**22**), 2,3-diepi-25-methyldolichosterone (**25**); (*v*) 3α-monohydroxylated: typhasterol (**7**), 2-deoxy-25-methyldolichosterone (**18**), 28-homotyphasterol (**37**), 28-nortyphasterol (**49**); (*vi*) 3β-monohydroxylated: teasterone (**8**), 3-epi-2-deoxy-25-methyldolichosterone (**19**), 28-homoteasterone (**34**), 28-norteasterone (**62**); (*vii*) 1β,2α,3α-trihydroxylated: 1β-hydroxycastasterone (**28**); (*viii*) 1α,2α,3β-dihydroxylated: 1α-hydroxy-3-epicastasterone (**29**); (*ix*) 2α,3α-epoxide: 2,3-diepisecasterone (**52**); (*x*) 2β,3β-epoxide: secasterone (**38**), 24-episecasterone (**46**); (*xi*) Δ^2-olefin: secasterol (**53**); (*xii*) 3β-conjugates: teasterone-3-myristate (**35**), teasterone-3-laurate (**44**), 3-O-β-D-glucopyranosylteasterone (**48**); (*xiii*) 23α-conjugates: 23-*O*-β-*D*-glucopyranosyl-25-methyldolichosterone (**26**), 23-*O*-β-*D*-glucopyranosyl-2-epi-25-methyldolichosterone (**32**), castasterone 23-phosphate (**60**); (*xiv*) 3-dehydro: 3-dehydroteasterone (**36**); (c) **6α-hydroxybrassinosteroids:** 6α-hydroxycastasterone (**47**); (d) **6-deoxobrassinosteroids:** (*i*) 2α,3α-dihydroxylated: 6-deoxocastasterone (**5**), 6-deoxodolichosterone (**6**), 6-deoxo-

28-homodolichosterone (**13**), 6-deoxo-25-methyldolichosterone (**31**), 6-deoxo-28-norcastasterone (**41**), 6-deoxo-24-epicastasterone (**42**); (*ii*) 2α,3β-dihydroxylated: 3-epi-6-deoxocastasterone (**30**); (*iii*) 3α-monohydroxylated: 6-deoxotyphasterol (**39**), 6-deoxo-28-nortyphasterol (**50**), 6-deoxo-28-homotyphasterol (**61**); (*iv*): 3β-monohydroxylated: 6-deoxoteasterone (**45**), 6-deoxo-28-norteasterone (**56**); (*v*) 3-dehydro: 3-dehydro-6-deoxoteasterone (**40**), 3-dehydro-6-deoxo-28-norteasterone (**57**).

3 Brassinosteroids Precursors

A series of papers revealed the main steps of brassinosteroids biosynthesis, from the plant sterols to the brassinosteroid lactones, especially that from campesterol (**CR**) or campestanol (**CN**) to brassinolide (**1**). From these studies it became clear that, if the natural brassinosteroids can be easily recognized from their chemical structures, similar observation does not happen with their precursors (see Fig. 1.3 and Table 1.2). The first experiments established the biosynthesis of brassinolide (**1**) from teasterone (**8**) via, sequentially, 3-dehydroteasterone (**36**), typhasterol (**7**), and castasterone (**2**) (Suzuki et al. 1993a, 1994a, c) (follow by Fig. 1.4). Soon after it was found that campesterol (**CR**) was converted to campestanol (**CN**) and to 6α-hydroxycampestanol (**63**), 6-oxocampestanol (**64**), 22α-hydroxy-6-oxocampestanol (**65**), named cathasterone, and this one to teasterone (**8**) (Fujioka et al. 1995b). The complete biosynthetic sequence of brassinolide starting from campesterol (**CR**) via cathasterone (**65**) is known as the early C-6 oxidation pathway (a route in which C-6 oxidation occurs earlier than 22α,23α-dihydroxylation).

The frequent isolation or detection of 6-deoxobrassinosteroids brought the suspicion that another biosynthetic route to brassinosteroid lactones could exist. Feeding experiments with labeled precursors established the sequence 6-deoxoteasterone (**45**), 3-dehydro-6-deoxoteasterone (**40**), 6-deoxotyphasterol (**39**), 6-deoxocastasterone (**5**), castasterone (**2**), brassinolide (**1**), which was called the late C-6 oxidation pathway (a route in which C-6 oxidation occurs later than 22α, 23α-dihydroxylation) (Choi et al. 1997). It was further demonstrated the conversion of campestanol (**CN**) to 6-deoxoteasterone (**45**) through 6-deoxocathasterone (**66**) (Bishop et al. 1999), and the presence of 3-epi-6-deoxocathasterone (**67**), a putative brassinosteroid precursor, in cultured cells of *Catharantus roseus* (Fujioka et al. 2000b).

A thorough examination of the sterols present in cultured cells of *C. roseus* and in *Arabidopsis* seedlings, conjugated with metabolic studies with deuterated substrates, revealed that the conversion of campesterol (**CR**) to campestanol (**CN**) occurs through campest-4-en-3-one (**4en3one**) and campestan-3-one (**3one**) (Fujioka et al. 2002). Moreover, it revealed the operation of intermediates in the conversion of campesterol (**CR**) to 6-deoxocathasterone (**66**), originating

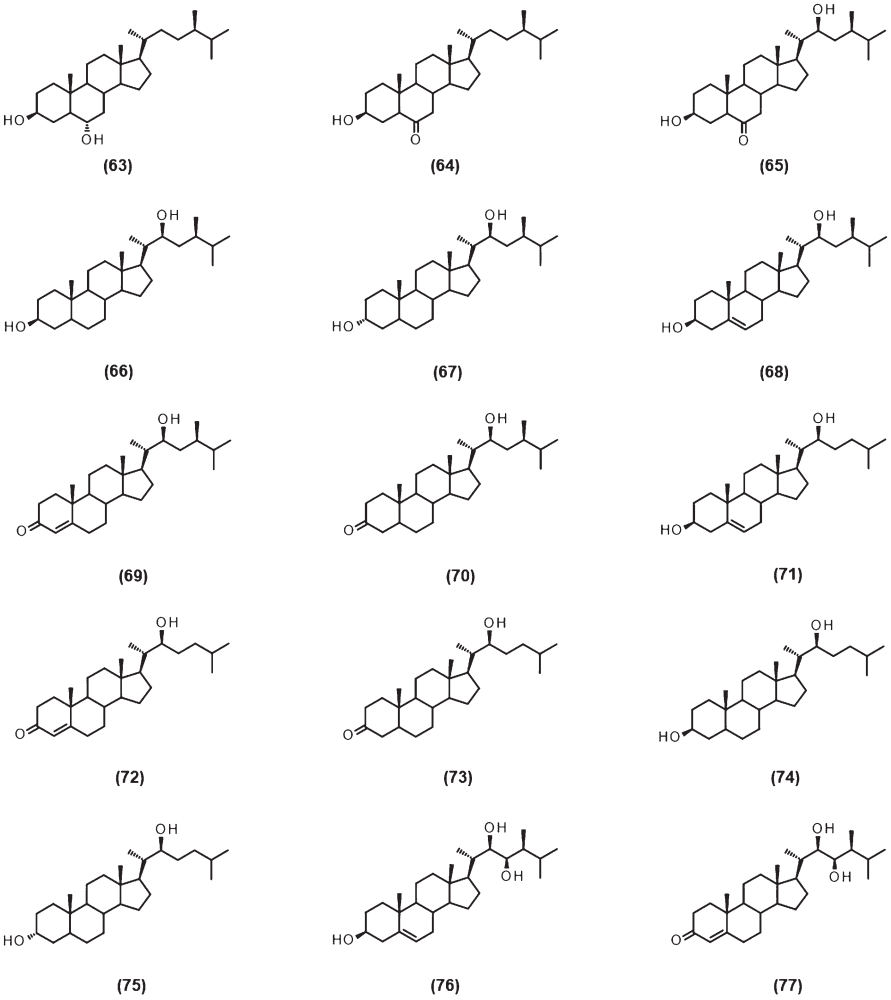

Fig. 1.3 Brassinosteroids precursors

22α-hydroxycampesterol (**68**), 22α-hydroxycampest-4-en-3-one (**69**), and 22α-hydroxy-5α-campestan-3-one (**70**) from, respectively, campesterol (CR), campest-4-en-3-one (**4en3one**) and campestan-3-one (**3one**). In the same extracts were found also the 28-norhomologues 22α-hydroxycholesterol (**71**), 22α-hydroxycholest-4-en-3-one (**72**), 22α-hydroxy-5α-cholestan-3-one (**73**), 6-deoxo-28-norcathasterone (**74**) and 3-epi-6-deoxo-28-norcathasterone (**75**). Later, studying the action of *Arabidopsis* CYP90C1 and CYP90D1, it was found that these enzymes act on 3-epi-6-deoxocathasterone (**67**), 22α-hydroxycampesterol (**68**), 22α-hydroxy-5α-campestan-3-one (**70**), and 22α-hydroxycampest-4-en-3-

Table 1.2 Brassinosteroid precursors

Compound	Trivial name	References
63	6α-Hydroxycampestanol	Fujioka et al. (1995b)
64	6-Oxocampestanol	Fujioka et al. (1995b)
65	Cathasterone	Fujioka et al. (1995b)
66	6-Deoxocathasterone	Bishop et al. (1999)
67	3-Epi-6-deoxocathasterone	Fujioka et al. (2000b)
68	22α-Hydroxycampesterol [(22*S*)-22-Hydroxycampesterol]	Fujioka et al. (2002)
69	22α-Hydroxycampest-4-en-3-one [(22*S*,24*R*)-22-Hydroxyergost-4-en-3-one]	Fujioka et al. (2002)
70	22α-Hydroxycampestan-3-one [(22*S*,24*R*)-22-Hydroxy-5α-ergostan-3-one]	Fujioka et al. (2002)
71	22α-Hydroxycholesterol [(22*S*)-28-Nor-22-hydroxycampesterol]	Fujioka et al. (2002)
72	22α-Hydroxycholest-4-en-3-one [(22*S*)-28-Nor-22-hydroxyergost-4-en-3-one]	Fujioka et al. (2002)
73	22α-Hydroxycholestan-3-one [(22*S*)-28-Nor-22-hydroxy-5α-ergostan-3-one]	Fujioka et al. (2002)
74	6-Deoxo-28-norcathasterone	Fujioka et al. (2002)
75	3-Epi-6-deoxo-28-norcathasterone	Fujioka et al. (2002)
76	22α,23α-Dihydroxycampesterol [(22*R*,23*R*)-22,23-Dihydroxycampesterol]	Ohnishi et al. (2006b)
77	22α,23α-Dihydroxycampest-4-en-3-one [(22*R*,23*R*)-22,23-Dihydroxycampest-4-en-3-one]	Ohnishi et al. (2006b)

one (**69**) to yield, respectively, 6-deoxotyphasterol (**39**), 22α, 23α-dihydroxycampesterol (**76**), 3-dehydro-6-deoxoteasterone (**40**), and 22α, 23α-dihydroxycampest-4-en-3-one (**77**), revealing a new shortcut in the biosynthesis of brassinosteroids. Compounds **63**-**77**, isolated from plant material, present side chains with no oxygen function or 22α-monohydroxylated or 22α,23α-dihydroxylated and rings A/B typical of common plant sterols (as 3β-hydroxy-Δ^5-sterols or 3β-hydroxy-5α-stanols) or less usual ones [like Δ^4-sten-3-ones (Franke et al. 2004; Georges et al. 2006; Pinto et al. 2002) or 5α-stan-3-ones (Guillen and Manzanos 2001)] or reflecting the steps for the construction of typical A/B rings of brassinosteroids (5α-stan-3β,6α-diols, 5α-stan-3β-ol-6-one, 5α-stan-3α-ol) (Fig. 1.5). None of these fragments, *per se*, can be attributed exclusively to brassinosteroids (Zullo 2018).

It is to note that only brassinosteroids precursors of campestane and cholestane skeletons had been isolated to date, what does not exclude the possibility of similar biosynthetic reactions can occur at the remaining skeletons (ergostane, sitostane, 24-methylenecholestane, 24-ethylydenecholestane, 25-methylcampestane and 24-methylene-25-methylcholestane), for all the possible sequences in the grid (as shown in Fig. 1.4) or through conversions of skeletons while functional-

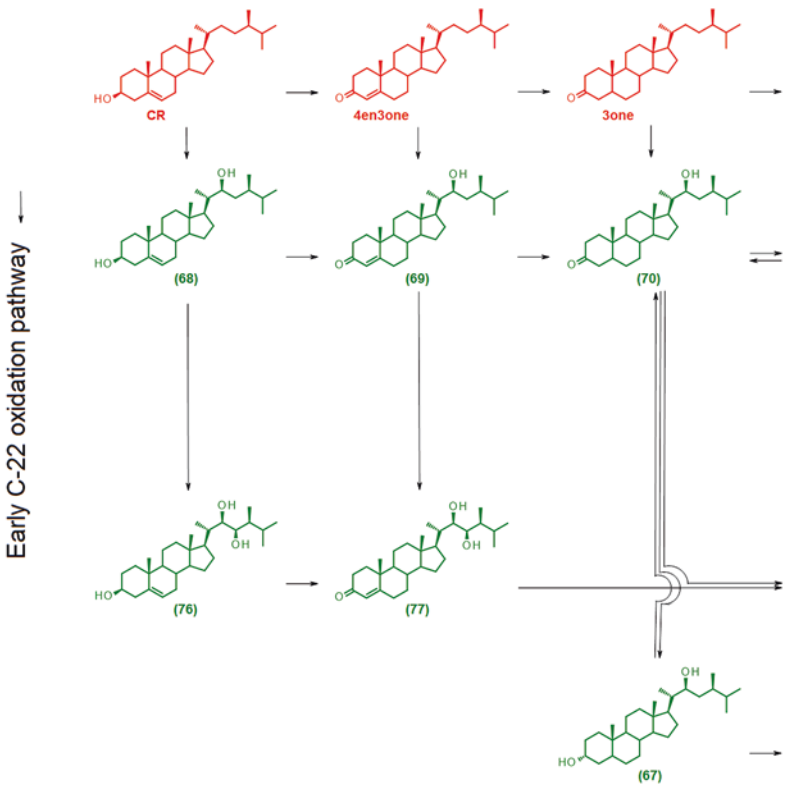

Fig. 1.4 Biosynthesis of brassinolide (1) from campesterol (red: sterols; green: brassinosteroids precursors; blue: brassinosteroids). Adapted from Zullo 2018

izing them towards the synthesis of castasterone-like or brassinolide-like brassinosteroids, what could explain the isolation or detection of brassinosteroids of different skeletons in the same plant materials. The fact that total sterols usually comprise 2–3 × 10^{-3} g/g of plant dry weight (Benveniste 2004) and that brassinosteroids are present usually in 10^{-12}–10^{-9} g/g fresh weight in plant material (Bajguz and Tretyn 2003; Takatsuto 1994), immersed in a matrix of tens of compounds of similar structure (and, hence, of similar polarity and similar chromatographic behavior), turns a very difficult task to determine the brassinosteroids profile of a given plant material, including the compounds of transient existence,

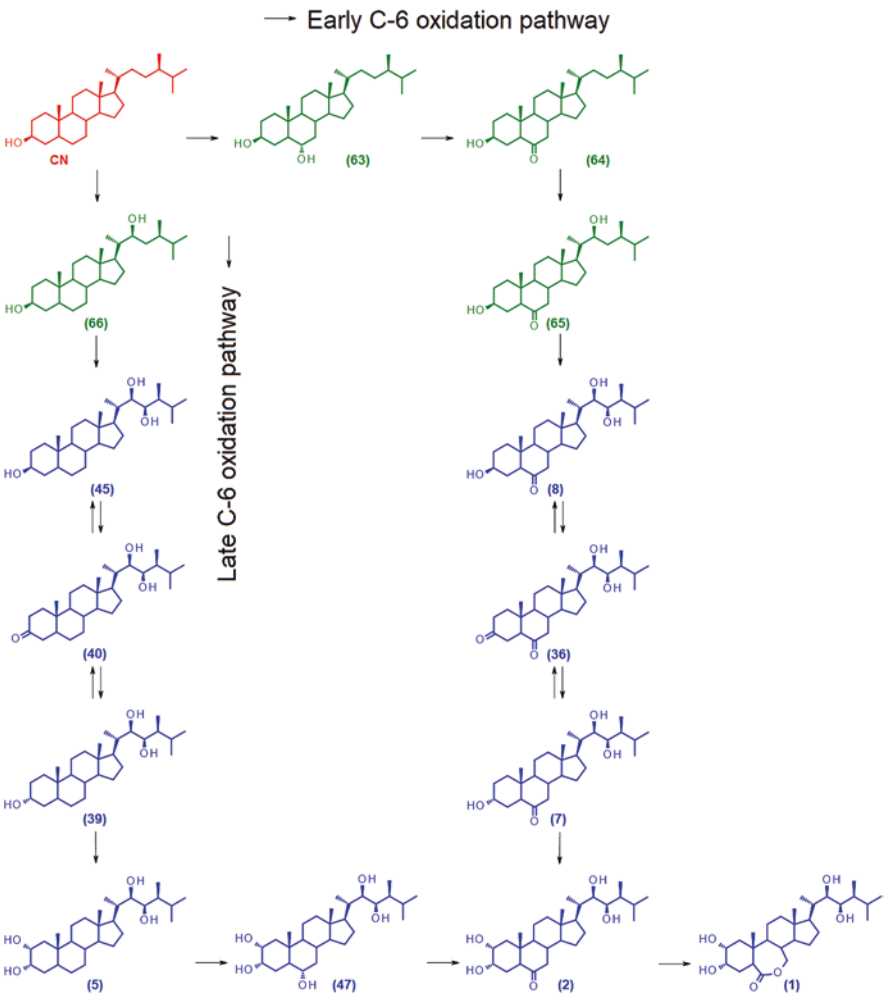

Fig. 1.4 (continued)

like their precursors, can explain why precursors of different skeletons have not been isolated yet.

4 Brassinosteroids with Partially Elucidated Structure

A few natural brassinosteroids were isolated in pure state in enough amount to identify them by the usual spectroscopic methods, but usually they are detected by comparison with authentic compounds prepared by synthesis. Sometimes, due to small

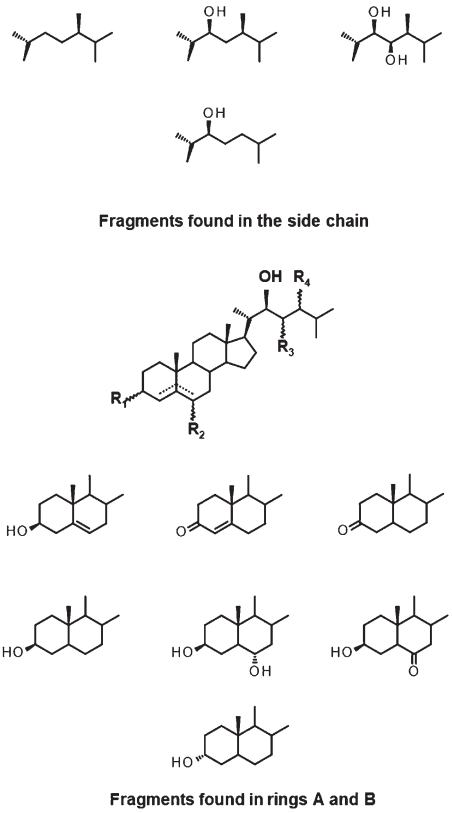

Fig. 1.5 Fragments found in brassinosteroids precursors

amounts of samples, to similar spectroscopic characteristics but different chromatographic behavior, it is not possible to determine the structure of all compounds present in a given brassinosteroids extract. Eventually the complete structure of one of these compounds is correctly elucidated.

One of the richest sources of brassinosteroids, the seeds of kidney beans, presents about 60 compounds of partially known structure (Hwang et al. 2006), for which some of them were described (Yokota et al. 1987c) (see Fig. 1.6). Among them is cited 1 isomer of 6-deoxo-28-homodolichosterone (**78**), 4 isomers of castasterone (**79**), 1 isomer of a hydroxylated castasterone (**80**), 2 isomers of 28-homocastasterone (**81**), 3 isomers of a homologue of dolichosterone (**82**), 1 isomer of a brassinolide derivative with 14 atomic units higher (**83**), 1 isomer of a brassinolide derivative with 44 atomic units higher (**84**), 1 isomer of dolicholide (**85**), 1 isomer of dolicholide with an extra oxygen (**86**), another one with an extra hydroxyl (**87**), a dolicholide derivative 28 atomic units higher (**88**), and another one

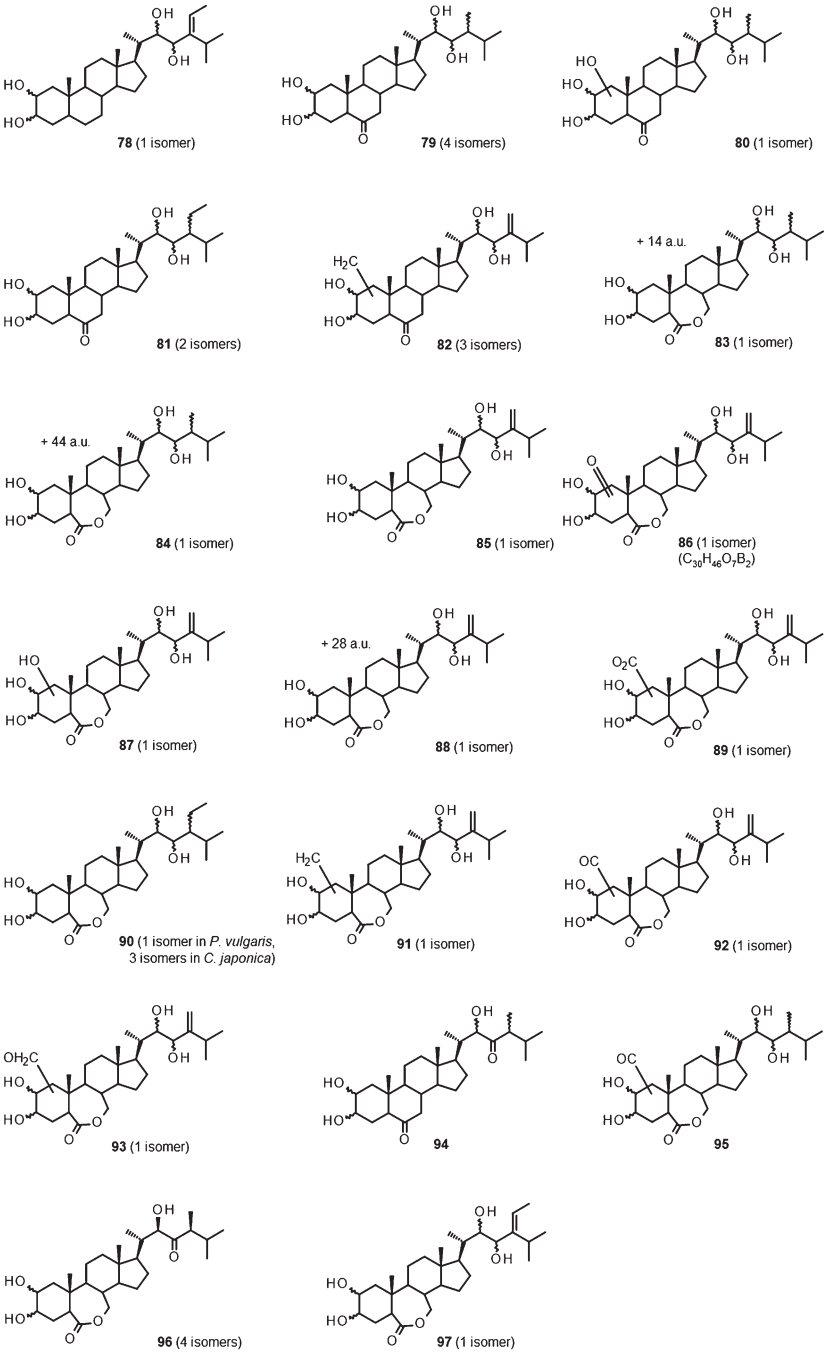

Fig. 1.6 Brassinosteroids with partially elucidated structure

with a carboxy group (**89**), an isomer of 28-homobrassinolide (**90**), an homologue of dolicholide (**91**) and its carbonyl derivative (**92**), a carbonyl homologue of dolicholide (**93**) (Yokota et al. 1987c). Two other brassinosteroids were reported in *Phaseolus vulgaris*, ξ-epi-23-dehydrocastasterone (**94**) and an homologue with a carbonyl group (**95**) (Kim 1991). Three isomers of 28-homobrassinolide (**90**), four isomers of 23-dehydrobrassinolide (**96**), and one isomer of 28-homodolicholide (**97**) were reported in pollen and anthers of *Cryptomeria japonica* (Yokota et al. 1998). 25-Methyldolichosterone (**16**) was later identified as one of the isomers of (**82**) (Kim et al. 1987), as well as cryptolide (**54**) as one of the four isomers of 23-dehydrobrassinolide (**96**) (Watanabe et al. 2000).

5 Occurrence of Brassinosteroids

Brassinosteroids have been isolated from different plant organs such as pollen, anthers, seeds, leaves, stems, roots, flowers, and grain as well as in insect and crown galls. The endogenous level of brassinosteroids varies from plant's organ and the age of the plant. Pollen and immature seeds are found to have the highest concentration of brassinosteroids, however, young growing tissues contain higher levels of brassinosteroids than mature tissues. The presence of some bioactive brassinosteroids viz., castasterone (**2**, BR_2), brassinolide (**1**, BR_1), 6-deoxocastasterone (**5**, BR_5), teasterone (**8**, BR_8), typhasterol (**7**, BR_7) and 3-dehydro-6-deoxoteasterone (**40**, BR_{40}) was confirmed in at least 103, 71, 40, 34, 28 and 28 plant species, respectively. Brassinolide (**1**) and castasterone (**2**) are widely distributed in algae and flowering plants, but only castasterone (**2**) was detected in lower non-flowering plants (liverwort, moss, lycophytes and ferns). Their presence in so many species, from the simplest algae to the more complex phanerogams, as well as the increasing detection in many new species indicates their ubiquitous distribution in the plant kingdom, what is expected from their role as plant hormones.

Table 1.3 lists the occurrence of brassinosteroids in plant species and Table 1.4 the occurrence of the established brassinosteroids precursors. It does not discriminate from which organ they were isolated or detected, or the concentration which they were found, so, primary source of information must be retrieved for proper use of their data.

Brassinosteroids were also found in plant derived products, as 24-epibrassinolide (**27**) in biodiesel cakes of *Brassica carinata* A. Braun or *Brassica napus* L. (Bardi and Rosso 2015); brassinolide (**1**), castasterone (**2**), typhasterol (**7**), teasterone (**8**) and 28-homocastasterone (**12**) in a vermicompost leachate (Aremu et al. 2015); and brassinolide (**1**), castasterone (**2**), 28-norbrassinolide (**14**) and 28-norcastasterone (**15**) in date (*Phoenix dactilifera* L.), medlar (*Eryobotrya japonica* Lindl.), milkvetch (*Astragalus* sp.), rape (*Brassica napus* L.) and robinia (*Robinia pseudo-acacia* L.) honeys, and also 28-homobrassinolide (**17**) in the last four honeys (Wang et al. 2017).

Table 1.3 Occurrence of natural brassinosteroids

Species	Family	Brassinosteroids	References
Acutodesmus acuminatus (Lagerh.) Tsarenko	*Chlorophyceae*	BR_1 BR_2 BR_7 BR_{12}	Stirk et al. (2013, 2018)
Acutodesmus incrassatulus (Bohlin) Tsarenko	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Aegle marmelos Correa	*Rutaceae*	BR_{27}	Sondhi et al. (2008)
Alnus glutinosa Gaertn.	*Betulaceae*	BR_1 BR_2	Plattner et al. (1986)
Amaranthus inamoenus	*Amaranthaceae*	BR_2	Takatsuto et al. (1999)
Apium graveolens L.	*Umbelliferae*	BR_{43}	Schmidt et al. (1995c)
Arabidopsis thaliana (L.) Henyh.	*Brassicaceae*	BR_1 BR_2 BR_4 BR_5 BR_7 BR_8 BR_{15} BR_{27} BR_{36} BR_{39} BR_{40} BR_{45} BR_{49} BR_{51} BR_{58} BR_{59} BR_{60}	Fujioka et al. (1996, 1997, 1998b, 2000a), Schmidt et al. (1997), Noguchi et al. (1999, 2000), Choe et al. (2001, 2002), Konstantinova et al. (2001), Nomura et al. (2001), Bancos et al. (2002, 2006), He et al. (2003), Kim et al. (2005a, 2006a, 2015), Carland et al. (2010), Shimada et al. (2003), Turk et al. (2003, 2005), Nakamura et al. (2005), Poppenberger et al. (2005), Takahashi et al. (2005), Chung et al. (2010), Beste et al. (2011), Schneider et al. (2012), Choi et al. (2013), Zhu et al. (2013), Best et al. (2016), Antonchick et al. (2006), Lee et al. (2006, 2010), Ohnishi et al. (2006b, 2012),

(continued)

Table 1.3 (continued)

Species	Family	Brassinosteroids	References
			Swaczynova et al. (2007), Katsumata et al. (2008), Huo et al. (2012), Villiers et al. (2012), Roh et al. (2012), Polko et al. (2013), Son et al. (2013), Xin et al. (2013), Singh et al. (2014), Lv et al. (2014), Kasote et al. (2016), Youn et al. (2016), Ding et al. (2016), Xu et al. (2016), and Chen et al. (2018)
Areca catechu L.	*Arecaceae*	BR_1	Wang and Lu (2008)
Atractylodes lancea	*Compositae*	BR_1	Ren et al. (2014)
Atryrium yokoscence (Fr. & Sav.) C. Ch.	*Woodsiaceae*	BR_2 BR_5 BR_8 BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Attalea vitrivir Zona	*Arecaceae*	BR_1 BR_2	Dias et al. (2017)
Banksia grandis Willd.	*Proteaceae*	BR_1 BR_2	Takatsuto (1994)
Beta vulgaris L.	*Chenopodiaceae*	BR_2 BR_9	Schmidt et al. (1994)
Brassica campestris var. *pekinensis*	*Brassicaceae*	BR_1 BR_2 BR_{12} BR_{14} BR_{15} BR_{17} BR_{27}	Abe et al. (1982, 1983), Ikekawa et al. (1984), Ikekawa and Takatsuto (1984), Pan et al. (2013), and Lv et al. (2014)
Brassica carinata A. Braun	*Brassicaceae*	BR_{27}	Bardi and Rosso (2015)
Brassica juncea L.	*Brassicaceae*	BR_2 BR_7 BR_8 BR_{27}	Kanwar et al. (2012, 2013, 2015)
Brassica napus L.	*Brassicaceae*	BR_1 BR_2 BR_4 BR_5 BR_7 BR_8 BR_{12} (or BR_{33}) BR_{14} BR_{15} BR_{27} (or BR_{51}) BR_{28} BR_{29} BR_{40} BR_{41} BR_{47}	Grove et al. (1979), Swaczynova et al. (2007), Ding et al. (2014a, b), Zhang et al. (2010), Pan et al. (2012), Ding et al. (2013a, b, 2016), Oklestkova et al. (2017), and Yu et al. (2017)
Brassica napus var. *oleifera*	*Brassicaceae*	BR_{27}	Bardi and Rosso (2015)
Butia capitata (Mart.) Becc.	*Arecaceae*	BR_1 BR_2	Dias et al. (2017)
Camellia sinensis (O) Kuntze (= *Thea sinensis* L.)	*Theaceae*	BR_5 BR_7 BR_{10} BR_{27} BR_{36} BR_{39} BR_{41} BR_{50} BR_{56} BR_{57}	Gupta et al. (2004) and Bhardwaj et al. (2007)
Cannabis sativa L.	*Cannabaceae*	BR_2 BR_8	Takatsuto et al. (1996b)

(continued)

Table 1.3 (continued)

Species	Family	Brassinosteroids	References
Castanea crenata Sieb. et Zucc	*Fagaceae*	BR_1 BR_2 BR_7 BR_8 BR_{15}	Park et al. (1994a), Yokota et al. (1982a), Abe et al. (1983), Ikeda et al. (1983), Arima et al. (1984), Ikekawa et al. (1984)
Catharanthus roseus Don.	*Apocynaceae*	BR_1 BR_2 BR_5 BR_7 BR_8 BR_{21} BR_{25} BR_{39} BR_{40} BR_{45} BR_{47}	Park et al. (1989), Yokota et al. (1990a), Choi et al. (1993, 1996, 1997), Suzuki et al. (1993a, 1994a, 1995), Fujioka et al. (1995b, 2000b), and Fujioka and Sakurai (1997)
Centella asiatica (L.) Urban	*Apiaceae*	BR_2	Sondhi et al. (2010)
Chlamydomonas reinhardtii P.A. Dang.	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Chlorella minutissima Fott et Nováková	*Trebouxiophyceae*	BR_1 BR_2 BR_{42}	Stirk et al. (2013, 2014a)
Chlorella pyrenoidosa Chick	*Trebouxiophyceae*	BR_1 BR_2	Stirk et al. (2013)
Chlorella vulgaris Beijerinck	*Trebouxiophyceae*	BR_1 BR_2 BR_5 BR_7 BR_8 BR_{12} BR_{39} BR_{40} BR_{45}	Stirk et al. (2013, 2018), Bajguz (2009), and Bajguz and Piotrowska-Niczyporuk (2013, 2014)
Chlorococcum ellipsoideum Deason et Bold	*Chlorophyceae*	BR_1 BR_2 BR_7 BR_{12}	Stirk et al. (2013, 2018)
Cistus hirsutum Theill.	*Cistaceae*	BR_1 BR_2	Takatsuto (1994)
Citrus sinensis Osbeck	*Rutaceae*	BR_1 BR_2	Motegi et al. (1994)
Citrus unshiu Marcov.	*Rutaceae*	BR_1 BR_2 BR_7 BR_8	Takatsuto (1994)
Coccomyxa sp.	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Coelastrum microporum Nägeli	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Cryptomeria japonica D. Don.	*Taxodiaceae*	BR_3 BR_7 BR_{10} BR_{17} BR_{36} BR_{54}	Watanabe et al. (2000), Takatsuto (1994), and Yokota et al. (1998)
Cucumis sativus L.	*Cucurbitaceae*	BR_1	Hou et al. (2017)
Cucurbita moschata Duchesne	*Cucurbitaceae*	BR_1 BR_2	Jang et al. (2000) and Pachthong et al. (2006)
Cupressus arizonica E. Greene	*Cupressaceae*	BR_1 BR_2 BR_4 BR_5 BR_7 BR_8 BR_{12} BR_{36} BR_{39} BR_{40}	Griffiths et al. (1995)

(continued)

Table 1.3 (continued)

Species	Family	Brassinosteroids	References
Cyrtomium laetevirens (Hiyama) Nakaike	*Dryopteridaceae*	BR_2 BR_{40} BR_{45}	Yokota et al. (2017)
Daucus carota ssp. *sativus* L.	*Apiaceae*	BR_1 BR_2 BR_9 BR_{27}	Schmidt et al. (1998), Swaczynova et al. (2007), and Lv et al. (2014)
Deparia japonica (Thunb.) M. Kato	*Woodsiaceae*	BR_2 BR_5 BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Desmodesmus armatus (R. Chodat) E. Hegewald	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Diospyros kaki Thunb.	*Ebenaceae*	BR_2	Takatsuto (1994)
Distylium racemosum Sieb et Zucc.	*Hammamelidaceae*	BR_1 BR_2 BR_7 BR_8 BR_{14} BR_{15} BR_{36}	Ikekawa et al. (1984), Ikekawa and Takatsuto (1984), and Abe et al. (1994)
Dolichos lablab Adans.	*Leguminosae*	BR_1 BR_2 BR_3 BR_4 BR_5 BR_6 BR_{10} BR_{11}	Yokota et al. (1982b, 1983b, 1984) and Baba et al. (1983)
Dryopteris crassirhizoma Nakai (1920)	*Dryopteridaceae*	BR_2 BR_5 BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Dryopteris erythrososa (D.C.Eaton) Kuntze	*Dryopteridaceae*	BR_2 BR_5 BR_8 BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Echium plantagineum L.	*Boraginaceae*	BR_1 BR_2	Takatsuto (1994)
Ecklonia máxima (Osbeck) Papenfuss	*Phaeophyceae*	BR_1 BR_2	Stirk et al. (2014b)
Elaeis guineenses Jacq. var. *tenera*	*Palmae*	BR_1	Habib et al. (2012)
Equisetum arvense L.	*Equisetaceae*	BR_2 BR_4 BR_5 BR_{14} BR_{15} BR_{39} BR_{40} BR_{45}	Yokota et al. (2017) and Takatsuto et al. (1990a)
Eriobotrya japonica Lindl.	*Rosaceae*	BR_2	Takatsuto (1994)
Erythronium japonicum Decne	*Liliaceae*	BR_7	Yasuta et al. (1995)
Eucalyptus calophylla R. Br.	*Myrtaceae*	BR_2	Takatsuto (1994)
Eucalyptus marginata Sn.	*Myrtaceae*	BR_4	Takatsuto (1994)
Fagopyrum esculentum Moench.	*Polygonaceae*	BR_1 BR_2	Takatsuto et al. (1990b)
Ginkgo biloba L.	*Gingkoaceae*	BR_8	Takatsuto et al. (1996a)
Gyoerffyana humicola Kol et Chodat	*Chlorophyceae*	BR_1 BR_2 BR_7 BR_{12}	Stirk et al. (2013, 2018)

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Table 1.3 (continued)

Species	Family	Brassinosteroids	References
Gypsophila perfoliata L.	*Caryophyllaceae*	BR_{27}	Schmidt et al. (1996)
Helianthus annuus L.	*Asteraceae*	BR_1 BR_2 BR_{15} BR_{27}	Takatsuto et al. (1989) and Pan et al. (2012)
Hordeum vulgare L.	*Poaceae*	BR_2 BR_{12} BR_{27}	Dockter et al. (2014) and Gruszka et al. (2016)
Humulus lupulus L.	*Cannabaceae*	BR_1 BR_2 BR_3 BR_7 BR_9 BR_{11} BR_{14} BR_{17} BR_{27} BR_{62}	Oklestkova et al. (2017) and Chen et al. (2018)
Hydrodictyon reticulatum (L.) Lagerheim	*Hydrodictyaceae*	BR_9 BR_{12}	Yokota et al. (1987b)
Klebsormidium flaccidum (Kütz.) P.C. Silva, K.R. Mattox et W.H. Blackw.	*Charophyceae*	BR_1 BR_2	Stirk et al. (2013)
Lagenaria ciceraria	*Cucurbitaceae*	BR_2	Takatsuto and Makiuchi (2000)
Lilium elegans Thunb.	*Araceae*	BR_1 BR_2 BR_7 BR_8	Suzuki et al. (1994b)
Lilium longiflorum Thunb.	*Araceae*	BR_1 BR_2 BR_7 BR_8 BR_{35} BR_{36} BR_{44} BR_{48}	Asakawa et al. (1994, 1996), Abe (1991), Abe et al. (1994), Soeno et al. (2000a, b)
Lolium perenne L.	*Poaceae*	BR_{33}	Taylor et al. (1993)
Luffa cylindrica (L.) M.J. Roem	*Cucurbitaceae*	BR_1 BR_2	Pachthong et al. (2007)
Lychnis viscaria L.	*Caryophyllaceae*	BR_9 BR_{46}	Friebe et al. (1999)
Lycopersicon esculentum Mill.	*Solanaceae*	BR_1 BR_2 BR_5 BR_7 BR_8 BR_9 BR_{14} BR_{15} BR_{27} BR_{39} BR_{40} BR_{41} BR_{42} BR_{45} BR_{47} BR_{50} BR_{60}	Yokota et al. (1997), Bishop et al. (1999), Koka et al. (2000), Nomura et al. (2001, 2005), Yokota et al. (2001), Van Meulebroek et al. (2012), Wu et al. (2013), and Kim et al. (2015)
Lygodium japonicum (Thunb.) Sw.	*Lygodiaceae*	BR_2 BR_5 BR_8 BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Malus prunifolia (Willd.) Borkh.	*Rosaceae*	BR_2 BR_5 BR_7 BR_8 BR_{39} BR_{40} BR_{45}	Pereira-Netto et al. (2009)
Marchantia polymorpha L.	*Marchantiaceae*	BR_2 BR_5 BR_8 BR_{40}	Kim et al. (2002) and Yokota et al. (2017)
Matricaria recutita L.	*Compositae*	BR_{17} BR_{34}	Pradko et al. (2015)
Matteuccia struthiopteris (L.) *Tod.*	*Woodsiaceae*	BR_2 BR_5 BR_{40}	Yokota et al. (2017)

(continued)

Table 1.3 (continued)

Species	Family	Brassinosteroids	References
Monoraphidium contortum (Thur.) Komárková-Legnerová	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Myrmecia bisecta Reisigl	*Trebouxiophyceae*	BR_1 BR_2	Stirk et al. (2013)
Nautococcus mamillatus Korschikov	*Chlorophyceae*	BR_1 BR_2 BR_7 BR_{12}	Stirk et al. (2013, 2108)
Nicotiana tabacum L.	*Solanaceae*	BR_2 BR_5 BR_8 BR_{39} BR_{40} BR_{45}	Ohnishi et al. (2006a)
Onoclea sensibilis L.	*Woodsiaceae*	BR_2 BR_5 BR_{40}	Yokota et al. (2017)
Ornithopus sativus Brot.	*Fabaceae*	BR_2 BR_5 BR_9 BR_{41} BR_{42}	Schmidt et al. (1993a) and Spengler et al. (1995)
Oryza sativa L.	*Poaceae*	BR_1 BR_2 BR_4 BR_5 BR_7 BR_8 BR_9 BR_{12} BR_{15} BR_{17} BR_{27} BR_{34} BR_{36} BR_{37} BR_{39} BR_{40} BR_{42} BR_{45} BR_{61}	Abe et al. (1995a, 1984b), Mori et al. (2002), Wu et al. (2008), Nakamura et al. (2006), Asahina et al. (2014), Tanabe et al. (2005), Sakamoto et al. (2006, 2012); Kim et al. (2008), Ding et al. (2013a, b, 2014b, 2016), Li et al. (2013), Xin et al. (2013, 2016), Wang et al. (2014), Joo et al. (2015), Yu et al. (2016), Qian et al. (2017), Deng et al. (2016), Tamiru et al. (2016), Yokota et al. (2017), Ikekawa and Takatsuto (1984), Abe (1991); Shim et al. (1996), Park et al. (1994b), and Chen et al. (2018)
Osmunda japonica Thunb.	*Osmundaceae*	BR_2 BR_5 BR_7 BR_8 BR_{36} BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Perilla frutescens Britton.	*Labiatae*	BR_2 BR_{10}	Park et al. (1994b)
Petunia hybrida line W138	*Solanaceae*	BR_1 BR_2 BR_5 BR_7 BR_8 BR_{36} BR_{39} BR_{40} BR_{45}	Verhoef et al. (2013)
Phalaris canariensis L.	*Poaceae*	BR_2 BR_8	Shimada et al. (1996)
Pharbitis nil (L.) Choisy	*Convolvulaceae*	BR_2 BR_5 BR_7 BR_8 BR_{39} BR_{45}	Suzuki et al. (2003)
Pharbitis purpurea Voigt	*Convolvulaceae*	BR_2 BR_{15}	Suzuki et al. (1985)

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Table 1.3 (continued)

Species	Family	Brassinosteroids	References
Phaseolus vulgaris L.	*Fabaceae*	BR_1 BR_2 BR_3 BR_4 BR_5 BR_6 BR_7 BR_8 BR_{11} BR_{12} BR_{13} BR_{14} BR_{16} BR_{18} BR_{19} BR_{20} BR_{21} BR_{22} BR_{23} BR_{24} BR_{25} BR_{26} BR_{27} BR_{28} BR_{29} BR_{30} BR_{31} BR_{32} BR_{37} BR_{55} BR_{62}	Yokota et al. (1983c), Kim et al. (1987), Yokota and Takahashi (1988); Park et al. (2009a, b), Yokota et al. (1987a, c, 1990b); Kim (1991); Kim et al. (2000c, 2006b); Hwang et al. (2006, 2007), Swaczynova et al. (2007), Lee et al. (2011), and Oklestkova et al. (2017)
Phoenix dactylifera L.	*Arecaceae*	BR_9	Zaki et al. (1993)
Physcomitrella patens (Hedw.) Bruch & Schimp.	*Funariaceae*	BR_2 BR_5 BR_8 BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Picea sitchensis (Bong.) Carr.	*Pinaceae*	BR_2 BR_7	Yokota et al. (1985)
Pinus silvestris Lour.	*Pinaceae*	BR_1 BR_2	Kim et al. (1990)
Pinus thunbergii Parl.	*Pinaceae*	BR_2 BR_7	Yokota et al. (1983a)
Pisum sativum L.	*Fabaceae*	BR_1 BR_2 BR_5 BR_7 BR_{36} BR_{39} BR_{40} $BR_{43}BR_{45}$	Yokota et al. (1996) and Nomura et al. (1997, 2001, 2004, 2007)
Poloidion didymos Pascher	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Protococcus viridis C. Agardh	*Chlorophyceae*	BR_1 BR_2 BR_7 BR_{12}	Stirk et al. (2013, 2018)
Protosiphon botryoides G.A. Klebs	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Psophocarpus tetragonolobus DC	*Fabaceae*	BR_1 BR_2 BR_5 BR_{12}	Yokota et al. (1991) and Takatsuto (1994)
Pteridium aquilinum (L.) Kuhn in Kersten (1879)	*Dennstaedtiaceae*	BR_2 BR_5 BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Pyrus communis L.	*Rosaceae*	BR_1 BR_2	Oikawa et al. (2015)
Raphanus sativus L.	*Brassicaceae*	BR_1 BR_2 BR_8 BR_{34}	Schmidt et al. (1991, 1993b)
Raphidocelis subcapitata (Korshikov) G. Nygaard, J. Komárek, Kristiansen et Skulberg	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)

(continued)

Table 1.3 (continued)

Species	Family	Brassinosteroids	References
Rheum rhabarbarum L.	*Polygonaceae*	BR_1 BR_2 BR_9	Schmidt et al. (1995a)
Robinia pseudo-acacia L.	*Fabaceae*	BR_2 BR_5 BR_7	Abe et al. (1995b)
Scotiellopsis terrestris (Reisigl) Pun_coch. et Kalina	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Secale cereale L.	*Poaceae*	BR_2 BR_5 BR_7 BR_8 BR_{12} BR_{15} BR_{20} BR_{21} BR_{38} BR_{52} BR_{53}	Schmidt et al. (1995b), Antonchick et al. (2003, 2005), and Pociecha et al. (2016)
Selaginella moellendorffii Hieronymus	*Sellaginellaceae*	BR_2 BR_5 BR_{40}	Yokota et al. (2017)
Selaginella uncinata (Desv. ex Poir.) Spring	*Sellaginellaceae*	BR_2 BR_5 BR_8 BR_{39}	Yokota et al. (2017)
Solidago altissima L.	*Asteraceae*	BR_1	Takatsuto (1994)
Spongiochloris excentrica Starr	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Sporobolus stapfianum Gand.	*Poaceae*	BR_1 BR_2 BR_3	Sasse et al. (1998)
Stichococcus bacillaris Nägeli	*Trebouxiophyceae*	BR_1 BR_2	Stirk et al. (2013)
Stigeoclonium nanum (Dillwyn) Kütz.	*Chlorophyceae*	BR_1 BR_2	Stirk et al. (2013)
Thea sinensis L. (= *Camellia sinensis* (O) Kuntze)	*Theaceae*	BR_1 BR_2 BR_7 BR_8 BR_{12} BR_{15}	Abe et al. (1983, 1984a), Morishita et al. (1983), and Ikekawa and Takatsuto (1984)
Thelypteris decursive-pinnata (H.C. Hall) Ching, 1936	*Thelypteridaceae*	BR_2 BR_5 BR_{39} BR_{40} BR_{45}	Yokota et al. (2017)
Thelypteris palustris Schott	*Thelypteridaceae*	BR_5 BR_8 BR_{40} BR_{45}	Yokota et al. (2017)
Triticum aestivum L.	*Poaceae*	BR_1 BR_2 BR_5 BR_7 BR_8 BR_{12} BR_{17} BR_{27} BR_{36}	Yokota et al. (1994) and Janeczko and Swaczynova (2010)
Tulipa gesneriana L.	*Liliaceae*	BR_7	Takatsuto (1994)
Typha latifolia Mey.	*Typhaceae*	BR_7	Schneider et al. (1983) and Yoshihara and Katou (1985)

(continued)

Table 1.3 (continued)

Species	Family	Brassinosteroids	References
Ulothrix sp.	*Ulvophyceae*	BR_1 BR_2	Stirk et al. (2013)
Vicia faba L.	*Fabaceae*	BR_1 BR_2 BR_5 BR_7 BR_{15} BR_{27} BR_{39} BR_{45}	Ikekawa et al. (1988), Park et al. (1987), Fukuta et al. (2004), and Pan et al. (2013)
Vitis vinífera L.	*Vitaceae*	BR_1 BR_2 BR_5	Xu et al. (2015)
Zea mays L.	*Poaceae*	BR_1 BR_2 BR_3 BR_5 BR_7 BR_8 BR_9 BR_{11} BR_{14} BR_{15} BR_{27} BR_{36} BR_{39} BR_{40} BR_{45} BR_{62}	Suzuki et al. (1986); Sekimoto et al. (1997), Kim et al. (2005b, 2006c), Hartwig et al. (2011), Pan et al. (2013), Yokota et al. (2017), and Oklestkova et al. (2017)
Zinnia elegans Jacq.	*Asteraceae*	BR_2 BR_5 BR_7 BR_{39} BR_{45}	Yamamoto et al. (2001, 2007)

Table 1.4 Occurrence of brassinosteroids precursors

Species	Family	Brassinosteroid precursors	References
Arabidopsis thaliana (L.) Henyh.	*Brassicaceae*	**63 64 65 66 67 68 69 70 71 72 73 74**	Fujioka et al. (2002), Lee et al. (2006), Ohnishi et al. (2006b), Shahnejat-Bushehri et al. (2016), Roh et al. (2012), and Zhu et al. (2013)
Attalea vitrivir Zona	*Arecaceae*	**64**	Dias et al. (2017)
Atryrium yokoscence (Fr. & Sav.) C. Ch.	*Woodsiaceae*	**65 66 67 69**	Yokota et al. (2017)
Butia capitata (Mart.) Becc.	*Arecaceae*	**64**	Dias et al. (2017)
Camellia sinensis (O.) Kuntze	*Theaceae*	**73**	Bhardwaj et al. (2007)
Catharanthus roseus Don.	*Apocynaceae*	**64 65 66 67 68 69 70 71 72 73 74**	Fujioka et al. (1995b, 2000b, 2002)
Chlorella minutissima Fott et Nováková	*Trebouxiophyceae*	**64**	Stirk et al. (2014a)
Cyrtomium laetevirens (Hiyama) Nakaike	*Dryopteridaceae*	**65 66 67**	Yokota et al. (2017)
Dryopteris crassirhizoma Nakai (1920)	*Dryopteridaceae*	**67 66**	Yokota et al. (2017)
Dryopteris erythrososa (D.C.Eaton) Kuntze	*Dryopteridaceae*	**65 66 67**	Yokota et al. (2017)

(continued)

Table 1.4 (continued)

Species	Family	Brassinosteroid precursors	References
Deparia japonica (Thunb.) M. Kato	*Woodsiaceae*	**65 66 67**	Yokota et al. (2017)
Equisetum arvense L.	*Equisetaceae*	**65 66 67 69**	Yokota et al. (2017)
Lycopersicon esculentum	*Solanaceae*	**73**	Yokota et al. (2001)
Lygodium japonicum (Thunb.) Sw.	*Lygodiaceae*	**65 66 67**	Yokota et al. (2017)
Malus prunifolia (Willd.) Borkh.	*Rosaceae*	**63 65**	Pereira-Netto et al. (2009)
Marchantia polymorpha L.	*Marchantiaceae*	**65 66 67**	Yokota et al. (2017)
Osmunda japonica Thunb.	*Osmundaceae*	**65 66 67 69**	Yokota et al. (2017)
Onoclea sensibilis L.	*Woodsiaceae*	**66**	Yokota et al. (2017)
Oryza sativa L.	*Poaceae*	**65 66 67 68 69 73**	Yokota et al. (2017), Wu et al. (2008), and Tamiru et al. (2016)
Petunia hybrida	*Solanaceae*	**65 66 67 69**	Verhoef et al. (2013)
Pteridium aquilinum (L.) Kuhn in Kersten (1879)	*Dennstaedtiaceae*	**65 66 69**	Yokota et al. (2017)
Physcomitrella patens (Hedw.) Bruch & Schimp	*Funariaceae*	**65 66 67**	Yokota et al. (2017)
Selaginella moellendorffii Hieronymus	*Sellaginellaceae*	**66**	Yokota et al. (2017)
Selaginella uncinata (Desv. ex Poir.) Spring	*Sellaginellaceae*	**65 66 67**	Yokota et al. (2017)
Thelypteris decursive-pinnata (H.C. Hall) Ching, 1936	*Thelypteridaceae*	**65 66 69**	Yokota et al. (2017)
Thelypteris palustris Schott	*Thelypteridaceae*	**65 66 67**	Yokota et al. (2017)
Zea mays L.	*Poaceae*	**65 66 67 69**	Yokota et al. (2017)

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Chapter 2
Brassinosteroids in Microalgae: Application for Growth Improvement and Protection Against Abiotic Stresses

Andrzej Bajguz

Abstract Brassinosteroids have been found in a broad spectrum of microalgae, their biological activities correspond to the function in higher plants. Studies on the endogenous brassinosteroids suggest that the operation of the early and late C6-oxidation pathways, lead to brassinolide existence in algae. The growth and development of algae under the influence of brassinosteroids are unusually dynamic, despite the application of micromolar concentrations. These compounds regulate every aspect of algal life, from formation during development *via* stimulation of metabolite synthesis to abiotic stress responses, such as heavy metal action, salt and thermal stress. The relationship between brassinosteroids and the other well-known plant hormones has been explored. This chapter summarizes the studies of brassinosteroids on algal cultures in the last three decades.

Keywords Activity · Anti-stress Protection · Biosynthesis · Distribution

1 Introduction

Algae are autotrophic, aquatic, rarely terrestrial plants which bodies range from unicellular to multicellular structures with no vasculature and little diversification into various tissue systems. They can be a single cell as small as 1 μm (e.g. *Micromonas* sp.) or a large seaweed which can grow up to more than 65 m in length (e.g. *Macrocystis pyrifera*). Algae can produce extracellular complexing agents including polysaccharides, proteins, peptides and small organic acids that are able to decrease the concentration of bioavailable metals in the immediate vicinity of the cell. Aquatic algae are found in both fresh and salt water with a wide tolerance for pH, temperature, oxygen, and CO_2 levels. Microalgae can be used to phytoremediation techniques due to their effective efflux mechanisms for metals and the ability to modify the chemical speciation of the metal through the expulsion of inert trace

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metal complexes (Safi et al. 2014; Sahoo and Seckbach 2015; Borowitzka et al. 2016).

Algae are an area of interest due to their usefulness as food for pigments, protein, dietary fiber, mineral, vitamins, lipids, antioxidants, other valuable products and as a potential feedstock for biofuels. Microalgae can be found in the market as food supplements, colourants and food emulsions. These products come in different forms such as capsules, tablets, extracts and powder. The algal biomass is used as a supplement to noodles, breads, biscuits, candies, ice cream, bean curd and other common foods to enhance their nutritional and health values, whereas the extracts are widely used to enrich liquid foods, such as health drink, soft drink, tea, beer or spirits. Some of algal products are currently commercialized by the pharmaceutical and cosmetic industries. Nevertheless, algae are considered as nutraceuticals instead of food products due to the lack of clear and official legislations in terms of quality and requirements regarding microalgae. Algae are also a good model for laboratory studies because they grow much faster than other plants (Liang et al. 2004; Fradique et al. 2010; Sivakumar et al. 2012; Zeraatkar et al. 2016; Singh et al. 2017; Wells et al. 2017).

Plant hormones play an important role in vascular plants, coordinating growth and stress responses and regulating most of physiological and biochemical processes. Recent studies have identified genes and enzymes involved in their biosynthesis and signalling pathways. Phytohormones, including auxins, cytokinins, gibberellins, ethylene, abscisic acid (ABA), polyamines, brassinosteroids (BRs), jasmonides, salicylates and signal peptides, have been found in a variety of algae (Bajguz and Tretyn 2003; Tsavkelova et al. 2006; Tarakhovskaya et al. 2007; Bajguz 2009b; Davies 2010; Stirk et al. 2013a, b, 2003; Stirk and Staden 2014; Tran and Pal 2014; Lu and Xu 2015). Here, the recent progress in BRs detection, biosynthesis and their application for improvement of growth and resistance to abiotic stresses in algal cultures has been described.

2 Occurrence

In 1968, the first scientific account of the novel phytohormones *viz.* BRs from the leaves of *Distylium racemosum* was reported (Marumo et al. 1968). Two years later, the first bioactive compound was identified from *Brassica napus* and was named as brassin (Mitchell et al. 1970). The breakthrough discovery of the future brassinosteroid's group was the isolation of brassinolide (BL) in 1979 from the pollen of *Brassica napus* (Grove et al. 1979). Castasterone (CS), as the second BR, was isolated from the insect galls of chestnut (*Castanea crenata*) (Yokota et al. 1982). Since then, more than 60 natural BRs have been isolated from various plant species. They have been reported in higher plant species that include gymnosperms, monocots and dicots. Similarly, they have also been found in some lower aquatic (algae)

and terrestrial (bryophytes and pteridophytes) plants (Bajguz and Tretyn 2003; Bajguz 2009b; Stirk et al. 2013a, b; Stirk and Staden 2014).

Although little is known about the physiological role of BRs in algae, bioactive compounds have been detected (Table 2.1). In 1987, 24-epiCS has been identified in *Hydrodictyon reticulatum* for the first time not only in algae but also in plant kingdom (Yokota et al. 1987). To date, the presence of BL and CS was detected in 25 algal species. In many algae, e.g. *Chlorella minutissima* and *Monoraphidium contortum*, BL was present in higher concentrations than CS (Stirk et al. 2013a). Seven BRs, such as BL, CS, teasterone (TE), typhasterol (TY), 6-deoxoTE, 6-deoxoTY and 6-deoxoCS occur in *Chlorella vulgaris*. These compounds are intermediates in the early and late C6-oxidation biosynthetic pathways of C_{28} BRs (Bajguz 2009b).

3 Detection

The detection of BRs was accomplished by gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS) techniques. Typically, single quadrupole analysis and selected ion monitoring were used, although gas chromatography tandem mass spectrometry (GC-MS/MS) method was becoming more prominent. Nevertheless, today, liquid chromatography (LC) coupled with tandem mass spectrometry (MS) has become a powerful tool for BR analysis. The most frequently used with LC methods are triple quadrupole or time-of-flight analyzers. It is due to its selectivity and sensitivity, substantial reduction of sample-treatment steps compared to the methods above, and its reliable quantification and confirmation at the low concentrations (Kanwar et al. 2017). Using ultra-high performance liquid chromatographic separation, BRs are detected in the highly selective multiple reaction monitoring mode. The detection limit for most of the BRs analyzed was close to 50 ng/g algal biomass (Tarkowská and Strnad 2017).

Therefore, BRs are present in very low amounts in algae and both extraction and purification are important steps in detection of these compounds. BRs as neutral compounds that display no ionic properties and a high hydrophobicity are most of- ten extracted in organic solvents, such as methanol (MeOH) or acetonitrile (ACN) (Tarkowská and Strnad 2017). Briefly, after homogenization (using liquid nitrogen and ball mill) algal material is first extracted with MeOH or ACN overnight. Then, the exctract is purified using a Discovery® DPA-6S cartridges (50 mg) and Isolute® C4 SPE cartridge (100 mg). After purification, plant extract is dried in the vacuum and reconstituted in 100% MeOH. The screening process is performed on MS equipped with an electrospray ionization source coupled with LC (Tarkowská et al. 2016).

Table 2.1 Occurrence of brassinosteroids in algae

Species[a]	Brassinosteroid[b]	References
Acutodesmus acuminatus	BL (125), CS (105)	Stirk et al. (2013a)
Acutodesmus incrassatulus	BL (125), CS (93)	
Chlamydomonas reinhardtii	BL (163), CS (154)	
*Chlorella minutissima***	BL (307), CS (215), CT (41), 6-deoxo-epiCS (1580)	Stirk et al. (2013a, 2014a)
Chlorella pyrenoidosa	BL (253), CS (158)	Stirk et al. (2013a)
*Chlorella vulgaris***	BL (70), CS (470), 6-deoxo CS (320), TY (390), TE (260), 6-deoxoTY (180), 6-deoxoTE (220)	Bajguz (2009a, b)
Chlorococcum ellipsoideum	BL (169), CS (106)	Stirk et al. (2013a)
Coccomyxa sp.	BL (206), CS (177)	
Coelastrum microporum	BL (199), CS (158)	
Desmodesmus armatus	BL (125), CS (109)	
*Ecklonia maxima**	BL (stipe: 12; frond: 5), CS (stipe: 13; frond: 9)	Stirk et al. (2014b)
Hydrodictyon reticulatum	24-epiCS (0.3), 28-homoCS (4)	Yokota et al. (1987)
Gyoerffyana humicola	BL (271), CS (201)	Stirk et al. (2013a)
Klebsormidium flaccidum	BL (549), CS (429)	
Monoraphidium contortum	BL (285), CS (195)	
Myrmecia bisecta	BL (202), CS (164)	
Nautococcus mamillatus	BL (116), CS (100)	
Poloidion didymos	BL (167), CS (173)	
Protococcus viridis	BL (211), CS (135)	
Protosiphon botryoides	BL (101), CS (74)	
Raphidocelis subcapitata	BL (59), CS (59)	
Scotiellopsis terrestris	BL (337), CS (236)	
Spongiochloris excentrica	BL (131), CS (108)	
Stichococcus bacillaris	BL (292), CS (243)	
Stigeoclonium nanum	BL (169), CS (145)	
Ulothrix sp.	BL (85), CS (74)	

[a] Time of algal cultivation is 1 day, except for algae with: *2 days, **4 days

[b] Amount (:> ng/g biomass, in brackets)

4 Biosynthesis

BRs, as triterpenes (C_{30}), are generated by the joining of two farnesyl (C_{15}) chains, derived from three five-carbon isopentane (isoprene) units. The isoprenoid precursor, i.e. isopentenyl diphosphate is synthesized either from acetyl-CoA *via* mevalonic acid (mevalonate pathway) or by pyruvate and glyceraldehyde 3-phosphate (non-mevalonate pathway; present in algae). Isoprene units condensed to squalene undergo conversion *via* some steps to campesterol (Lichtenthaler 1999; Buchanan et al. 2005). Because BL and CS have the methyl group at C-24*S* position, they are synthesized from campesterol in several steps. The presence of two parallel pathways of C_{28} BR from campesterol to castasterone, named as the early and late C-6 oxidation pathways, was revealed in *Chlorella vulgaris* (Fig. 2.1) (Bajguz 2009b). These reactions are similar to pathways which exist in higher plants (Zhao and Li 2012; Chung and Choe 2013; Youn et al. 2018). Furthermore, study by Bajguz and Asami (2004) demostrates that brassinazole (Brz), specific BR biosynthesis inhibitor, inhibits the algal growth, however, the inbition effect was reversed by exogenous BL. It is known that Brz blocks the conversion of campestanol to 6-deoxocathasterone, 6-deoxocathasterone to 6-deoxoteasterone, 6-oxocampestanol to cathasterone, and cathasterone to teasterone. It suggests that the presence of endogenous BRs in algae is indispensable for their normal growth.

In *Chlorella minutissima*, 6-deoxo-epicastasterone and cathasterone occur; their initial endogenous levels increase irrespective of the presence or absence of light between 10 and 15 h of cultivation. After 15 h, a decline in BR content was observed. It suggests that light is not a controlling factor in BR biosynthesis. Moreover, a slight decrease of BR level on dark-grown *Chlorella minitussima* was observed with little increase in biomass (Stirk et al. 2014a).

5 Regulation of Growth and Metabolite Synthesis

The chemical structure of BRs is the factor differentiating the algal response on their growth and level of primary metabolites. BRs with 7-oxalactone B-ring, such as BL, 24-epiBL and 28-homoBL, are more effective than 6-ketone compounds, such as CS, 24-epiCS and 28-homoCS. BRs stimulate algal cell divisions intensively leading to an increase in the number of *Chlorella vulgaris* cells. They increase by two to three times the efficiency of the developmental cycle of *Chlorella vulgaris* and increase net photosynthetic rate and chlorophylls, carotenoids, sugar, protein, organic and inorganic phosphorus contents. BRs increase not only the content of primary metabolites in algal cells but also the intensity of sugar and glycolate extracellular secretion (Bajguz and Czerpak 1996, 1998; Bajguz 2000b). 24-epiBL has a meaningful impact on the increase of chlorophyll α and β and carotenoids such as α-, β-carotene, cryptoxanthin, lutein, zeaxanthin, astaxanthin, neoxanthin, violaxantin, content in *Acutodesmus obliquus*. 24-epiBL also inhibits the formation of

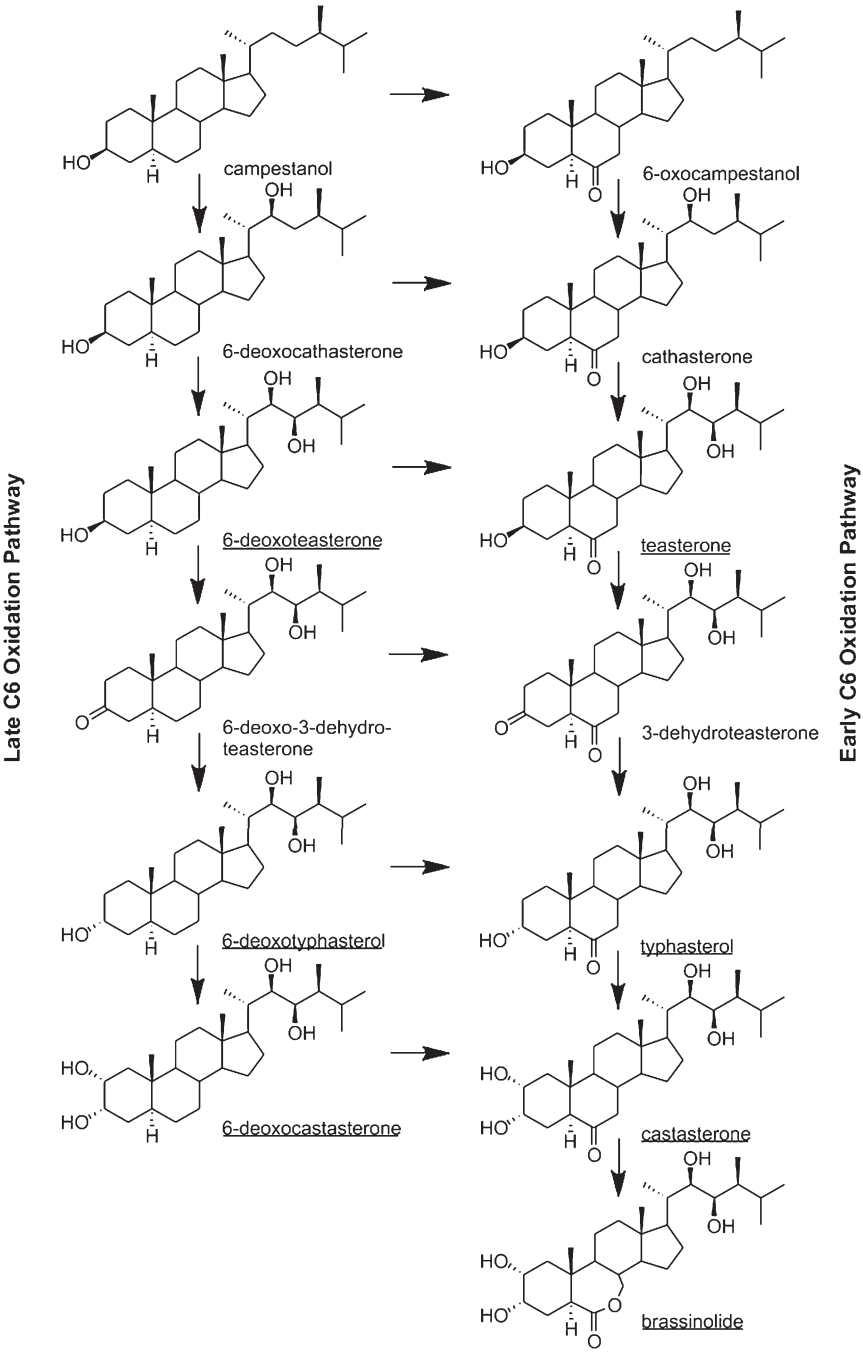

Fig. 2.1 Biosynthetic pathways of brassinosteroids (compounds detected in *Chlorella vulgaris* are underlined) (Bajguz 2009b)

reactive oxygen species such as hydrogen peroxide and oxidative damage as evidenced by a decrease of the lipid peroxidation (expressed as malondialdehyde level). The positive effect of 24-epiBL resulting from the cellular oxidative state can be alleviated by antioxidants such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD) and ascorbate which levels were increased by exogenous BR (Talarek-Karwel et al. 2018). BL and 24-epiBL, stimulate an increase in *Scenedesmus quadricauda* cell size. The effect was observed only at 5 nM for BL, but was seen at most of the tested concentrations for 24-epiBL. At 50 nM and higher for BL and at 100 nM for 24-epiBL reduction of cell size was observed. Both BRs increase biomass production of *Scenedesmus quadricauda* and the content of chlorophyll and carotenoids. BRs stimulate fatty acids accumulation in *Scenedesmus quadricauda*. The fatty acids profile was dependent on the type of BR and their concentration. Increasing concentrations of 24-epiBL significantly induce production of palmitic, oleic and γ-linolenic acids. Only in 5 nM, BL induces the accumulation of oleic, palmitic and palmitoleic acids. These results suggest that BRs are also important phytohormone which could be used to manipulate the fatty acids profile in the biofuel and pharmaceutical industries (Kozlova et al. 2017). Brassinazole (Brz), an inhibitor of BR biosynthesis, suppresses the growth of *Chlorella vulgaris* with a decrease in RNA, protein, sugar and carotenoids contents. The inhibitory effect of Brz was partially reversed with the co-application of BL (Bajguz and Asami 2004).

The relationship between BRs and the other phytohormones has been studied not only in vascular plants (Hardtke et al. 2007; Choudhary et al. 2012; Gallego-Bartolome et al. 2012; Hofmann 2015; Tian et al. 2018) but also in microalgae. BR induces the synthesis of ABA in *Chlorella vulgaris* cells (Bajguz 2009a). Exogenous indole-3-acetic acid (IAA) and *trans*-zeatin (*t*Z) stimulate the endogenous content of BRs in *Chlorella vulgaris* (Table 2.2). It suggests a possibility that auxin and cytokinin regulate directly the biosynthesis of BRs. Auxin and cytokinin also cooperate synergistically with BRs stimulating cell proliferation and endogenous level of protein, chlorophylls and monosaccharides in a dose-effect relationship in *Chlorella vulgaris* cells (Bajguz and Piotrowska-Niczyporuk 2013, 2014).

Table 2.2 Enhancement of brassinosteroids level by auxin and cytokinin in *Chlorella vulgaris* after 48 h of cultivation

Brassinosteroid content (fg/cell)			
	Control	50 mM IAA[a]	10 nM *tZ*[b]
6-Deoxoteasterone	0.151	0.175	0.196
6-Deoxotyphasterol	0.129	0.135	0.134
6-Deoxocastasterone	0.223	0.241	0.173
Teasterone	0.191	0.213	0.294
Typhasterol	0.251	0.267	0.245
Castasterone	0.329	0.339	0.319
Brassinolide	0.085	0.098	0.447

[a] Bajguz and Piotrowska-Niczyporuk (2013)
[b] Bajguz and Piotrowska-Niczyporuk (2014)

Application of 24-epiBL enhances the stress tolerance (e.g. temperature, light, salt stress) by increasing the level of astaxanthin in *Haematococcus pluvialis*. The eight carotenogenic genes (*ipi*-1, *ipi*-2, *psy*, *pds*, *lyc*, *crt*R-B, *bkt* and *crt*O) were up-regulated by using different concentration of 24-epiBL. In the concentration of 25 mg/L 24-epiBL had a greater influence on the transcriptional expression of *ipi*-1, *ipi*-2, *crt*R-B, *lyc* and *crt*O than on *psy*, *pds*, *bkt*. In turn, at 50 mg/L 24-epiBL had a greater effect on the transcriptional expression of *ipi*-2, *pds*, *lyc*, *crt*R-B, *bkt* and *crt*O than on *ipi*-1 and *psy*. Furthermore, in culture treated with 24-epiBL the biosynthesis of astaxanthin (Fig. 2.2) was up-regulated by *ipi*-1 and *psy* at the post-transcriptional level, *pds*, *lyc*, *crt*R-B, *bkt* and *crt*O at the transcriptional level and *ipi*-2 at both levels. BRs, jasmonic acid (JA) and salicylic acid (SA), as anti-stress hormones, can enhance the level of astaxanthin but they have different regulatory profiles (Table 2.3) (Gao et al. 2013). Astaxanthin is used as a source of pigmentation for fish (salmons and trouts), shrimps, lobsters and crayfishes in aquaculture and for eggs in the poultry industry. Moreover, it has a higher antioxidant activity than other carotenoids. Application of this carotenoid has health benefits, such as strong antioxidant, anti-inflammatory, anti-cancer and cardiovascular effects. Astaxanthin also protects the skin against UV-induced photo-oxidation (Panis and Carreon 2016; Shah et al. 2016).

The observed increases in protein, chlorophylls and carotenoids contents due to the effects of exogenously applied BRs to the growth media would be of value in algal cultivation for commercial production of feed or bioproducts. Thus, despite the recent findings on the positive influence of BRs on algal biomass production and synthesis of valuable biomolecules, there are several gaps in our understanding of the impact of phytohormones on various features of microalgal physiology. Considering the importance of rapid growth and high metabolite content in microalgal cultivation, more study to gain a better understanding of BRs is warranted (Tate et al. 2013).

6 Anti-stress Protection

Environmental stresses are the most major natural limiting factors for plant growth and development. Most stress conditions in plants cause an accumulation of reactive oxygen species (ROS), e.g. superoxide ion, hydrogen peroxide, oxygen-containing radicals. ROS detoxification involves the combined action of both antioxidant enzymes, such as SOD, APX, CAT and glutathione reductase (GR), and metabolites, such as ascorbate, glutathione and tocopherols. Furthermore, BRs have been implicated in abiotic stress responses. Enhancement of plant resistance to various stresses by BRs has been evaluated aiming at finding practical applications for BRs in aquaculture (Bajguz and Hayat 2009; Rajewska et al. 2016).

The role of BRs in alleviating the adverse effects of stresses in algae was studied. BRs, as anti-stress substances, have generated considerable practical interest for aquacultural uses. In particular, endogenous level of BRs can be informative to

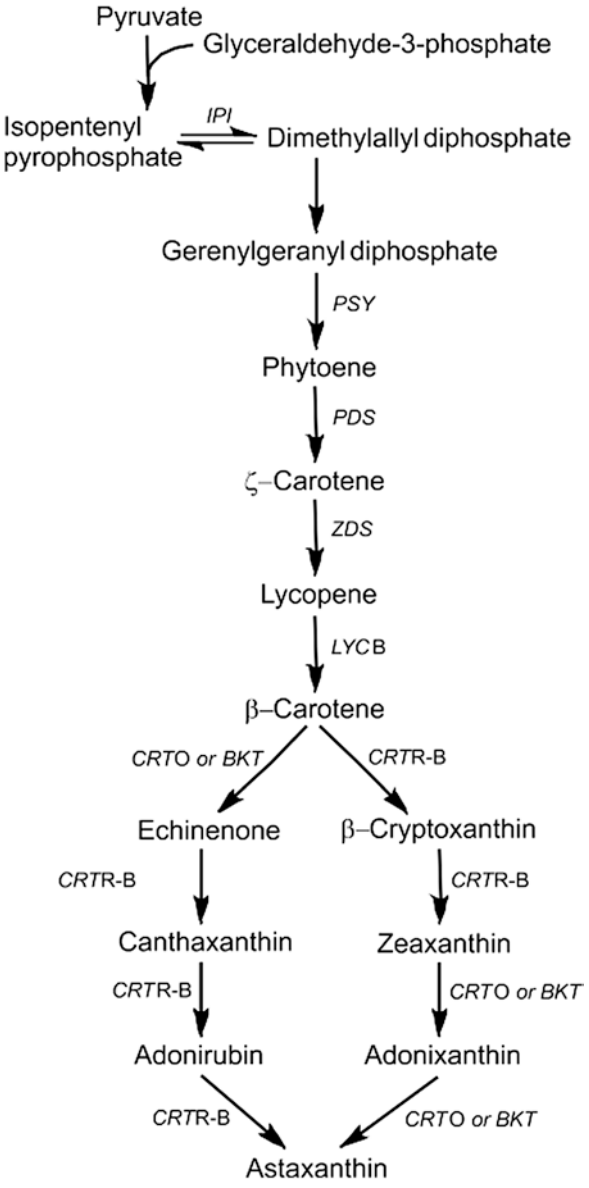

Fig. 2.2 Biosynthesis of astaxanthin in *Haematococcus pluvialis* (Gao et al. 2013). Enzyme abbreviations are as follows: *BKT* β -carotene ketolase, *CRTO* β -carotene oxygenase, *CRTR*-B β -carotene 3,3′-hydroxylase, *IPI* isopentenyl diphosphate isomerase, *LYCB* lycopene β –cyclase, *PDS* phytoene desaturase, *PSY* phytoene synthase, *ZDS* ζ -carotene desaturase

reveal key links between these hormones and stress protection as well as crosstalk with other phytohormones. Exogenously applied BL enhances the ABA content in *Chlorella vulgaris* cultures in response to short-term (3 h) heat stress (30–40 °C). BL has no significant effect on the number of cells and the content of chlorophyll and sugar in *Chlorella vulgaris* cells (Bajguz 2009a). Exogenous BL also partially overcomes the inhibitory effect of heavy metals on *Chlorella vulgaris*, decreasing

Table 2.3 Regulation of astaxanthin biosynthesis by stress-related phytohormones (Gao et al. 2013)

	Transcription level			Post-transcriptional level		
Gene	BR	JA	SA	BR	JA	SA
ipi-1	•	•	•	•	•	
ipi-2	•	•	•	•	•	
psy		•	•	•		
pds	•	•	•			•
lyc	•	•				•
*crt*R-B	•	•	•			
bkt	•	•	•			
*crt*O	•	•	•			

Gene designations are according to the corresponding enzymes, which are shown in the title of Fig. 2.2

the accumulation of heavy metals in the cells and increasing ABA, IAA and zeatin content although there was no change in the endogenous BL content (Bajguz 2011). Endogenous level of BRs increases in response to salt and low temperature (15 °C) stress in *Chlorococcum ellipsoideum*, *Gyoerffyana humicola*, *Nautococcus mamillatus*, *Acutodesmus acuminatus*, *Protococcus viridis* and *Chlorella vulgaris*. The response of algal cultures was observed within 30 min of the salt shock. The higher level of BRs, mainly CS with lower amounts of BL, 28-homoCS and TY, was shown. Furthermore, the temperature stress had a slight effect on the BRs content in these algae (Stirk et al. 2018).

The application of exogenous 24-epiBL shows increasing the content of lipids in *Chlorella vulgaris* culture under high temperature (30 °C). At the temperature of 25 °C the maximum growth rate was reached. The highest lipid content was obtained in culture treatment with 24-epiBL and growing at 30 °C. It indicates that BR significantly increases the lipid content of algae subjected to the stress induced by high temperature (Liu et al. 2018).

BL inhibits the degradation of lipids resulting from the overproduction of ROS and increase the activity of antioxidative enzymes (SOD, APX, GR, CAT) and content of antioxidants (glutathione, ascorbate) in *Chlorella vulgaris* cells treated with heavy metals (cadmium, lead, copper) (Bajguz 2010). Exogenous BRs cause the rapid response in *Chlorella vulgaris* by acceleration of phytochelatins (PC) synthesis. PC are metal-binding cysteine-rich compounds, which can facilitate the chelation of metal ions. BRs accelerate the synthesis of PC in the following order: BL > 24-epiBL > 28-homoBL > CS > 24-epiCS > 28-homoCS. Application of BRs to *Chlorella vulgaris* cultures reduces the impact of heavy metals stress on growth and enhances the chlorophyll, sugar and protein contents (Bajguz 2002, 2011). Another heavy metal detoxification mechanism is biosorption, which is dependent on pH solution. The optimum pH of metal ions sorption is between 4 and 6. Lowering the pH in cell wall spaces stimulates the growth of *Chlorella vulgaris* under the influence of BRs (Bajguz and Czerpak 1996; Bajguz 2000a). These results indicate the ameliorative influence of BRs on the inhibitory effect of heavy metals. The increase

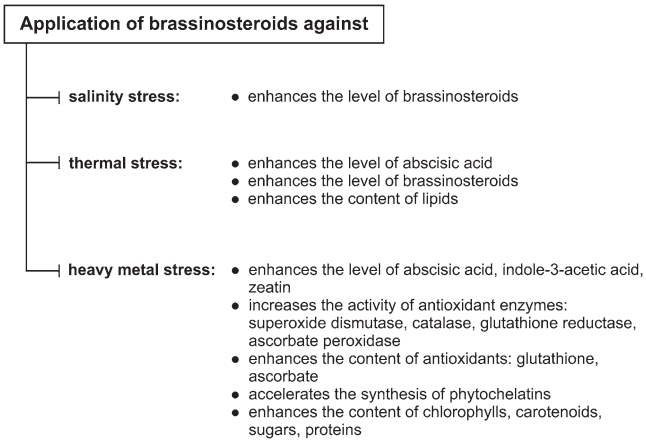

Fig. 2.3 Brassinosteroids in response to abiotic stresses in algae

of resistance due to application of BRs was reflected in the improvement of algal growth in the present of heavy metals. However, BRs are not involved by synthesising de novo in response of algal growth under heavy metal stress but can interact *via* enhancing the content of other phytohormones, i.e. auxin, cytokinin and ABA (Bajguz 2011).

Although algae have several self-defense mechanisms to survive in stressful condition, BRs regulate stress response by a complex sequence of biochemical reactions. They accelerate these processes and mitigate the negative effect of stresses in algae (Fig. 2.3).

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Chapter 3
Brassinosteroids in Cereals – Presence, Physiological Activity and Practical Aspects

Anna Janeczko

Abstract Brassinosteroids (BRs) are plant steroid hormones that are characterised by a sterane skeleton of four rings with a number of functional groups attached (mainly hydroxyl). The first species from the *Poaceae* family in which BRs were found was rice (*Oryza sativa* L., cv. Arborio J1) – castasterone (13.6 pg g^{-1} F.W.) and dolichosterone (8.4 pg g^{-1} F.W.). BRs were also found in corn, wheat, rye, barley as well as *Phalaris canariensis* L. or ryegrass. There are significant differences between the different cereals in the types of BRs that are present and in their concentration. In agricultural and biological experiments whose aim was to clarify the role of these compounds in cereals, exogenous 28-homobrassinolide and 24-epibrassinolide and less often, brassinolide or other BRs were most commonly used. Recently, however, the number of articles in which BR-biosynthetic deficient mutants or BR-signalling mutants are being used in studies has increased. BR mutants of cereals include mutants of rice (i.e. *d61*), barley (i.e. *uzu*) and corn (*Brd1*). It is worth emphasising that in the case of cereal plants, studies on mutants have confirmed lot of the physiological functions of BRs that have previously been reported in works in which exogenous BR was applied. One can also mention the participation of BRs in regulating plant growth, CO_2 assimilation, proline and sugar production, their protective effects on the PSII (under stress conditions) or their participation in a complicated network of connections with other plant hormones. In addition to being a good model for studies of the role of BRs in cereals, mutants of cereal crops can be used in agricultural practice, i.e. to create new dwarf cultivars. This chapter will review the knowledge about brassinosteroids in cereals – their presence, physiological activity and practical applications.

Keywords Antioxidants · Brassinosteroid content · Photosynthesis · Plant stress response · Plant growth and development · *Poaceae*

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1 Introduction – Chemistry of Brassinosteroids

Brassinosteroids (BRs) are plant steroid hormones that are isolated from oilseed rape pollen (Grove et al. 1979). BRs have sterane as the main skeleton in the molecule; BRs are also defined as polyhydroxysteroids because they contain many hydroxyl groups per molecule. Low quantities (ng or pg g^{-1} fresh weight [F.W.]) of BRs are present in plants in a free form or in the form of conjugates (glycosides, conjugates with fatty acids). There are three main groups of BRs: C_{27}, C_{28} and C_{29}. They differ in the number of carbons in a molecule. An example of C_{27} is 28-norcastasterone, an example of C_{28} is 24-epibrassinolide or brassinolide, while 28-homobrassinolide represents C_{29} (Fig. 3.1). Synthetic analogues of BRs such as biobrass-6 (BB-6, Mazorra et al. 2004) are also known. Interestingly, some epoxybrassinosteroids (e.g. secasterone) have been discovered in the *Poaceae* family

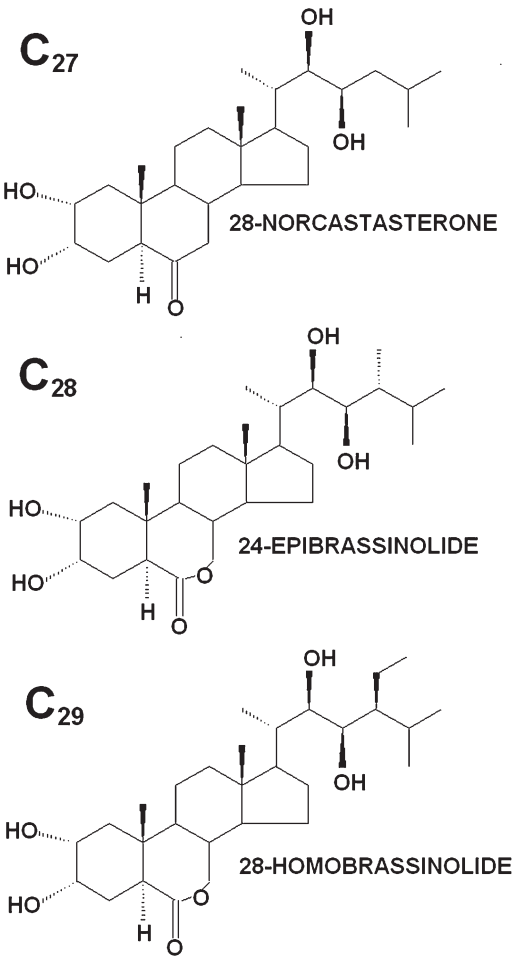

Fig. 3.1 Examples of the brassinosteroids that represent three structural groups: C_{27}, C_{28} and C_{29}, which are present in the *Poaceae* family. (Gamoh et al. 1990; Janeczko and Swaczynová 2010). 24-Epibrassinolide and 28-homobrassinolide are the BRs that are most often used in experiments using an exogenous application to plants from this family

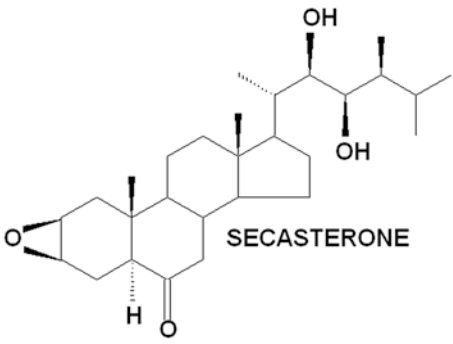

Fig. 3.2 Secasterone, the first naturally occurring 2,3-epoxybrassinosteroid was named after the species (*Secale cereale* L.) in which it was discovered. (Schmidt et al. 1995)

(*Secale cereale* L.) (Fig. 3.2). Sterols are the precursors of BR biosynthesis in plants. The first BR biosynthetic pathway that was discovered was described for brassinolide. The pathway starts with sterol – campesterol and goes through cathasterone, teasterone, typhasterol and castasterone in the early C6 oxidation pathway (Fujioka and Yokota 1997). BR receptors are present in cell membranes and are well described together with the signal transduction pathways (Clouse 2011). However, specific brassinosteroid binding has been reported in studies using the so-called radioligands not only in different cell membrane fractions, but also in the cytosol (Xu et al. 1994). This binding was weakened by trypsin, which indicates the protein nature of the binding structures. Hence, perhaps similar to animals and humans, plant steroid hormones have cytoplasmic or nuclear receptors. The primary function of BRs is the regulation of growth processes – plant mutants with a disturbed BR production show a dwarf phenotype (Morinaka et al. 2006; Makarevitch et al. 2012; Janeczko et al. 2016). BRs regulate the developmental processes (Yang et al. 2011) including fruit development (Symons et al. 2006). BRs also participate in the plant response to environmental stress (Krishna 2003).

Poaceae plants include many species that are very important from an agricultural point of view such as rice, wheat, maize or barley. These cereals, which are consumed in the form of groats, flakes, bread and other baked goods, are an important source of carbohydrates in the human diet. Research on the mechanisms that control the processes of the growth and development of these species as well as their resistance to stress factors is very important from a practical point of view (for farmers or plant breeders). In turn, brassinosteroids, which are hormones that have multidirectional physiological effects, are also of interest to many researchers. This chapter will review the knowledge about brassinosteroids in cereals – their presence, physiological activity and some possibilities for their practical application in agriculture.

2 Occurrence of Brassinosteroids and Their Changes in Plants of the *Poaceae* Family

BRs were discovered by Grove et al., in oil rapeseed pollen in 1979, while rice was the first species in the family *Poaceae* (5 years later) in which the occurrence of BR was confirmed (Abe et al. 1984). To date, the presence of BRs among *Poaceae* has been confirmed in wheat, maize, rye, barley, *Phalaris canariensis* L. and perennial ryegrass (Table 3.1). Some of the studies that have been carried out were qualitative analyses of BRs and several works have provided quantitative analyses. Brassinosteroids have been determined using gas chromatography coupled with mass spectrometry (GC-MS) (Abe et al. 1984, 1995; Suzuki et al. 1986; Yokota et al. 1994; Park et al. 1994; Schmidt et al. 1995; Antonchick et al. 2003; Kim et al. 2005), using liquid chromatography with fluorometric detection (Gamoh et al. 1990), using immunochemical methods (Taylor et al. 1993), using liquid chromatography coupled with mass spectrometry (Antonchick et al. 2005) and using high and ultra performance liquid chromatography coupled with tandem mass spectrometry with electrospray ionisation (HPLC or UHPLC-ESI-MS/MS) (Janeczko and Swaczynová 2010; Janeczko et al. 2010, 2011, 2013, 2015; Dockter et al. 2014; Pociecha et al. 2016; Gruszka et al. 2016a, b). Only small amounts of plant material are needed for an HPLC analysis usually (even less than 1 g of tissue), while for a GC analysis, more material (even kilograms) is required.

The level and profile of BRs in *Poaceae* varies and there are many factors that modify them. Differences have been found between individual families, plant species and cultivars (Table 3.1) as well as between plant organs (Asahina et al. 2014). Mutations are an important factor that causes changes in the BR content. Mutants with BR biosynthesis disorders are usually characterised by a reduced content of these compounds, whereas mutants with BR-perception disorders usually accumulate more of these compounds than the wild type (Dockter et al. 2014, Table 3.1).

The content and profile of BRs in cereals may be influenced by the exogenous application of BRs. In wheat, 24-epibrassinolide, when applied *via* plant spraying in the heading stage or *via* presowing seed soaking, did not accumulate in grains that were collected but did change the profiles of the BRs (Janeczko et al. 2010). 24-Epibrassinolide, when applied to the heading plants, decreased content of its precursor (24-epicastasterone) in the grains, which might be the result of negative feedback in the biosynthesis pathway of these BR. Interestingly, in this experiment it was also found that the solvent for the 24-epibrassinolide – ethanol, which was present in the working solutions, modified the composition of the BRs in the plants, for example, it increased the amount of brassinolide in the collected seeds (Janeczko et al. 2010). Changes in the content and profile of endogenous BRs *via* the exogenous application of BR were also noted in the study of Janeczko and Swaczynová (2010). The impact of exogenous BR on fluctuations in the endogenous BR content in a plant may be explained by their metabolising to other BRs (Joo et al. 2015) or the direct influence of the applied BRs on the biosynthetic pathways (e.g. on the basis of the aforementioned feedback).

Table 3.1 Brassinosteroids in plants from *Poaceae* family

Publication	Species	Organ	BRs
Abe et al. (1984)	**Rice** (*Oryza sativa* L.) cv. Arborio J1	Shoots	castasterone (13.6 pg g^{-1} F.W.), dolichosterone (8.4 pg g^{-1} F.W.)
Suzuki et al. (1986)	**Maize** (*Zea mays* L.)	Pollen	castasterone (120 ng g^{-1} F.W.), typhasterol (6.6 ng g^{-1} F.W.), teasterone (4.1 ng g^{-1} F.W.)
Gamoh et al. (1990)	**Maize** (*Zea mays* L.)	Pollen	castasterone (27.2 ng g^{-1} F.W.), 28-norcastasterone (18.3 ng g^{-1} F.W.), dolichosterone (16.9 ng g^{-1} F.W.)
Taylor et al. (1993)	**Perennial ryegrass** (*Lolium perenne* L.)	Pollen	25-methylcastasterone
Yokota et al. (1994)	**Wheat** (*Triticum aestivum* L.) cv. Chihoku	Bran	castasterone, 3-dehydroteasterone, teasterone, typhasterol, 6-deoxocastasterone
		Flour	kastasteron, teasterone, typhasterol, 6-deoxocastasterone
Park et al. (1994)	**Rice** (*Oryza sativa* L.) cv. Tongjinbyeo	Young seeds	castasterone, teasterone, 6-deoxocastasterone
Schmidt et al. 1995	**Rye** (*Secale cereale* L.)	Seeds	secasterone, castasterone, 28-homocastasterone, 28-norcastasterone, 6-deoxocastasterone, typhasterol, teasterone
Abe et al. (1995)	**Rice** (*Oryza sativa* L.) cv. Koshihikari	Bran	28-homotyphasterol, 28-homoteasterone, 6-deoxocastasterone
Shimada et al. (1996)	**Canary grass** (*Phalaris canariensis* L.)	Seeds	castasterone (5 ng g^{-1} seeds), teasterone (0.7 ng g^{-1} seeds)
Antonchick et al. (2003)	**Rye** (*Secale cereale* L.) cv. Sorom	Leaves of 18-d-old seedlings	secasterone (52 pg g^{-1} F.W.), 2,3-diepisecasterone (20 pg g^{-1} F.W.)
		Roots of 18-d-old seedlings	secasterone (107 pg g^{-1} F.W.), 2,3-diepisecasterone (32 pg g^{-1} F.W.)
Antonchick et al. (2003)	**Rye** (*Secale cereale* L.) cv. Petka	Leaves of 18-d-old seedlings	2,3-diepisecasterone (102 pg g^{-1} F.W.)
		Roots of 18-d-old seedlings	2,3-diepisecasterone (22 pg g^{-1} F.W.)

(continued)

Table 3.1 (continued)

Publication	Species	Organ	BRs
Antonchick et al. (2005)	**Rye** (*Secale cereale* L.) cv. Sorom	Seeds	castasterone (574 pg g^{-1} seeds), 2-epicastasterone 201 pg g^{-1} seeds), 3-epicastasterone (115 pg g^{-1} seeds)
		Leaves of 14-d-old seedlings	castasterone, 2-epicastasterone, 3-epicastasterone
Kim et al. (2005)	**Maize** (*Zea mays* L.) cv. Golden cross bantam	Primary roots	6-deoxocathasterone (0.1 ng g^{-1} F.W.), 6-deoxoteasterone (1.0 ng g^{-1} F.W.), 6-deoxotyphasterol (9.0 ng g^{-1} F.W.)
Wu et al. (2008)	**Rice** (*Oryza sativa* L.) wild type	Flag leaves collected after beginning of flowering	6-deoxocathasterone (1.06 ng g^{-1} F.W.), 3-epi-6-deoxocathasterone (2.23 ng g^{-1} F.W.), 6-deoxoteasterone (0.18 ng g^{-1} F.W.), 6-deoxo-3-dehydroteasterone (1.18 ng g^{-1} F.W.), 6-deoxotyphasterol (8.96 ng g^{-1} F.W.), 6-deoxocastasterone (1.84 ng g^{-1} F.W.), teasterone (0.027 ng g^{-1} F.W.), typhasterol (1.47 ng g^{-1} F.W.), castasterone (0.68 ng g^{-1} F.W.)
		Seeds collected 15 days after pollination stage	6-deoxocathasterone (0.48 ng g^{-1} F.W.), 3-epi-6-deoxocathasterone (0.045 ng g^{-1} F.W.), 6-deoxoteasterone (0.085 ng g^{-1} F.W.), 6-deoxo-3-dehydroteasterone (0.075 ng g^{-1} F.W.), 6-deoxotyphasterol (0.14 ng g^{-1} F.W.), 6-deoxocastasterone (0.115 ng g^{-1} F.W.), teasterone (0.040 ng g^{-1} F.W.), typhasterol (0.08 ng g^{-1} F.W.), castasterone (0.08 ng g^{-1} F.W.)
Janeczko and Swaczynová (2010)	**Spring wheat** (*Triticum aestivum* L.) cv. Cytra)	10-d-old seedlings (first + second leaf)	brassinolide (303 pg g^{-1} F.W.), 24-epibrassinolide (258 pg g^{-1} F.W.), castasterone (traces)
		Third leaf of 21-d-old seedlings	brassinolide (885 pg g^{-1} F.W.), castasterone (785 pg g^{-1} F.W.)
Janeczko et al. (2010)	**Spring wheat** (*Triticum aestivum* L.) cv. Torka	Mature seeds	brassinolide (127 pg g^{-1} F.W.), castasterone (159 pg g^{-1} F.W.), 24-epicastasterone (535 pg g^{-1} F.W.)
Hartwig et al. (2011)	**Maize** (*Zea mays* L.) wild type	Shoots of 4-week old plants	6-deoxocathasterone (0.27 ng g^{-1} F.W.), 6-deoxoteasterone (0.03 ng g^{-1} F.W.), 3-dehydro-6-deoxoteasterone (0.28 ng g^{-1} F.W.), 6-deoxotyphasterol (1.89 ng g^{-1} F.W.), 6-deoxocastasterone (5.72 ng g^{-1} F.W.), cathasterone (n.d.), teasterone (n.d.), typhasterol (0.045 ng g^{-1} F.W.), castasterone (1.14 ng g^{-1} F.W.), brassinolide (n.d.)

(continued)

Table 3.1 (continued)

Publication	Species	Organ	BRs
	na1 mutant	Shoots of 4-week old plants	6-deoxocathasterone (0.025 ng g^{-1} $_{F.W.}$), 6-deoxoteasterone (0.01 ng g^{-1} $_{F.W.}$), 3-dehydro-6-deoxoteasterone (n.d.), 6-deoxotyphasterol (0.115 ng g^{-1} $_{F.W.}$), 6-deoxocastasterone (0.235 ng g^{-1} $_{F.W.}$), cathasterone (n.d.), teasterone (0.045 ng g^{-1} $_{F.W.}$), typhasterol (0.14 ng g^{-1} $_{F.W.}$), castasterone (0.065 ng g^{-1} $_{F.W.}$), brassinolide (n.d.)
Janeczko et al. (2011)	**Spring barley** (*Hordeum vulgare* L.) cv. Sezam	Seventh leaf	brassinolide (700 pg g^{-1} $_{F.W.}$), castasterone (930 pg g^{-1} $_{F.W.}$), 24-epibrassinolide (traces)
Dockter et al. (2014)	**Spring barley** (*Hordeum vulgare* L.)	Aerial part of 14-d-old seedlings	
	cv. Bowman		castasterone (1245 pg g^{-1} $_{F.W.}$)
	mutant BW084		castasterone (167 pg g^{-1} $_{F.W.}$)
	mutant BW091		castasterone (232 pg g^{-1} $_{F.W.}$)
	mutant BW333		castasterone (390 pg g^{-1} $_{F.W.}$)
	mutant BW033		castasterone (2097 pg g^{-1} $_{F.W.}$)
	mutant BW312		castasterone (4357 pg g^{-1} $_{F.W.}$)
	mutant BW885		castasterone (3448 pg g^{-1} $_{F.W.}$)
Asahina et al. (2014)	**Rice** (*Oryza sativa* L.) cv. Koshihikari	Aerial part of 7-d-old seedlings growing at white light	6-deoxocathasterone (605 pg g^{-1} $_{F.W.}$), 6-deoxoteasterone (177 pg g^{-1} $_{F.W.}$), teasterone (40 pg g^{-1} $_{F.W.}$), 6-deoxo-3-dehydroteasterone (549 pg g^{-1} $_{F.W.}$), 6-deoxotyphasterol (2897 pg g^{-1} $_{F.W.}$), typhasterol (463 pg g^{-1} $_{F.W.}$), 6-deoxocastasterone (900 pg g^{-1} $_{F.W.}$), castasterone (329 pg g^{-1} $_{F.W.}$)
		Roots of 7-d-old seedlings growing at white light	6-deoxocathasterone (723 pg g^{-1} $_{F.W.}$), 6-deoxoteasterone (288 pg g^{-1} $_{F.W.}$), teasterone (248 pg g^{-1} $_{F.W.}$), 6-deoxo-3-dehydroteasterone (546 pg g^{-1} $_{F.W.}$), 6-deoxotyphasterol (3904 pg g^{-1} $_{F.W.}$), typhasterol (780 pg g^{-1} $_{F.W.}$), 6-deoxocastasterone (142 pg g^{-1} $_{F.W.}$), castasterone (34 pg g^{-1} $_{F.W.}$)

(continued)

Table 3.1 (continued)

Publication	Species	Organ	BRs
Janeczko et al. (2015)	**Spring wheat** (*Triticum aestivum* L.) cv. Katoda	Aerial part of 7-d-old seedlings	brassinolide (4000 pg g^{-1} F.W.), castasterone (80 pg g^{-1} F.W.)
Janeczko (2016)	**Spring wheat** (*Triticum aestivum* L.) cv. Katoda	Flag leaf of well-watered plants	castasterone (21 ng g^{-1} F.W.)
		Flag leaf of drought-stressed plants	castasterone (5.5 ng g^{-1} F.W.)
	cv. Monsun	Flag leaf of well-watered plants	castasterone (19 ng g^{-1} F.W.)
		Flag leaf of drought-stressed plants	castasterone (6.5 ng g^{-1} F.W.)
Pociecha et al. (2016)	**Winter rye** (*Secale cereale* L.) cv. Dańkowskie Złote cv. Stach	Leaves of 3-week-old plants -before cold hardening	castasterone (2473 pg g^{-1} F.W.) castasterone (2088 pg g^{-1} F.W.)
	cv. Dańkowskie Złote cv. Stach	-after 3 weeks of cold hardening at +4 °C	castasterone (6389 pg g^{-1} F.W.) castasterone (4872 pg g^{-1} F.W.)
	cv. Dańkowskie Złote cv. Stach	-after 6 weeks of cold hardening at +4 °C	castasterone (6575 pg g^{-1} F.W.) castasterone (7577 pg g^{-1} F.W.)
Gruszka et al. (2016a)	**Barley** (*Hordeum vulgare* L.)	Leaves of 14-d-old seedlings	
	cv. Delisa		castasterone (3619 pg g^{-1} F.W.)
	mutant *brd1-a*		castasterone (1485 pg g^{-1} F.W.)
	mutant *brd1-b*		castasterone (1299 pg g^{-1} F.W.)
	cv. Sebastian		castasterone (2413 pg g^{-1} F.W.)
	mutant *brd1-c*		castasterone (1021 pg g^{-1} F.W.)
	mutant *brd1-d*		castasterone (742 pg g^{-1} F.W.)
Gruszka et al. (2016b)	**Barley** (*Hordeum vulgare* L.)	Third and fourth leaf of optimally watered plants in fifth-leaf stage of growth	
	cv. Bowman		castasterone (5800 pg g^{-1} F.W.) 28-homocastasterone (52,690 pg g^{-1} F.W.)

(continued)

Table 3.1 (continued)

Publication	Species	Organ	BRs
	mutant BW084		castasterone (1160 pg g^{-1} F.W.) 28-homocastasterone (86,220 pg g^{-1} F.W.)
	mutant BW091		castasterone (1296 pg g^{-1} F.W.) 28-homocastasterone (93,405 pg g^{-1} F.W.)
	mutant BW333		castasterone (5220 pg g^{-1} F.W.) 28-homocastasterone (75,682 pg g^{-1} F.W.)
	mutant BW312		castasterone (9600 pg g^{-1} F.W.) 28-homocastasterone (43,110 pg g^{-1} F.W.)
	mutant BW885		castasterone (7540 pg g^{-1} F.W.), 28-homocastasterone (62,270 pg g^{-1} F.W.) 24-epibrassinolide (1200 pg g^{-1} F.W.)
	cv. Bowman	Third and fourth leaf of drought stressed plants in fifth-leaf stage of growth	castasterone (9280 pg g^{-1} F.W.) 28-homocastasterone (47,900 pg g^{-1} F.W.) 24-epibrassinolide (1186 pg g^{-1} F.W.)
	mutant BW084		castasterone (2273 pg g^{-1} F.W.) 28-homocastasterone (71,850 pg g^{-1} F.W.) 24-epibrassinolide (1104 pg g^{-1} F.W.)
	mutant BW091		castasterone (2270 pg g^{-1} F.W.) 28-homocastasterone (72,808 pg g^{-1} F.W.) 24-epibrassinolide (1296 pg g^{-1} F.W.)
	mutant BW333		castasterone (7540 pg g^{-1} F.W.) 28-homocastasterone (75,790 pg g^{-1} F.W.) 24-epibrassinolide (928 pg g^{-1} F.W.)
	mutant BW312		castasterone (19,428 pg g^{-1} F.W.) 28-homocastasterone (28,740 pg g^{-1} F.W.) 24-epibrassinolide (1200 pg g^{-1} F.W.)
	mutant BW885		castasterone (14,208 pg g^{-1} F.W.) 28-homocastasterone (57,480 pg g^{-1} F.W.) 24-epibrassinolide (912 pg g^{-1} F.W.)
Janeczko, Oklestkova, Novak, unpublished data 1	**Spring wheat** (*Triticum aestivum* L.) cv. Katoda	Aerial part of 21-d-old well-watered seedlings	28-homocastasterone (9 ng g^{-1} F.W.)
		Aerial part of 21-d-old drought-stressed seedlings	28-homocastasterone (13 ng g^{-1} F.W.)

(continued)

Table 3.1 (continued)

Publication	Species	Organ	BRs
	cv. Monsun	Aerial part of 21-d-old well-watered seedlings	28-homocastasterone (7 ng g^{-1} F.W.)
		Aerial part of 21-d-old drought-stressed seedlings	28-homocastasterone (12 ng g^{-1} F.W.)
Janeczko, Oklestkova, Novak, unpublished data 2	**Barley** (*Hordeum vulgare* L.) cv. Delisa	Aerial part of 7-d-old untreated plants	castasterone (0.42 ng g^{-1} F.W.) 28-homobrassinolide 137 ng g^{-1} F.W.) teasterone (1.12 ng g^{-1} F.W.)
		Aerial part of 7-d-old plants treated with brassinazole (brassinosteroid biosynthesis inhibitor)	castasterone (0.36 ng g^{-1} F.W.) 28-homobrassinolide (117 ng g^{-1} F.W.) teasterone (0.60 ng g^{-1} F.W.)

Original data expressed in pmol from part of articles have been recalculated to pg or ng (unification for the table purpose)

Stress is a very important factor that affects the BR content in *Poaceae* plants. For example, drought causes changes in the amount of individual BRs in wheat. The amount of 28-homocastasterone increased in aerial part of two cultivars of 21-day-old seedlings after a period of drought compared to plants that were optimally watered (Janeczko, Oklestkova, Novak, unpublished data 1, Table 3.1). The same phenomenon was observed in barley by Gruszka et al. (2016b, Table 3.1). On the other hand content of castasterone in flag leaf of drought stressed wheat plants was lower than in well-watered control (Janeczko 2016, Table 3.1). The BR content in tissues is also regulated by the plant growth temperature. Barley (genotype BW885) growing at 14 °C was characterised by a lower BR content (castasterone) than that growing at 26 °C (Dockter et al. 2014). The castasterone content increased in two rye cultivars (cv. Dańkowskie Złote and cv. Stach) during a few weeks of growth in the cold (cold-hardening process) (Pociecha et al. 2016). The presence or absence of light and its wave length also modifies the BR content in cereals (Asahina et al. 2014). For example, the castasterone content in the aerial parts of rice seedlings that were kept in the dark was on average 90 pg g^{-1} F.W. The authors considered this value to be 1 in order to make it easier to compared with the results that were obtained for plants growing in light. Plants cultured in far red light had 0.86 of the value that was observed in the dark, it was 1.68 for red light, 4.53 for blue light and plants growing in white light reached 4.30.

Finally, the content of BRs in *Poaceae* plants can be changed by using BR biosynthesis inhibitors such as brassinazole (BRZ). In about one-week-old barley seedlings, BRZ, which was applied *via* root watering on Petri dish, decreased the castasterone content by about 14% (Janeczko, Oklestkova, Novak, unpublished data 2, Table 3.1). The content of 28-homobrassinolide was decreased by about 15% and

content of teasterone was lowered by about 46% (Janeczko, Oklestkova, Novak, unpublished data 2, Table 3.1).

3 Uptake and Transport of Brassinosteroids in Plants of the *Poaceae* Family

In agricultural and biological experiments on cereal plants, BRs are most often applied by spraying the aerial parts of the plant (Ramraj et al. 1997; Shahbaz and Ashraf 2007; Kroutil et al. 2010) and much less often through the root system (plant watering) (Janeczko and Swaczynová 2010) or through presowing seed soaking (Sairam 1994a). It has been found that the uptake and transport of BRs depend on method of their application. BRs that are applied by spraying are poorly transported or are immobile in all of the plant. The ^{14}C-labeled brassinosteroids (e.g. 24-epibrassinolide), when applied on the leaf of rice or wheat seedlings, were not transported to the other leaves, although they could penetrate inside the tissues at the application site or even slightly translocate within the leaf (Yokota et al. 1992; Nishikawa et al. 1994). In this case, BR transport seems to be partly dependent on the concentration in the working solution. Higher compound concentrations may promote greater uptake efficiency. After spraying wheat (two-leaf stage of growth) with 24-epibrassinolide (0.1 μM), this compound was not detected in the third leaf (Janeczko and Swaczynová 2010). When a higher concentration (2 μM) was used, 24-epibrassinolide was detected in trace amounts in the third leaf. We assume theoretically in this case that the 24-epibrassinolide that was detected in the leaves was the same as that applied exogenously to the plants. The protective barrier covering the leaf on which drops of the working solution containing the hormone flow down may be a factor that limits BR penetration into the leaf. This problem can be eliminated by using the so-called infiltration method. The BR solution is pumped directly into the apoplast under pressure (Janeczko et al. 2011). The introduction of 24-epibrassinolide at 0.005 and 0.25 mg dm^{-3} concentrations to the apoplast of 12-day-old barley seedlings with two leaves resulted in an increased concentration of this compound in the seventh leaf, which formed later, compared to the control. It is interesting, however, that a similar content of this BR was found in the leaves of older plants regardless of the concentration of 24-epibrassinolide in the solution that was applied to seedlings. Therefore, possible BR transport was under the control of internal homeostasis mechanisms, thus preventing the penetration of non-physiological BR concentrations into the developing leaves.

More efficient uptake and subsequently, BR transport can be obtained using the root application because roots are organs that are designed to uptake substances from the soil solution. After the root application of radiolabelled 24-epibrassinolide to wheat and brassinolide or castasterone to rice, radioactivity was detected in the aerial parts of plants (Nishikawa et al. 1994; Yokota et al. 1992). According to Yokota et al. (1992), radioactivity in the aerial parts of plants was detected 6 h after

the root application and the majority of the determined there brassinosteroid pool was unmetabolised BRs. Wheat seedlings that were grown on Petri dishes and watered with a solution containing 24-epibrassinolide (0.1 and 2 μM) on the third day of vegetation accumulated an increased amount of this steroid in the leaves (Janeczko and Swaczynová 2010). The BR transport was also disproportionate to the applied concentration in this case. The root application of 24-epibrassinolide at a lower concentration caused amount of this compound in the leaves to increase 2-fold compared to the control. The quantity of 24-epibrassinolide in the leaves only increased 3-fold after the application of a 20-fold higher concentration (Janeczko and Swaczynová 2010). According to Nishikawa et al. (1994), BR transport probably occurs through the phloem. However, BRs induce physiological changes that involve the entire plant organism regardless of whether the BR transport takes place at a lower or higher efficiency (after root application) or whether it is applied locally (after spraying).

4 Selected Aspects of the Physiological Activity of Brassinosteroids in the *Poaceae* Family

Among the known brassinosteroids, exogenous 28-homobrassinolide and 24-epibrassinolide are the ones that are most commonly used in experiments on *Poaceae* plants, while brassinolide and other BRs are used less frequently. The experiments have mainly been carried out on popular species such as wheat, maize, rice and barley but also on less known finger millet. Currently, the availability of mutants, among others, rice and barley as well as maize mutants with BR biosynthesis or signalling disorders, allows for a more detailed explanation of some of the mechanisms of action of BR.

4.1 Plant Growth, Development and Yield

4.1.1 Plant Growth

Plant growth stimulation by brassinosteroids is the first known physiological activity of these compounds (Grove et al. 1979). Exogenous BRs stimulate the growth of plants in a concentration-dependent manner. For example, 24-epibrassinolide, when applied to two-week-old wheat seedling in concentration range from 0.005 to 2 mg dm^{-3}, most efficiently stimulated growth at concentrations of 0.25 and 0.5 mg (Janeczko et al. 2010) (Fig. 3.3). After root application of brassinolide solution (0.1–2 mg dm^{-3}) to germinated wheat seedlings, the most efficient growth stimulation of the aerial parts and roots was observed at a 1 mg dm^{-3} concentration (El-Feky and Abo-Hamad 2014).

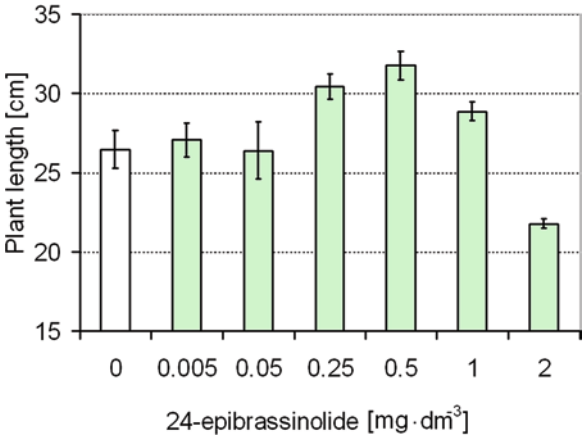

Fig. 3.3 Length of the aerial part of 4-week-old wheat seedlings 2 weeks after being sprayed with 24-epibrassinolide – a dose response curve. Mean values ± SE. (Based on Janeczko et al. 2010, modified)

The mutants of barley, rice and maize with brassinosteroid biosynthesis and signalling disorders were characterised by dwarfism, which confirms the significance of BRs for the growth processes of plants from the *Poaceae* family (Fig. 3.4a–d). Semi-dwarf mutants (*uzu*) were described in barley for the first time (Saisho et al. 2004). A monogenic, recessive mutation of the *HvBRI1* (*Uzu1*) gene encoding the transmembrane BR receptor was responsible for the mutant's phenotype. Recently, new mutations of the *uzu1* gene have been identified in barley. Mutations were induced *via* chemical and physical mutagenesis. Such mutants permitted a more detailed functional analyses of the gene and the encoded BR receptor. All of the mutations were 'missense type' mutations and resulted in substitutions of amino acids in different BR receptor domains, which is associated with BR-binding disorders (Gruszka et al. 2011a; Dockter et al. 2014). Mutants with BR biosynthesis disorders are also known in barley. Example are the semi-dwarf 522DK and 527DK mutants that were obtained by chemical mutagenesis (collection of the University of Silesia (Poland); Gruszka et al. 2011b, Fig. 3.4a, b). The mutants had missense mutations in the *HvDWARF* gene, which caused disturbances of the C6-oxidase activity in the BR biosynthetic pathway (Gruszka et al. 2011b). The mutants had a reduced content of endogenous castasterone compared to the wild type, i.e. 42% and 36% of the wild type values in the 522DK and 527DK, respectively (Janeczko et al. 2016). These mutants were more or less about 30% shorter than the wild type (Delisa) at every growth stage – from the coleoptile stage to the heading plants (Janeczko et al. 2016).

A dwarf maize mutant with a mutation in the *Brd1* gene encoding C-6 oxidase, which is the key enzyme responsible for BR conversions in the final steps of their biosynthesis, was described by Makarevitch et al. (2012). Plants with the mutation in this gene were five times shorter than the wild type and were also characterised by disturbances in their leaf and flower morphology.

A *d61* mutant phenotype, which is connected to the loss of function of the *OsBRI1* gene (BR receptor mutation), was described in rice (Morinaka et al. 2006). The identified alleles of this gene were numbered from 1 to 9 (mutants *d61-1–d61-9*). This

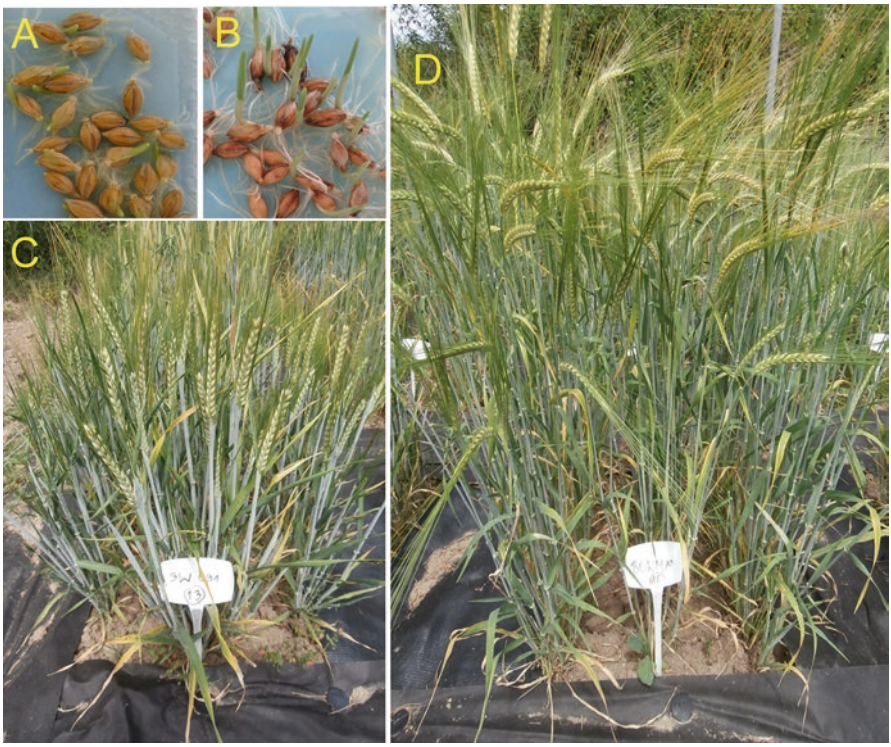

Fig. 3.4 Germination of the BR-deficient barley mutant 522DK (**a**) and the wild type cv. Delisa (**b**). Dwarf BR-deficient barley mutant BW091 (**c**) and wild type cv. Bowman (**d**) growing in a field. (Photo by A. Janeczko)

mutation is associated with the occurrence of dwarfism as well as with erect leaves. Earlier, Yamamuro et al. (2000) proved that OsBRI1 was responsible, among others, for internode elongation (inducing the formation of the intercalary meristem and the longitudinal elongation of internode cells) or for skotomorphogenesis in rice. Simultaneously, BRs also control leaf erectness in *Poaceae* (Sun et al. 2015). This mechanism is associated with the inhibition of the proliferation of specific abaxial cell groups in the lamina joint parts by BRs in rice. A U-type cyclin (CYC U4;1), whose activity and expression is controlled by BR signalling, has also been identified (Sun et al. 2015). Cyclins are engaged in the cell cycle as well as the activity of cyclins and cyclin-dependent kinases determine the progression of the cell cycle. Crosstalk with typical growth hormones is another mechanism by which BR influence *Poaceae* growth. In rice, BRs regulate cell elongation by modulating the metabolism of gibberellins (GAs) (Tong et al. 2014). BRs regulate the expression of the GA metabolic genes (i.e. by inducing *D18*/*GA3ox-2* expression), thereby promoting GA1 accumulation and cell elongation in rice. Excess BRs inactivate GAs through the upregulation of the GA inactivation gene (*GA2ox-3i*) and additionally repress BR biosynthesis, which results in growth inhibition. GAs may also inhibit

BR biosynthesis and BR response. On the other hand, barley BR mutants produced lower levels of cytokinins, thus the crosstalk between these hormones and BRs may also be important for growth regulation in *Poaceae* (Janeczko et al. 2016). Finally, cell elongation in *Poaceae* (rice) may also be simultaneously induced by BR and IAA (Yang et al. 2006). The authors characterised a brassinolide upregulated gene in rice, *OsBLE3*, which was expressed in the roots and leaf sheaths and this expression was BR dose-dependent. The reduced *OsBLE3* expression (in *OsBLE3* antisense transgenic rice) was accompanied by growth retardation. The presence of auxin response elements in the 5′-flanking region of the *OsBLE3* gene indicated that the expression of this gene was under the control of auxin. Simultaneously, the *OsBLE3* transcript level was reduced in the BR-deficient mutant compared to the wild type. According to Yang et al. (2006), OsBLE3 is engaged in cell elongation in rice through a dual regulation by brassinosteroid (brassinolide) and auxin (IAA).

4.1.2 Plant Development and Yield

The action of brassinosteroids on the development of plants of the *Poaceae* family is multidirectional. The application of 24-epibrassinolide in winter wheat during suboptimal vernalisation (low-temperature treatment required for the induction of development) slowed down the generative development by delaying plant entry into the heading stage compared to the control (Janeczko et al. 2015). Plants that had been treated with a BR biosynthesis inhibitor (brassinazole) headed faster than control. Plants that had been treated with the inhibitor, the effect of which was simultaneously compensated for the addition of exogenous 24-epibrassinolide, headed at a similar time as the controls. This suggests that the BRs in wheat may be a negative regulator in the generative development induction process (Janeczko et al. 2015). On the other hand, plant heading time was delayed 20 days in spring barley that had BR biosynthesis disorders and a decreased castasterone content (mutants 522DK and 527DK) (Janeczko et al. 2016). The role of BRs in the induction of generative development in *Poaceae* requires further research and differences between species must be taken into account. The action of BR, however, is also observed at later developmental stages – e.g. during pollen development. Holá et al. (2010) sprayed three maize lines with 24-epibrassinolide and one synthetic analogue of castasterone (2α,3α,17β-trihydroxy-5α-androstan-6-one) in field. The BRs were used in development stages V3/4 and V6/7 (i.e. 41 and 55 days from sowing) at concentrations of 10^{-8}–10^{-14} M. The spraying in stage V3/4 delayed male anthesis and silking, whereas spraying in stage V6/7 accelerated these processes regardless of the BR concentration and genotype. In turn, the final number of ears that were developed by each plant at the end of the flowering was dependent on the BR concentration and the time of application. The most effective were BRs in the 10^{-14} M concentration that were applied in V3/V4, while the application of BRs in V6/V7 decreased the ear number/plant. The work carried out by Czech researchers drew attention to the fact that the use of BR in field maize cultivation not only requires the precise determination of the BR concentrations, but also the selection of the most suitable plant

developmental stage for BR application and even taking into account the specificity of the line/cultivar.

The importance of natural BRs in the later stages of maize development was also confirmed in mutant studies of this species (Hartwig et al. 2011). Authors studied maize dwarf mutant *nana plant1* (*na1*), which has feminised male flowers. The mutant carried a loss-of-function mutation in the *DET2* homologue, which is a gene in the BR biosynthetic pathway and accumulated (24R)-24-methylcholest-4-en-3-one. It was accompanied by a decrease of the downstream BR metabolites. The expression of *na1* throughout their development, especially in the anthers, allowed a hypothesis that BRs promoted the masculinity of the male inflorescence and participated in the sex determination process in maize to be formulated.

Finally, BRs in *Poaceae* may participate in final steps of development – grain production and filling. Wu et al. (2008) created a transgenic rice expressing the gene encoding sterol C-22 hydroxylases. The enzyme controlled the BR levels and the obtained plants were characterised by an increased BR content downstream of 6-deoxocathasterone. For example, the content of 3-*epi*-6-deoxocathasterone was doubled, as was the content of 6-deoxotyphasterol. Transgenic plants with an increased BR level produced more tillers and seeds than the wild type. Seed yield increased by 15–44% depending on the growth conditions. The glucose pool was higher in the flag leaves and the plants had an increased glucose accumulation compared to the starch in the seeds. The significance of BRs for the production of grains and more broadly biomass by a plant was also demonstrated in the work of Morinaka et al. (2006). The authors compared biomass and grain production in the wild-type and the *d61-7* dwarf rice mutant (BR receptor mutation). The wild-type biomass was 38% higher than *d61-7* at the standard planting density, but the *d61-7* biomass was 35% higher than the wild type at a high planting density. Erect leaves of this mutant allowed for better light penetration in the field in this case. The small size of the *d61-7* grains, however, did not allow a higher yield to be obtained than for the wild type.

Research on mutants and transgenic plants (Morinaka et al. 2006; Wu et al. 2008), which provide information about the role of BRs in the processes of biomass accumulation and yielding, confirmed the results that had been obtained earlier in the experiments using exogenous BRs in rice by Ramraj et al. (1997) or Fujii and Saka (2001). The study of Fujii and Saka (2001) showed that exogenous brassinolide influenced the transport/accumulation of assimilates in the grains, thereby increasing the concentration of starch and sucrose in the forming seeds. On the other hand, an increase in the rice yield was found in a field experiment (India) after the application of 28-homobrassinolide (Ramraj et al. 1997). A double BR spraying at a concentration of 1 mg dm^{-3} was the most effective. The control yield was 4.90 t/ha, while 6.27 t/ha was obtained from the plants that had been treated with 28-homobrassinolide. The BR-sprayed fields were characterised by an increased number of panicles per square metre. Brassinosteroids also stimulate yield of wheat. 28-Homobrassinolide stimulated the wheat yield in field and pot experiments (Sairam 1994a, b). The field experiment (India) compared the wheat culture in a season with frequent droughts to artificially irrigated plants and found an increased number of seeds in the ears, the number of ears per m^2 and 1000 seed weight in both

groups of plants under the influence of 28-homobrassinolide. The compound was applied by seed soaking (6 h before sowing) and by spraying 25-day-old seedlings with 28-homobrassinolide (0.01 and 0.05 ppm) (Sairam 1994a). The cultivar C306 responded better to 28-homobrassinolide than HD2329 in that experiment. For example, the number of seeds that were collected from m^2 was 328 in the C306 under artificial irrigation, while 456 seeds were obtained in the plants after 0.05 ppm steroid spraying (Sairam 1994a). In a 3-year field experiment (India), 28-homobrassinolide stimulated the yield of wheat cv. Lok-1 (Ramraj et al. 1997). The average control yield from three seasons was 5.70 t/ha and an average of 6.70 t/ha was obtained for the best 28-homobrassinolide combination (spraying in two developmental stages, a concentration of 0.5 mg dm^{-3}) (Ramraj et al. 1997). Another BR – 24-epibrassinolide – when applied to wheat (plant spraying or seed priming) also increased the yield of this species (Ali et al. 2008; Hnilička et al. 2007; Janeczko et al. 2010). However, the effect of 24-epibrassinolide on the chemical composition of the grain was low and additionally depended on the cultivar and growth conditions (Hnilička et al. 2007, Janeczko et al. 2010). An increase in the content of soluble sugars in seeds (by 25% after hormonal seed priming), but not the starch content, was found in a pot experiment (Janeczko et al. 2010). A decrease in the fat content was observed (34% after 24-epibrassinolide spraying), but no significant changes in the soluble protein content were found. The influence of the hormone on the content of carbohydrates, proteins and lipids was very slight in a field cultivation (Janeczko et al. 2010). Hnilička et al. (2009) observed a weak, although in most cases positive effect, of 24-epibrassinolide spraying (10^{-9} M at the beginning of the flowering stage) on the protein, lipid and starch content in six wheat cultivars, which had been subjected to drought and a temperature increase to 33 °C (in the late stage of stem growth) in a pot experiment. Calorimetric analysis of the amount of energy that was accumulated in the grains (determined based on the combustion of a grain sample in an oxygen atmosphere in a calorimetric vessel) showed that its greater resources were stored by the plants that had been treated with 24-epibrassinolide. The action of BR in other *Poaceae* plants was also tested in maize and *Eleusine coracana* L. (finger millet). 24-Epibrassinolide and a castasterone analogue influenced the yield of field-grown maize that had a strong dependence on the cultivar, concentration and yield parameter (Holá et al. 2010). For example, the application of the castasterone analogue (10^{-14} M) increased the dry weight of the whole ear and cob in line 2023 when the plants were treated with the hormone in stage V3. The effect in line CE704 was the opposite. An increased yield from 1636 kg/ha (control) to 1990 kg/ha was found in the *Eleusine coracana* L. plants, to which BR had been applied by 8-h seed soaking (0.1 ppm) before sowing (Nithila et al. 2007).

It seems that the effect of BRs on the yield in *Poaceae* is mainly based on the regulation of the processes that are related to photosynthesis efficiency and, as was mentioned above, the transport of assimilates. An increase in the chlorophyll content that was caused by BR was found in wheat (Sairam 1994a, b), which is important in terms of the efficiency of solar energy absorption and the performance of the photosynthetic light reactions. Barley mutants with a reduced BR level also had a lower chlorophyll content in the leaves (Janeczko et al. 2016). The effect of BR on accumulation of photosynthetic pigments and the photosynthetic light reactions is

different in maize. According to Rothová et al. (2014), the application of 24-epibrassinolide and a castasterone analogue increased photosynthetic pigment accumulation and selected parameters that characterise PSII efficiency. For example, a positive effect on the oxygen-evolving complex (OEC) was observed. On the other hand, PSI efficiency in maize was not affected by these two steroids (Honnerová et al. 2010). An increased maximum quantum yield of primary photochemistry of PS II (Fv/Fm) was shown in transgenic rice with an increased BR accumulation (Wu et al. 2008) compared to the wild type. This rice was also characterised by an increased CO_2 assimilation in the photosynthetic dark reactions. The increased activity of the CO_2-binding enzyme Rubisco (carboxylase-ribulose-1,5-bisphosphate carboxylase) and net photosynthesis were previously recorded in wheat after exogenous BR application (Braun and Wild 1984; Sairam 1994a, b; Hnilička et al. 2008). Simultaneously, BR-deficient barley mutants were characterised by a lower Rubisco activity (Janeczko et al. 2016). These mutants also had a reduced sucrose accumulation along with increased glucose and fructose levels, thereby suggesting that BR could also affect the enzymatic system that is involved in sugar (sucrose) biosynthesis. This is consistent with studies in which exogenous BRs increased the production of sugars and their transport (Fujii and Saka 2001; Wu et al. 2008). These phenomena are an important element of the mechanism by which BRs stimulate the biomass accumulation, including yield. As presented, BRs act during the entire plant life cycle in the *Poaceae* family and are responsible for the direct or indirect regulation of many growth, developmental and yield processes.

4.2 Plant Stress Response

During the vegetation period, plants are naturally exposed to different environmental factors – biotic (pathogens) and abiotic (drought or excess of water, too low or too intense light, cold, frost, too high temperatures etc.) The occurrence of these stress factors during the growth of the crop plants of the *Poaceae* family (such as rice, maize, wheat) and especially their higher severity can cause significant damage to crops that result in yield losses. Brassinosteroids are one of the plant hormones that stimulate the processes that counteract the negative effects of stress.

There are many publications that show that BRs counteract the effects of many types of stresses in the species of the *Poaceae* family. In this review, only a few examples will be given, together with an explanation of some of the mechanisms of action of BR.

4.2.1 Salt Stress

Salinity is a problem of agricultural soils in many countries, hence much work has been devoted to research that is aimed at improving the conditions of plant growth under this stress. Brassinosteroids alleviate the negative effects of salt stress on

Poaceae plants. An example is 28-homobrassinolide that was applied by presowing seed soaking (12 h, 10^{-4}–10^{-8} M concentrations) in maize (Arora et al. 2008). The hormone increased the activity of the antioxidant enzymes (superoxide dismutase (SOD), guaiacol peroxidase, catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (ASP) in the leaves of 30-day-old maize that had been exposed to salt stress (NaCl – 25, 50 and 75 mM). The hormone reduced the peroxidation of cell lipids (measured by the accumulation of malondialdehyde (MDA) and increased the protein content (Arora et al. 2008). According to the authors, 28-homobrassinolide alleviated the oxidative stress in the salt-treated maize plants. The ameliorative effects of another BR – 24-epibrassinolide – in mitigating the phytotoxicity of NaCl stress in the seedlings of maize were also reported by Agami (2013). The application of the hormone improved growth, increased photosynthetic pigment and proline content as well as the antioxidant activity of CAT and peroxidases. In addition to changes in the efficiency of the antioxidant system, BRs regulated maize's hormone metabolism under salt stress. Brassinolide used for seed soaking and plant spraying (0.25 ppm) abolished the adverse effect of salinity on plant hormone production (IAA, GA3 and zeatin) (El-Khallal et al. 2009). In wheat, spraying plants with 24-epibrassinolide stimulated biomass production and increased the leaf surface area under saline conditions in two cultivars – S-24 – saline-resistant and MH-97 – susceptible to this stress factor (Shahbaz et al. 2008). The application of this hormone to the roots in wheat growing in a hydroponic culture under saline conditions also resulted in an increase in the total yield (among others, through an increase of the 100-seed weight) in the two tested wheat cultivars (Ali et al. 2008). The best effects were reported for 0.104 and 0.052 μM concentrations (Ali et al. 2008). The results of Tofighi et al. (2017) were also interesting, as these authors claimed that BR increased wheat salinity tolerance by cooperating with arbuscular mycorrhizal fungi (*Glomus mosseae*). BR prevented a decrease in chlorophyll and increased the nitrate reductase activity in rice growing under saline conditions (Anuradha and Rao 2003). This compound also increased the content of the proline osmoprotectant, proteins and the activity of antioxidant enzymes as well as reduced the damage to cell membranes (Sharma et al. 2013). An increased activity of antioxidant enzymes in rice growing in salt stress was noted also under the influence of one of the BR analogues (BB-16) (Núñez et al. 2003).

4.2.2 Drought Stress

Water deficiency is one of the most important factors that limits crop yield. Drought excludes agricultural cultivation in many areas. Regions with sufficient water resources may also endure years with periodic droughts due to changes in climate. BRs are one of the regulators that can minimise the effects of drought on plant growth and yield. The effect of 24-epibrassinolide on the yield of spring wheat cv. Torka was evaluated in a field experiment conducted in the climatic conditions of central-eastern Europe (Poland) (Janeczko et al. 2010). 24-Epibrassinolide was administered *via* 48-h presowing seed soaking (1 mg dm^{-3}) and spraying the plants

in the heading stage (0.25 mg dm^{-3}). Although the average rainfall for July in Polish climatic conditions usually reaches 90 mm/month and is sufficient for plants, drought unexpectedly occurred in that month during an experiment in 2006 (rainfall 14 mm/month). This allowed the effect of the hormone to be evaluated under natural drought conditions. BR raised the crop yield in the field cultivation by about 20% compared to the untreated control. The basis for the increase in the yield was the formation of a higher number of seeds by the plant (Janeczko et al. 2010). Hnilička et al. (2007) also observed a slight increase in the seed and straw yield in six wheat cultivars that had been sprayed with 24-epibrassinolide (10^{-9} M concentration, greenhouse conditions) under drought stress and that were then subjected to an increased temperature of 33 °C (in the late stage of stem growth). Sairam (1994a, b) conducted research on selected mechanisms of BR action in wheat in drought conditions. Author found, among others, that in wheat BR (28-homobrassinolide) stimulated the activity of the enzymes that are associated with nitrogen metabolism: nitrate reductase and glutamate synthetase. This compound also decreased stress-induced cell membrane damage. Farooq et al. (2009, 2010) described a beneficial effect of BR on rice plants in drought conditions. BRs possibly enhanced plant growth because of the improved assimilation of carbon. The BR-treated plants were also characterised by an ability to maintain a better tissue water status. While drought increased H_2O_2 and MDA production, BRs counteracted this effect, among others, by enhancing the capacity of the antioxidant system. Of the two BRs that were used, 24-epibrassinolide and 28-homobrassinolide, the former was more active. Moreover, the application *via* spraying was more effective than seed priming. The study of Janeczko et al. (2016) characterised physiologically and biochemically BR-deficient barley mutants (522DK and 527DK) and the wild type Delisa. The aim of the study was to answer the question of whether/how disturbances in the production of brassinosteroids in barley affect the plant's metabolism under drought. In drought conditions, BR synthesis disorders were accompanied by a decrease in the production of other plant hormones (ABA and cytokinins), although this effect was not observed for auxins. The mutants produced less osmoprotectant proline compared to the wild type during drought. They also accumulated less sucrose, although the Rubisco activity was at a similar level in both the mutants and the wild type. The accumulation of the transcript of the gene encoding the protective protein – hsp90 – from the heat shock protein group was statistically significantly reduced in the 527DK mutant. A reduced kestose accumulation (one of the fructans considered to be cell membrane stabilising factor) was revealed in 527DK under drought. Finally, PSII efficiency in conditions of drought was lower in the mutants – especially in 527DK. The findings of Gruszka et al. (2016b) for drought-stressed barley mutants with disturbances in BR biosynthesis and signalling can serve as an interesting conclusion to this chapter. The authors proved that all of the mutants and the wild type plants increased the production/accumulation of BRs in drought conditions, which may support the presented data and show that BRs play an important role in protecting plants against drought.

4.2.3 Heavy Metal Stress

Many heavy metals such as copper, manganese, iron or cobalt are naturally present in living organisms and are often components of the enzyme and protein molecules that are required for cell function. However, an excess of these elements is toxic to cells. Many works have shown that the negative effects of heavy-metal poisoning were alleviated or limited by BRs in *Poaceae* plants. 24-Epibrassinolide (0.1 mg dm^{-3}), which was sprayed on maize plants that were then subjected to the stress of a high manganese concentration in soil (150–750 mg kg^{-1}, a phenomenon that is particularly dangerous in acidic soils), reduced the unfavourable physiological changes that are caused by excess of this element (Wang et al. 2009). This was manifested by an increase in the chlorophyll content, net photosynthesis intensity and dry matter accumulation. A decreased accumulation of H_2O_2 was observed in plants together with an increased activity of antioxidant enzymes (including SOD, CAT, GR, ASP). Bhardwaj et al. (2007) studied the effects of 28-homobrassinolide on maize seedling growth, lipid peroxidation and antioxidative enzyme activities under nickel stress. The hormone reduced the toxicity of the heavy metal on seedling growth and also influenced the protein content. Lipid peroxidation was increased under the heavy metal stress, but decreased in the BR-treated plants. The hormone also increased the activity of the antioxidant enzymes (except SOD). The application of 24-epibrassinolide was effective in ameliorating the stress that was caused by chromium in rice (Sharma et al. 2016). The application of the hormone as a pre-soaking treatment resulted in better plant growth, a lower accumulation of chromium by the tissues and a strengthened defense system by upregulating the gene-encoding antioxidant enzymes such as Mn-SOD, Cu/Zn-SOD, CAT or GR.

4.2.4 Temperature Stress

Among abiotic stresses, temperature stress is a particularly serious problem in agriculture and horticulture. Some species such as maize are very sensitive to cold, while frost, especially when there is insufficient snow cover on fields, can cause significant yield losses of winter cereals. High-temperature stress is dangerous when combined with drought during the vegetation season. Many hormones control the plant response to high or low temperatures and brassinosteroids appear to be among them. An increased concentration of abscisic acid (ABA) – a stress hormone – occurred in maize as a defensive response to cold stress (Janowiak et al. 2003). Moreover, the cold-tolerant cultivars of maize accumulated more of this hormone (Janowiak et al. 2003). Studies related to changes in the level of brassinosteroids in plants under temperature fluctuations are scarce. The content of one of the BRs, castasterone, which was measured in the barley line BW885 at 14 °C, was 7.43 pmol g^{-1} F.W., but increased to 10.31 pmol g^{-1} F.W. after the plants were moved to 26 °C (Dockter et al. 2014). Simultaneously, Pociecha et al. (2016)

observed an increased castasterone content from 4–5 pmol g^{-1} F.W. (control) to 14–16 pmol g^{-1} F.W. in winter rye (*Secale cereale* L.) plants after 6 weeks of plant cold hardening. The described changes may suggest that BRs play a role in the processes of acclimation to changing temperature conditions.

4.2.4.1 Frost

Sudden drops in temperature during winter followed by the periods of higher, dehardening temperature in countries that cultivate winter cereals is a factor that causes frost damage (especially in the absence of snow cover) that later affects yielding. Research conducted by Pociecha et al. (2016) showed that 24-epibrassinolide (0.25 mg dm^{-3}), when applied before the cold hardening of winter rye, significantly increased frost tolerance. Plants had less frost damage and a higher survival rate. 24-Epibrassinolide also improved frost tolerance in winter wheat (Janeczko 2016, Fig. 3.5). Wheat seedlings that had been sprayed with the hormone, cold-acclimated at +5 °C and then exposed to −12 °C had a better survival rate than untreated plants (Fig. 3.5). According to Pociecha et al. (2016), 24-epibrassinolide increased the Rubisco activity in both of the cultivars that were tested as well as the sucrose content (but in a cultivar-dependent manner). An increased sucrose concentration is a well-known phenomenon in the process of cold hardening and its function is to reduce the freezing point of the cell aqueous solution, which improves survival in frost conditions. In one of the cultivars that was tested, BR also stimulated the accumulation of protective fructooligosaccharide (nystose) by 55% compared to the cold-hardened plants that had not been sprayed with BR.

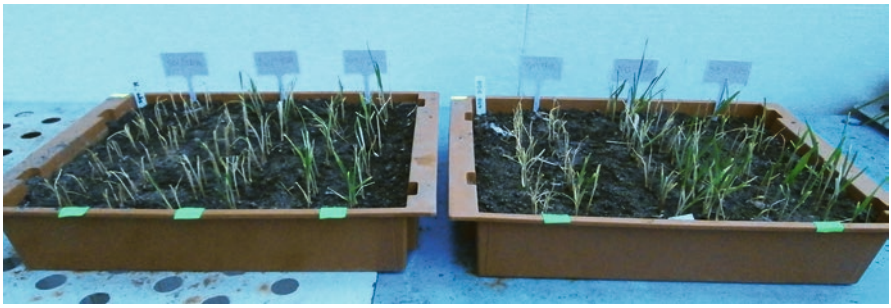

Fig. 3.5 Regrowth of winter wheat after exposure to −12 °C. Dying plants of cv. Bystra (low frost tolerance) and cv. Nutka (moderate frost tolerance), visible in control (*left pot*); only plants of the highly tolerant cv. Smuga survived. BR application (*right pot*) before the low temperature treatment increased the survival of the cv. Nutka plants and even some plants of cv. Bystra also regrew. (Janeczko 2016, data from project 2013/09/B/NZ9/01653). Order of cultivars in pot: cv. Bystra - first two rows, cv. Nutka - rows 3 and 4, cv. Smuga - rows 5 and 6.

4.2.4.2 Cold

Among the plants of the *Poaceae* family, maize is particularly cold sensitive. Temperatures below 10 °C can seriously damage young maize seedlings. An experiment of Singh et al. (2012) exposed maize seedlings to cold stress (net house with a maximal temperature of 17.6–24.5 °C and a minimal temperature of 2.8–7.4 °C; 21 days). The authors showed a decrease in plant height by about 35% and F.W. by about 24%; the data were compared to the controls that were growing in a green house (25/18 °C (d/n)). The application of 24-epibrassinolide (1 μM) to the plants that were growing in the net house increased plant height, fresh and dry weight (15, 36 and 2%, respectively) compared with the plants without the application of BR. Seedlings that were exposed to the cold in the net house had a slightly increased glucose, starch and sucrose content compared to the control plants that were cultured in the controlled conditions of the greenhouse. Additionally, 24-epibrassinolide elevated the content of these sugars (15–45%) compared to the stressed plants without the BR treatment. Cold also decreased the chlorophyll content in the maize in the net house, but this effect was neutralised by 24-epibrassinolide.

4.2.4.3 High Temperatures

High temperature in natural conditions, when associated with drought stress, is a very important cause of the limitation of photosynthesis and inhibition of growth. Thussagunpanit et al. (2015a, b) studied the effect of high temperature on rice plants. The decrease in the chlorophyll content was milder in rice that had been treated with 24-epibrassinolide prior to exposure to high temperature (40/30 °C; 7 days). After hormone application, the heat-stressed rice had a better PSII performance and significantly improved electron transport rate (Thussagunpanit et al. 2015a). The protective effect of 24-epibrassinolide (0.25 mg dm^{-3}, leaf infiltration before heat stress) on PSII performance was also found in barley seedlings (Janeczko et al. 2011). Energy absorption by the antennas, energy transferred to the reaction centre and energy transferred to the electron transport chain were higher in the first leaves of the seedlings by 23, 49 and 69%, respectively, when compared to the values that were recorded in the stressed plants without the application of BR. In rice, high temperature also decreased the leaf net CO_2 assimilation and transpiration parameters by 17 and 31%, respectively, and increased the leaf internal CO_2 concentration by 8% compared to non-stressed plants (Thussagunpanit et al. 2015a, b). The application of 24-epibrassinolide counteracted this effect.

4.2.5 General Comments

In opinion of author of this chapter, of all of the mechanisms of the action of BRs, two appear to be the most important and especially help *Poaceae* plants to achieve a better stress tolerance: (1) a modulation of the antioxidant cell system (Xia et al. 2015) and

(2) physicochemical modifications of the properties of the cell membrane (Filek et al. 2017). Of course, we should not forget that BRs regulate the expression of many genes as well as cooperate with other hormones, but it is the stabilisation of the cell membrane and the ability to maintain the proper redox balance in a cell that provide a favourable environment for the functioning of all of the other biochemical processes.

The cell membranes are involved in thermal sensing (Horváth et al. 2012) and are generally responsible for the cell-environment contacts. The proper functioning of the membranes affects all of the processes that are localised in the membranes such as the light phase of photosynthesis (the proper structure of the photosynthetic antennas, the efficiency of the photosystems) and also some parts of the dark phase (e.g. the aquaporin channels that enable CO_2 transport are located in the membranes). As was mentioned earlier, although the action of BR on the membranes in stress conditions is manifested by a reduction in membrane permeability and lipid peroxidation, BRs also modulate the physicochemical properties of the cell membranes (Filek et al. 2017). Two brassinosteroids with different chemical structures, 24-epibrassinolide and 24-epicastasterone, when introduced into lipid the monolayers, changed their physicochemical properties. Studies were performed using a Langmuir bath to analyse the monolayer formation of lipids that had been isolated from wheat leaves growing at 20 °C and in the cold (5 °C). 24-epibrassinolide increased the area per lipid molecule in the monolayers, which resulted in the formation of more flexible surface structures. This effect is very similar to the effect of sterols on membranes and is associated with a higher fluidity of membranes, which guarantees (especially in low temperatures) a better stress tolerance for the entire plant. Interestingly, the second BR that was studied, 24-epicastasterone, induced the different effects, which showed the importance of the BR chemical structure for their interaction with cell membranes and further physiological effects. Xia et al. (2015) described the maize model of interaction between BRs and the antioxidant system and the effects of these interactions in plants – growth or stress response (Fig. 3.6). According to Xia et al. (2015), activation of the BR receptor led to the production of reactive oxygen species (ROS) (e.g. H_2O_2). However, the temporal and spatial changes in their levels depended on the BR concentrations (the stress factors increase the accumulation of BRs (Gruszka et al. 2016b; Pociecha et al. 2016). High BRs levels cause the long-term accumulation of reactive oxygen species, which in turn triggers the miogen-activated protein kinase phosphorylation cascade. In this case, ROS and kinase stimulate the ABA biosynthesis – the main hormone that is associated with the induction of stress tolerance. Low BRs levels, on the other hand, cause a transient increase in the ROS concentration, which stimulates a cell's antioxidant system, which ultimately leads to shifting the redox balance of the cell towards the reducing processes. This acts as a signal, e.g. for the stimulation of the photosynthesis and growth processes.

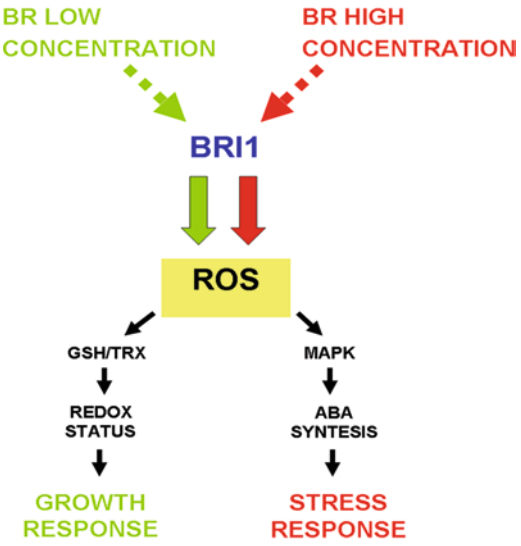

Fig. 3.6 BR concentration-dependent model of growth and stress response in maize (Xia et al. 2015, modified). *BRI1* brassinosteroid receptor domain, *MAPK* specific mitogen activated protein kinase, *GSH/TRX* glutathione/thioredoxin systems

5 Future Perspectives

The discussed experiments that tested the effects of exogenously applied BR treatments provide an overview of the physiological functions of these compounds in *Poaceae*. Recently, the number of studies that use research models that involve biosynthesis and perception BR mutants of *Poaceae* plants has also significantly increased. Importantly, studies on mutants confirmed lot of BR physiological functions previously reported in works that used exogenous BRs. For instance, the role of BRs in the regulation of CO_2 assimilation, proline and sugar production or their protective effect on the PSII complex under stress conditions can be mentioned. Simultaneously, the results from studies in which BRs were exogenously applied on plants growing in stress conditions were a good starting point for the production of agrochemicals that contain BRs – natural and biodegradable substances (Khripach 2010). Such agrochemicals could be useful for protecting cereal crops in changing climatic conditions. On the other hand, manipulating the endogenous BR levels or elements of its signalling pathways (classical breeding methods or genetic engineering) may help to obtain new cereal cultivars – dwarfs or those with a higher resistance to stress (Morinaka et al. 2006; Dockter et al. 2014).

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Chapter 4
The Importance of Fluoro and Hydroxyl Substitutions in Brassinosteroids for Shooting-Control: The Use of *In Vitro*-Grown Shoots as Test Systems

Adaucto B. Pereira-Netto

Abstract Biologically active brassinosteroids (BRs) induce a broad spectrum of responses, including stimulation of longitudinal growth of tissues via cell elongation and division, besides stimulation of vascular differentiation, the last one a developmental process critical for shoot elongation. We have been using *in vitro*-grown plants, especially the marubakaido apple rootstock, as test systems to probe into the ability of BRs, mainly new synthetic analogs, to control shooting. Replacement of 5α-H or 3α-OH groups of the steroidal structure of BRs by 5α-F, 3α-F or 5α-OH groups, respectively, has led to significant changes in the abilities of parent compounds such as homocastasterone to control shoot formation and their further elongation, being the effect species and organ-specific, besides being also dependent on the type, i.e., hydroxy or fluoro, position of the substitution. In this chapter, it will also be discussed how treatment of *in vitro*-grown shoots with new synthetic BR analogs has helped to: (1) Enhance our understanding about the relevance of selected functional groups for the BRs's action mechanism(s); (2) Get an insight into the morphological responses of shoots, grown *in vitro*, to the application of BRs and synthetic analogs; (3) Improve micropropagation techniques for clonal propagation, especially of woody species, in which new shoot formation and its further elongation is typically a constrain for efficient micropropagation; (4) Guide the development of novel BR analogs for higher activity, at a lower cost.

Keywords *Malus prunifolia* · Marubakaido · Rootstock, · Micropropagation · Brassinosteroid analog

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1 Introduction

Brassinosteroids (BRs), are highly oxygenated, low-abundance, plant steroids of ubiquitous occurrence in plants. Molecular genetic analysis has demonstrated that the ability to synthesize, perceive and respond to BRs is essential for normal plant growth and development. Biologically active BRs are known to play critical roles in a broad range of physiological processes, when exogenously supplied at very low concentration, at the nanomolar to micromolar levels. These processes include stimulation of longitudinal growth via cell elongation and cell division, and enhancement of phloem and xylem differentiation, all required for shoot elongation, especially of young tissues. A large number of reports have shown that BRs can improve yield and quality of crops, especially under stress conditions, besides being environmentally friendly, for example, by ameliorating toxic effects derived from heavy metals, including aluminum, copper, nickel and plumb. In addition, BRs are known to reduce the need for fertilizers and to accelerate metabolism of herbicides, fungicides and insecticides, and consequently reducing their residual levels in crops. Because of this BR-driven reduction of the risks for human health and environment, BRs have sparked great interest in green agricultural uses.

Similar to steroid hormones in animals, the structures of BRs consist of a cholesterol skeleton with various hydroxyl substitutions and attached functional groups. Sixty two chemical structures of naturally occurring BRs have been confirmed so far. All natural bioactive BRs, like brassinolide (BL, Fig. 4.1), castasterone (CS) and typhasterol (TY) present a vicinal 22*R*, 23*R* diol structural functionality, which are essential for high biological activity. The elucidation of the co-crystal structure of BL bound to BRI1, the leucine-rich repeat receptor kinase that is involved in perception and transduction of BR signaling at the cell membrane, shows that this diol moiety is engaged in a hydrogen-bonding net work within the hydrophobic pocket where the alkyl chain of the hormone fits (Hothorn et al. 2011; She et al. 2011). BL, the end product of the BR biosynthetic pathway, is widely considered to present higher biological activity than any other natural BRs. However, the synthesis of the naturally occurring BL is expensive. In addition, the rapid metabolism of natural BRs in plants and the consequent reduction in their biological activity is a major constrain for a broader commercial use of natural BRs, such as BL, in economical

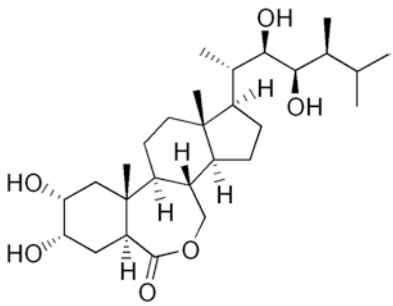
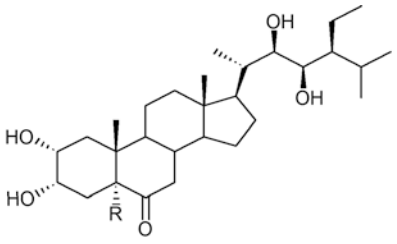


Fig. 4.1 Structural formulae of brassinolide (BL)

activities such as agriculture, horticulture and forestry. The easier to synthesize 24-epibrassinolide (24-epiBL), the stereoisomer of BL, has been the most widely used BR to date. However, 24-epiBL is also expensive, which limits its popularization and practical applications (Lei et al. 2017). Thus, the development of lower cost novel synthetic derivatives, besides enabling studies of structure-activity relationships, biosynthesis and metabolism of BRs, is an effective way to overcome the rapid metabolism of natural BRs in plants once synthetic derivatives have been demonstrated to be more difficult to be metabolized by plants. Such high biological activity new derivatives are expected to allow a broader commercial use of BRs.

Slight structural changes in ring A and B as well as in the side chain of BRs are known to result in moderate to drastic differences in plant growth activity (Liu et al. 2017). Substitution of a hydrogen atom by fluorine in what was originally a carbon–hydrogen bond, causes only a small increase in size of the BR molecule, but it significantly increase electronegativity and hydrogen bonding potential. Thus, fluorination of BRs can change their ability to bind to BRI1, the BR receptor, changing consequently the biological activity of the parent compound. The degree of response elicited by a given BR depends on the position of functional groups in the carbon skeleton. For example, the presence of C-2α hydroxyl, and especially C-3α hydroxyl, in ring A are needed for enhancement of biological activity. Furthermore, it is known for quite some time that alteration of the functional groups in the carbon skeleton affects the degree of response elicited by a given compound. The carbon-fluorine bond is physic chemically similar to the C–OH bond, rather than the C–H bond. Thus, fluorine could be considered as being equivalent to the oxygen of the hydroxyl group. In an attempt to enlarge studies on the effects of BRs and synthetic analogs on bioactivity, the naturally occurring BRs homocastasterone (HCS) and homotyphasterol (HTY), along with derivatives in which the 5α-H group of HCS and HTY was replaced by a 5α-F and/or a 5α-OH group, or the 3α-OH group has been replaced by a 3α-F group (Figs. 4.2 and 4.3) were applied to *in vitro*-grown shoots of the marubakaido apple rootstock or a clone of a hybrid between *Eucalyptus grandis* and *E. urophylla*. In this chapter, we describe the effects of these compounds on new shoot formation and further elongation, along with their consequences for the *in vitro* multiplication rate.



R=H 28-homocastasterone (28-HCS)
R=F 5α-fluoro-28-homocastasterone (5F-HCS)
R=OH 5α-hydroxy-28-homocastasterone (5OH-HCS)

Fig. 4.2 Structural formulae of 28-homocastasterone (HCS), 5α-fluoro-28-homocastasterone (5F-HCS) and 5α-hydroxy-28-homocastasterone (5OH-HCS)

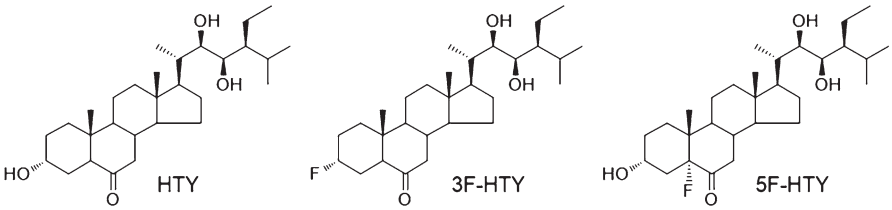


Fig. 4.3 Structural formulae of homotyphasterol (HTY), 3α-fluoro-homotyphasterol (3F-HTY) and 5α-fluoro-homotyphasterol (5F-HTY)

2 Effects of Brassinolide on *In Vitro*-Grown Shoots of the Marubakaido Apple Rootstock

Progressive increase in *in vitro*-grown marubakaido shoot length is related to increased doses of BL (Pereira-Netto et al. 2009). A statistically significant increase of 12% and 25%, respectively, for the main (shoots originating directly from the initial shoot segment) and primary lateral (shoots originating from the main shoots) shoot length has been found for shoots treated with 1.25 μg.shoot^{-1} BL, compared to untreated shoots. Shoot treatment with BL also results in enhanced formation of main and primary lateral shoots. Maximum enhancement in the formation of new main (23%) and primary lateral shoots (46%) were found for shoots treated with 0.25 and 0.50 μg.shoot^{-1} BL, respectively.

3 Effects of Homocastasterone and Hydroxy and Fluoro Synthetic Analogs on *In Vitro*-Grown Shoots of the Marubakaido Apple Rootstock

Twenty eight-homoethylcastasterone (HCS) has been widely employed in field trials because of its greater synthetic accessibility compared to the BL. However, studies in our laboratory have shown that leaf application (5 μL) of HCS, in which a fluoro group was introduced in alpha configuration at C-5, to *in vitro*-grown marubakido shoots results in enhanced formation of new main shoots, but especially enhanced formation of primary lateral and secondary lateral (shoots originating from the primary lateral shoots) shoots (Schaefer et al. 2002). These enhanced shoot formation is followed by enhanced elongation of main and primary lateral shoots (Pereira-Netto et al. 2006b). This shoot proliferation results in a 112% increase on multiplication rate for *in vitro*-grown marubakaido shoots treated with 500 ng per shoot 5F-HCS. Differently from 5F-HCS, which induced remarkable changes in the arquitecture of *in vitro*-grown marubakaido shoots, 28-HCS and 5OH-HCS applications result in no statistically significant change in formation or elongation of newly formed shoots (Pereira-Netto et al. 2006b).

Since both hydrogen and fluorine atoms are small, univalent and contribute rather little to total molecular polarizabilities, physical properties are less affected by equating fluorine and hydrogen than most of the chemical properties (Liebman 1988). Considering that effects of BRs and analogues on shoot formation and further elongation depend on the extents to which these molecules satisfy the structural requirements of the receptors and/or enzymes, the differential responses found for 28-HCS and its 5α-fluoro substituent-treated shoots of marubakaido described in this paper suggests differences on metabolic routes, higher chemical stability for 5F-HCS or higher affinity and/or binding time of 5F-HCS for the receptor sites of BRI1, the receptor for BRs in this biological system.

Since fluorine and hydroxyl are similar, regarding electronegativity, and the C-F bond is physicochemically similar to the C-OH bond, it was somewhat surprisingly to find that the 5F-HCS effectively promote shooting in the marubakaido apple rootstock, while 5OH-HCS shows no effect. This reduced bioactivity of 5OH-HCS might be due to formation of an H-bonding between the 3α and the 5α-hydroxy groups, an event that might reduce the ability of the hydroxylated compound to bind to the active site of the receptor through its C-3 hydroxyl group.

4 Effects of Homocastasterone and Its 5A-Monofluoro Analog on *In Vitro*-Grown Shoots of a Hybrid Between *E. GRANDIS* and *E. urophylla*

Stimulation of main shoot formation and further elongation is found for shoots of a hybrid between *E. grandis* and *E. urophylla* immersed in solutions of 28-HCS. For shoots treated with the 5-fluoro analog of 28-HCS, no significant change in either main shoot formation or further elongation is observed. Differently from what is seen for main shoots, treatment with 28-HCS lead to inhibition of both primary lateral shoots formation and their further elongation. Conversely, enhancement in the average length of primary lateral branches is found for shoots treated with 5F-HCS, although primary shoot formation is inhibited by 5F-HCS treatment. The extent in which 5F-HCS stimulated primary lateral shoots elongation does not differ significantly from the extent in which 28-HCS stimulats main shoots elongation. Multiplication rate raises significant 34% for shoots treated with 10 $mg.l^{-1}$ 28-HCS, compared to shoots treated with acetone, only (control), being the effect due essentially to the 28-HCS-driven enhancement in the formation of new main shoots. For shoots treated with 5F-HCS, decrease in the multiplication rate is observed as a consequence of the reduced formation of both, main and primary lateral branches. When seen together, these data clearly show that 28-HCTS and its 5α-monofluoro analog differentially change shoot architecture in *in vitro*-grown shoots of the hybrid between *E. grandis* and *E. urophylla* used in our laboratory.

Fluorination-driven changes in biological properties of compounds like gibberellins, tetracyclic diterpenoids that control stem elongation, depend upon the degree

of fluorination and differ according to the type of bioassay used to access the biological activity. Because of their high electronegativity, monofluoro analogues occasionally bind enzymes irreversibly, which might have deleterious effects on the organism. However, monofluoro analogues of gibberellins are shown to present higher biological active in assays such as the lettuce hypocotyls elongation, when compared to their parental counterparts. In our laboratory, the finding that 28-HCTS is able to stimulate elongation and formation of main branches, and consequently to enhance *in vitro* multiplication rate of the *E. grandis* X *E. urophylla* hibrid, prompted us to test the hypothesis that a 5 α-fluoro derivative might be able to amplify the stimulatory effect of 28-HCTS on elongation and formation of main branches. However, 5F-HCTS is unable to either stimulate elongation and formation of new main branches, or enhance multiplication rate, although it stimulates elongation of primary lateral branches. The reason(s) for these differential responses of 28-HCTS and 5F-HCTS does not appear to be straightforward. A possible formation of a hydrogen bond involving fluorine and a consequent reduced ability to bind to the BR receptor might explain the inability of 5F-HCTS to stimulate elongation and formation of main branches in the *E. grandis* X *E. urophylla* hybrid, differently than what is seen for the marubakaido apple rootstock.

Finally, the enhancement in the multiplication rate found for 28-HCTS-treated shoots in this study demonstrate that BRs can be used for the improvement of protocols used for *Eucalyptus* micropropagation (Patent BR 0403642-5). In addition to that, the results presented in this paper indicate that BRs might be useful to manage branching in field-grown *Eucalyptus* trees.

5 Effects of 5F-Homotyphasterol on *In Vitro*-Grown Shoots of the Marubakaido Apple Rootstock

Enhancement over a hundred percent on the number of newly formed primary lateral shoots (shoots originating directly from the main branches) is observed for *in vitro*-grown marubakaido shoots treated with 5F-HTY, tough no significant change on the number of newly formed main shoots (shoots originated directly from an original shoot, i.e., shoot treated with 5F-HTY) is observed for shoots treated with 5F-HTY (Pereira-Netto et al. 2019, in press). Enhancement on the average length is also found for both main and primary lateral shoots treated with 5F-HTY. These observed changes in shoot architecture, especially on formation and further elongation of primary lateral shoots, result in significantly higher, i.e. over 80%, multiplication rate (MR) for shoots treated with 5F-HTY.

Because BL is widely considered to present higher biological activity than any other natural BRs, along with its widespread occurrence in the plant kingdom, BL is commonly used as positive control to evaluate the biological activity of BR

analogs. Because of the structural similarity, the closer the intermediate in the pathway to BL, the greater is its activity. For example, the biological activity of typhasterol, which is considered to be an intermediate to CS and BL in the BR biosynthetic pathway, is typically only one tenth of that presented by BL in bioassays. In our laboratory BL has been shown to significantly stimulate elongation of both, main and primary lateral shoots, besides inducing a 46% increase in the formation of new primary lateral shoots in the marubakaido apple rootstock (Pereira-Netto et al. 2009). Since BL is the most potent natural BR, it is somewhat surprisingly to find that the 5α-monofluoro derivative of homo-TY (5F-HTY) is much more effective towards stimulation of primary shoot formation, compared to BL, inducing an over a 100% increase in the number of newly formed primary lateral shoots. Furthermore, 5F-HTY significantly stimulates both, main and primary lateral shoot elongation, tough in a more effectively way, compared to BL. These findings are especially relevant once: 1. homoBRs, like HBL typically show similar or reduced biological activity when compared to their counterparts, like BL; 2. 7-oxalactone BRs such as BL and HBL generally present stronger biological activity when compared to 6-oxo BRs, such as HTY. The reason(s) why BL was less effective towards stimulation of primary lateral shoots formation, compared to 5F-HTY, is (are) not clear. It is possible that these differential effects might result from differences between these different BRs at satisfying the structural requirements of BR receptors. For example, an enhanced affinity of the 5F-HTY for the receptor or an increased binding time of the 5F-HTY to the BR receptor, as a result of an eventually stronger hydrogen-bonding network within the hydrophobic pocket where the alkyl chain of the BR fits, might explain the ability of 5F-HTY to more effectively stimulate formation of primary lateral shoots in our system, compared to BL. However, that does not explain why the fluoro HTY did not stimulate formation of main shoots, compared to BL. Differences in response to the tested BRs might also be due to eventual differences in the way(s) that these BRs might influence BR biosynthetic enzymes. However, other possibilities such as an eventually higher susceptibility of the natural BL to inactivation, compared to 5F-HTY, a synthetic BR, can not be ruled out. Besides the promotive effect of BL on shoot formation in the marubakaido apple rootstock, we have also previously shown that BL significantly stimulated elongation of both, main and primary lateral shoots (Pereira-Netto et al. 2009). Noteworth, 5F-HTY significantly stimulated both, main and primary lateral shoot elongation, very likely as BL did. And again, similarly to what we have previously shown for BL, the 5F-HTY growth-promotive effects are more effective for primary lateral shoots compared to main shoots. Thus, when seen together, data for the effects of 5F-HTY on marubakaido shoots and data for *Eucalyptus* shoots (Pereira-Netto et al. 2006a), along with data for marubakaido shoots (Pereira-Netto et al. 2006b) treated with 28-HCS and 5F-HCS, respectively, clearly demonstrate that 5F-HTY and other BRs, affect differentially the morphogenetic potential of main and primary lateral shoots.

6 Comparative Effects of the 3α and 5α-Monofluoro Derivative of Homotyphasterol and the Parent Compound

Considering that 5F-HTY effectively promotes new shoot formation and further elongation of both main and primary lateral shoots in the marubakaido apple rootstock, the potential effects of the 3α-monofluoro analog of homotyphasterol (3F-HTY) and the parent compound (HTY) were probed in our laboratory against the formerly tested 5α-monofluoro analog of homotyphasterol (5F-HTY) as a way to investigate if the presence of the fluoro atom in α configuration at C5 was or not a requirement for the homotyphasterol to present strong biological activity. Neither 3F-HTY nor HTY are able to significantly stimulate new shoot formation, regardless the kind of shoot, i.e. main or primary lateral shoot. However, both, HTY and 3F-HTY effectively stimulated main shoot elongation, tough neither HTY nor 3F-HTY were effective towards stimulation of primary lateral shoot elongation. Considering that neither 3F-HTY nor HTY are capable to stimulate new shoot formation, it was not unexpected to realize that none of those compounds were able to enhance the multiplication rate of the *in vitro*-grown marubakaido rootstock. So, the presence of a fluoro atom in α configuration at C5 seems to be a requirement for the stimulation of new shoot formation but not shoot elongation in the marubakaido apple rootstock.

In the rice lamina inclination assay, HTY has been shown to present about 1.7 times less activity when compared to TY, which suggests that the activity of 24-ethyl BRs is increased by C-28 demethylation to the 24-methyl BRs (Joo et al. 2015). In our laboratory, 5F-HTY presents activity 2.35 times higher than HTY towards formation of new primary shoots, indicating that fluorination at C5 might mimic, with advantages, C-28 demethylation in HTY regarding stimulation of primary lateral shoot formation in our system. Data from our laboratory also demonstrate that HTY presents similar activity, towards stimulation of main shoots elongation, compared to the effect of BL on main shoot elongation (Pereira-Netto et al. 2009). However, differently from BL, HTY presents no activity towards main or primary lateral shoot formation, or towards primary shoot elongation in our system. As mentioned previously here, 5F-HTY presents higher biological activity towards primary shoot formation, compared to both, HTY and our previously reported data on the effect of BL on shooting in the marubakaido rootstock. Since BL has been shown to usually presents higher activity, when compared to HBL (Khripach et al. 2000), all of these data, seen together, provide support to the idea that 5F-HTY might be active per se towards primary shoot formation in the marubakaido rootstock, not requiring its conversion to other forms of BRs, downstream the BRs biosynthetic pathway, in order to present high biological activity. In addition, fluorination at C-5 of HTY might prevent its enzymatic inactivation which might in turn enhance its chemical stability and consequently prolong its activity, compared to natural BRs, potentially more susceptible to enzymatic inactivation. Noteworth, when probed in the rice lamina inclination (RLI) test, 5F-HTY presented only moderately higher activity, when compared to the parent compound HTY (Ramirez et al. 2000).The results

from RLI test and our results (stimulation of primary shoots formation) are significantly different, demonstrating that a single BR might exhibit different activities, depending on the testing system. Thus, our data on the biological activity of BL (Pereira-Netto et al. 2009), and HTY (Pereira-Netto et al. 2019 in press) and HCS (Pereira-Netto et al. 2006b; Pereira-Netto et al. 2012), and their F-derivatives provide support to the idea that biological activities of BRs can not be discussed in a single bioassay system.

As previously mentioned in this chapter, the closer the intermediate in the BL biosynthetic pathway, the greater is its activity, and bioactivities for homoBRs present the same trend. TY, one of the two immediate precursors of castasterone (CS) in the BL biosynthetic pathway, is converted to CS, an activation step in the BL pathway, in a reaction catalyzed by the cytochrome P450 CYP90C1. As expected, in bioassays such as the rice lamina inclination bioassay, HTY has been shown to present much less biological activity when compared to homoCS (HCS, Joo et al. 2015), which suggested that C2 α-hydroxylation of HTY was important to express a strong BR activity. In our system, i.e., the *in vitro*-grown marubakaido apple-rootstock, HCS is not able to stimulate new shoot formation (Pereira-Netto et al. 2012) or shoot elongation (unpublished data). Thus, surprisingly, differently from HCS (Pereira-Netto et al. 2003), the three compounds HTY, 3F-HTY and 5F-HTY, are all able to significantly stimulate main shoot elongation, tough only 5F-HTY was able to stimulate primary lateral shoot elongation. Neither the parent HTY nor 3F-HTY or 5F-HTY is able to significantly stimulate main shoot formation. Thus, the stimulation of main shoot elongation driven by the parent HTY or its two monofluoro analogs tested might not rely on C2 α-hydroxylation of these compounds by the marubakaido apple rootstock. Furthermore, since HTY is not considered to show high biological activity per se, 5F-HTY present comparable activity towards promotion of primary lateral shoot elongation, compared to BL, and 5F-HTY stimulates primary lateral shoot formation more effectively than BL, it is reasonable to consider that 5F-HTY might be biologically active per se *in vitro*-grown marubakaido shoots.

The metabolic stability of a C-F bond often prevents chemical reactions of the carbon attached to fluorine atom. Thus, one might assume that introduction of a 3α-F or 5α-F group in HTY might reduce the biological activity of HTY due to a reduced conversion of 3F-HTY or 5F-HTY into compounds downstream of the BR biosynthetic pathway, like HBL, once, for example, 3F-HTY has been shown not to be hydroxylated at C2 to CS, the immediate precursor of HBL (Galagovsky et al. 2001). In fact, this predicted reduced activity is actually seen when 3F-HTY is probed against HTY in the rice lamina inclination test (Galagovsky et al. 2001). Somewhat unexpectedly, we have observed that 3F-HTY is as effective as HTY on the stimulation of main shoot elongation in our system, demonstrating that the introduction of the 3α-F group in HTY did not change the biological activity of HTY.

Length of main shoots is enhanced by HTY and their two monofluoro derivatives used in this study. However, length of primary shoots is enhanced by 5F-HTY and unaffected by HTY and 3F-HTY. It might imply that these BRs might have different activities towards stimulation of shoot elongation, depending on the kind of shoot

considered, i.e., main or primary lateral shoot, or that elongation of main and primary lateral shoots might be controlled by different mechanisms.

7 Conclusions

In this chapter, we report on the evaluation of the biological activity of brassinolide (BL), and homocastasterone (HCS) and homotyphasterol (HTY) and synthetic fluoro analogs towards shooting stimulation in the marubakaido apple-rootstock and a hybrid between *E. grandis* and *E. urophylla*. The results reported here provide an insight into the morphological responses of *in vitro*-grown shoots to several natural BRs and fluoro and hydroxyl substitutions, in alpha configuration, in HCS and HTY on the sterol structure of exogenously supplied BRs. The biological activity of the synthetic analogs mentioned in this chapter is clearly dependent on the type, i.e. fluoro or hydroxyl, and position of the substitution. For example, fluorination at C5 but not at C3 significantly increases formation and further elongation of primary lateral shoots of the marubakaido apple rootstock, which results in effective enhancement of its *in vitro* multiplication rate. This BR-driven enhancement on the MR is an effective way to improve the micropropagation technique for the marubakaido rootstock and possibly for other plant systems as well, especially for woody species, in which new shoot formation and elongation is typically a constrain for efficient micropropagation protocols.

The growth promotive effect of fluoro substitution is organ and species specific once eucalyptus shoots respond differently compared to marubakaido shoots to the position of the fluoro substitution. This differences in specificity of the growth promotive effect of the fluoro substitution is an indicative that BR receptors in different plant organ and species might have, at least slight, differences in structural requirements to bind to the ligand BR. Furthermore, the effects of exogenous BRs on both, shoot elongation and formation, mentioned in this chapter demonstrate that modification of the allocation of growth among the various types of shoots can be effectively achieved at the biochemical/physiological level, at least in the *in vitro*-grown shoots mentioned here through applications of BRs.

Besides being capable of effectively enhancing shooting and also being nontoxic, BRs are environmentally friendly which bring vast perspectives for the application of compounds like 5F-HCS and 5F-HTY in agriculture, forestry and horticulture. In horticulture, for example, practical applications for findings described here include the 5F-HTY-driven enhancement of the multiplication rate for *in vitro*-grown marubakaido. In addition, the 5F-HTY-shooting stimulation reported in this chapter is potentially useful to improve micropropagation techniques for clonal propagation of other plant species as well, especially woody species, in which shoot formation and further elongation is typically a constrain for commercial micropropagation. In producing orchards, potential benefits include promotion of shooting, especially diverting allocation of growth from the main to lateral shoots, which is expected to enhance fruit production.

Finally, because of the evident difference in responsiveness of the *in vitro*-grown shoots, especially of the marubakaido rootstock, to fluorinated and non-fluorinated BRs, this *in vitro* system seems to be potentially useful to probe into the biological activity of BRs bearing fluorine atoms, especially at C5 in α configuration.

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Chapter 5
Role of Brassinosteroids in the Plant Response to Drought: Do We Know Anything for Certain?

Dana Hola

Abstract Brassinosteroids (BRs) are considered to be major players in the plant response to unfavourable conditions. They have been reported to alleviate stress symptoms and to enhance plant tolerance to various abiotic and biotic stressors including drought. However, our current knowledge of the role of BRs in the plant drought response should perhaps be limited only to the statement that the treatment of plants with BRs can mitigate the negative effects of this stress factor. No clear conclusions on the role of these phytohormones in the plant drought response should be inferred from the currently available data, because the results of BR/drought studies often differ quite substantially. This chapter attempts to provide a critical evaluation of the information available on this topic, *i.e.*, data obtained either from plants treated with exogenously applied BRs or mutants in BR biosynthesis/perception. The existing studies are considered from several viewpoints regarding important aspects of their experimental design and attention is also drawn to some of their shortcomings. The question of whether BRs truly function as *specific* regulators of drought-induced response or whether the observed effects of BRs on drought-stressed plants are of a more general character remains unanswered.

Keywords Brassinosteroids · Drought · Stress · Exogenous application · Mutants · Gene expression · Photosynthesis · Cell damage and protection · Plant morphology · Design of experiments

1 Introduction

Brassinosteroids (BRs) are phytohormones that occur naturally in higher plants and even some algae. One of the main roles of BRs in plants seems to be their participation in plant response to an unfavourable environment. Treatment with exogenously applied BRs is frequently proposed as an efficient means for mitigation of the

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negative effects of various stress factors on plants and for the improvement of crop yield.

A shortage of water is without doubt the major stress factor currently affecting plant life on Earth and limiting agricultural production on a global scale. Since the first analyses of the effects of BRs in drought-stressed plants (published almost 30 years ago), the number of studies dealing with this topic has gradually grown. It would seem that we have at our disposal a sufficient amount of data on this topic and could thus form some definite conclusions on the role of BRs in the plant drought response. However, is this truly the case? In the following sections of this chapter, I will attempt to critically evaluate various studies dealing with the BR/drought relationship and will briefly consider their strong points and shortcomings from several points of view.

2 How to Examine the BR/Drought Relationship?

The papers dealing with this topic can be mostly divided into two main categories. The majority are based on the exogenous application of BRs to plants subjected to conditions simulating drought and the subsequent analysis of some morphological, physiological, biochemical or other parameters associated with known aspects of the plant drought response. However, studies performed with BR mutants or transgenic plants have also started to appear (particularly during the last 3 or 4 years). Both these approaches have their advantages and disadvantages.

2.1 Mutants or Transgenic Plants in Genes Associated with BR Biosynthesis or BR Signalling

The utilisation of mutants in genes coding for BR-biosynthetic enzymes certainly ensures that the level of active BRs in plants experiencing drought is changed and maintained in the changed state during the whole life of the plants. This cannot be ensured by the application of exogenous BRs, particularly given the usual mode of such treatment and the limitations of BR transport between different plant organs. The majority of work with this type of experimental material has been performed with BR-deficient mutants of barley, maize, tomato, pea or *Arabidopsis*. Genes coding for enzymes participating in the early (Jäger et al. 2008; Gruszka et al. 2016, 2018) or late (Janeczko et al. 2016; Gruszka et al. 2016, 2018; Northey et al. 2016; Castorina et al. 2018; Lee et al. 2018) steps of BR biosynthesis are disabled in these plants. The study by Han et al. (2017), who prepared transgenic *Arabidopsis* plants overexpressing the gene for the enzyme that catalyses the conversion of BR intermediates to inactive acylated conjugates, thus resulting also in diminished levels of active BRs, could perhaps also be included in this category. The results of all these studies are somewhat ambiguous. The majority of these mutants or transgenics

performed better under drought conditions compared with *wild type* (*wt*) (Northey et al. 2016; Gruszka et al. 2016, 2018; Han et al. 2017; Castorina et al. 2018). However, other mutants were more sensitive to drought stress than their *wt* counterparts (Janeczko et al. 2016; Lee et al. 2018) or displayed a similar drought sensitivity to *wt* plants (Jäger et al. 2008). No clear-cut relationship between BR deficiency and plant response to drought can thus be inferred from these studies.

Five papers also examined mutants in the BR-signalling pathway. With the exception of Koh et al. (2007), who worked with the knockout mutant of a rice orthologue to *Arabidopsis* BIN2 kinase, all other work has been performed with mutants in the gene coding for the BRI1 receptor (Jäger et al. 2008; Feng et al. 2015; Gruszka et al. 2016, 2018). Again, the ambiguity of results does not allow for any definite conclusions: some mutants displayed better performance under water stress (Koh et al. 2007; Feng et al. 2015), and others did not greatly differ from *wt* plants (Jäger et al. 2008; Gruszka et al. 2016).

I believe that the assessment of BR/drought relationships using mutants in genes associated with BR biosynthesis or BR signalling faces two major challenges. All mutants described thus far display either a dwarf phenotype (with associated diverse morphological and anatomical changes involving the development of the vein system, size, thickness and general architecture of leaves, the distribution and development of stomata, *etc.*) or at least are significantly smaller than *wt*. The size/general morphology of the shoot is a very important factor in the plant drought response. The dwarf phenotype can cause better resistance to drought simply because these plants experience a less intensive water shortage. This phenomenon can be caused by reduced water loss from the shoot (associated with the diminished leaf size and irregular behaviour of stomata), resulting in more water in the soil available to the mutant plants compared with their *wt* counterparts. Northey et al. (2016) demonstrated that the soil water content in pots containing *wt* plants and dwarf BR-biosynthesis mutants of *Arabidopsis thaliana* after 9 days of withholding water differed quite substantially (10% *vs* 35–50%). Although this does not have to be the case for all BR mutants, it should certainly be taken into an account and the soil water content should always be determined in such experiments.

The second challenge of any approach that utilises the BR-deficient mutants consists of an entirely different matter. It is very difficult to differentiate between the effects of BRs *per se* and the effects of the changed levels of other phytohormones in such plants. These changes occur even under non-stressed conditions. Although Jäger et al. (2008) reported similar abscisic acid (ABA) levels in leaves of non-stressed dwarf mutants of pea displaying a BR deficiency compared with their *wt* counterparts, it seems that the levels of this phytohormone were in fact slightly reduced. A BR-deficient dwarf mutant of tomato contained significantly reduced amounts of ABA and auxins (Li et al. 2016). Semidwarf barley mutants in BR genes were characterised by reduced levels of ABA, cytokinins, gibberellins, salicylic and jasmonic acid (Gruszka et al. 2016; Janeczko et al. 2016). Thus, although species-specific differences evidently exist, such mutants display quite complex imbalances of various phytohormones that participate in plant development and the drought response. It remains unknown which effects observed in BR mutants can then be

attributed specifically to BRs and which to other phytohormones, the contents of which are also *apriori* significantly changed.

An interesting option of overcoming the problem of dwarf mutants lies in creating transgenic plants with *elevated* expression of the BR-biosynthetic gene(s). Such plants do not display dwarfism; in contrast, they are usually larger compared with *wt* (Sahni et al. 2016; Zhou et al. 2016; Duan et al. 2017). This phenomenon could lead to opposite issues; however, it seems that their larger shoot size is accompanied by an equally large increase in the root system (Sahni et al. 2016; Duan et al. 2017). Additionally, at least the ABA content did not seem to differ from *wt* plants in transgenic spinach with elevated expression of the *CYP90A1/CPD* gene (Duan et al. 2017). In all three cases examined thus far, such an artificial elevation of endogenous BR contents resulted in better plant resistance to drought/osmotic stress, which would argue in favour of the positive role of elevated BR contents. This finding is mostly consistent with the results of studies performed with drought-stressed BR-treated plants and further confuses the issue of BR-deficient mutants displaying increased drought resistance.

2.2 Plants Treated with Exogenously Applied BRs

Both the major issues of working with BR dwarf mutants under drought conditions mentioned above would seem to turn the scale in favour of the exogenous application of BRs. There is great variability regarding plant species analysed in such studies. Thus far, 10 monocot and 34 dicot species of angiosperms were examined and some work was also performed with gymnosperms. Considering that many scientists performing this type of analysis are interested in BR/drought relationship from a purely practical perspective, it is not surprising that crop plants (particularly cereals, legumes or main vegetables) have strongly prevailed, with wheat, maize and tomato leading the list (Tables 5.1, 5.2, 5.3, 5.4, and 5.5).

While the approach using BR-treated plants presents several advantages over work with BR mutants, it also has some drawbacks. In addition to possible problems with BR penetration into plants (incidentally, most authors do not state whether they used some surfactant!) and uncertainty regarding the precise amounts of BRs received by plants, the exogenously applied BRs are probably not transported from the site of their application to other organs (Nishikawa et al. 1994; Symons and Reid 2004; Symons et al. 2008; Janeczko and Swaczynová 2010). The effect of exogenously applied BRs is thus probably locally limited. Very little is also known about the metabolism of exogenously applied BRs and their effect on the contents of endogenous BRs. Based on several studies (Janeczko and Swaczynová 2010; Janeczko et al. 2010, 2011a, b), such treatment *can* affect the levels of endogenous BRs. However, the changes can be either positive or negative, and they depend on the concentration or the application mode of the respective BR, the plant species and the developmental stage.

Table 5.1 Studies dealing with the effect of brassinosteroids (BRs) on plants subjected to drought stress simulated by cessation of watering. Major aspects of the experimental setups (if mentioned in the respective study) are presented and the results are summarised using the following symbols: ○… differences between the respective BR-treated and non-treated plants (or BR mutants/transgenics and wild type plants) were not significant; ▲, resp. ▼ … BR treatment/gene mutation/gene overexpression led to an increase, resp. a reduction in the values of the respective parameter. In case more than one type of effect was observed in at least 25% of the measurements (depending, *e.g.,* on the plant genotype, the time of measurement, BR concentration, *etc.*), the symbols signify: ► … either an increase or no significant difference; ◄ … either a reduction or no significant difference; ♦… either an increase or a reduction; ✕… presence of all three types of effects. The black background refers to stressed plants, the grey background refers to stressed and then rewatered plants; the white background refers to non-stressed plants (in case they were included in the respective study)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment /start of the stress period / measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Cicer arietinum (1)	Greenhouse; pots (20 × 17 cm) with soil; 1 plant/pot	21, 35 or 46 d	2×10^{-9} M EBL; leaf spraying (3× in 7-d intervals)	14 d/7 d/28, 42 or 56 d	Root and shoot FM, DM, root length, plant height, stem diameter, no. of leaves ▲; no. of nodules, NR activity in leaves and roots○	Singh et al. (1993)
Triticum aestivum (1)	NS; pots (30 × 30 cm) with sandy loam soil and manure (6:1); NPK fertiliser; 4 plants/pot	7 d (*also some time after rewatering*). Leaf RWC 81% (*control 94%*)	2×10^{-6} and 2×10^{-7} M HBL; leaf spraying (2× in a 2-d interval)	30 d/Anthesis/Anthesis +7 d + NS time after rewatering, or after harvest	Total Chl and total proteins contents, NR and glutamine synthase activities ▲▲▲; P_N ▲○▲; RWC ▲○○; E ▲◄▼; g_S ►○◄; no. of ears per plant ▲▲; grain yield per plant ▲►; no. of grains per ear ►►; harvest index ▲○; 1000 grain weight○○	Sairam (1994a)

(continued)

Table 5.1 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment /start of the stress period / measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Triticum aestivum (1DS, 1DR)	Outdoor; pots (35 × 35 cm) with sandy loam soil and manure (6:1); NPK fertiliser; 4 plants/pot	7 d. Leaf RWC 67–69% (*control 82–85%*)	10^{-7} M and 2×10^{-8} M HBL; seed soaking or leaf spraying (1×, both leaf sides)	Seeds and 25 d/ Anthesis/Anthesis +7 d	Total plant DM, leaf area, P_N, Chl content, NR activity ▲▲; RWC ▶▶; EL ▼	Sairam (1994b)
Sorghum bicolor (1)	Greenhouse; pots with loamy soil; NPK fertiliser; 4 plants/pot	19, 28, 33 or 39 d (*also 12 or 20 d after rewatering*)	2×10^{-7} M EBL; leaf spraying (4× in 3-d intervals)	V3 + 1 d/V3/V6 or V8 to V10	Root length ▲▶; root DM ▶○; total plant and shoot DM, plant height ◉▶; no. of roots, root/shoot DM and length ratios, total plant biomass ◉○; plant survival ○	Xu et al. (1994a)
Sorghum bicolor (1)	Greenhouse; pots with loamy soil; NPK fertiliser; 4 plants/pot	20 d. Leaf RWC 77%	2×10^{-7} M EBL; leaf spraying (4× in 3-d intervals)	11 d/7 d/27 d (V6)	Time with open stomata after cessation of watering ▲; leaf glacuousness, RWC, cuticular E ◉; stomatal E ▼	Xu et al. (1994b)
Pinus banksiana (1)	Greenhouse, then GC; pots? with peat, vermiculite and sphagnum moss	12 d	10^{-11} M HBL; injection into plant stem (7× in 1-d interval)	1.5 year +11 d/1.5 year +18 d/1.5 year +30 d	Growth rate, ethylene content ▲; xylem Ψ_p, E, g_S, ci/ca, P_N, WUE, EL ◉	Rajasekaran and Blake, 1999

Cucumis sativus (1)	Greenhouse; pots? with soil	11 d; at the end of this period soil WC reached 6.2% (*control 55–57%*). Leaf WC 76% (*control 83%*)	10^{-6}, 10^{-8}, 10^{-9} and 10^{-11} M EBL; leaf spraying (1×)	V3 (floral bud formation stage) / V3 + 10 d/ V3 + 21 d	Thermotolerance ▲◆; total amino acids content ▲○; individual amino acids content ▲X; WC, water retention capacity ○○; proline content ▼▲	Pustovoitova et al. (2001)
Solanum lycopersicum (1)	NS; pots? with soil and manure (1:1)	3 d. Leaf RWC 77% (*control 88%*), leaf Ψ −0.49 MPa (*control −0.16 MPa*)	10^{-7} and 2×10^{-8} M BIOBRAS-6; seed soaking (for 8 h)	Seeds/30 d/33 d	Ψ ▶○; total plant, root, leaf and stem DM ○▲; total proteins content ○▶; plant height, stem diameter, NR activity ○○; Chl content◀▲; RWC ◀○; proline content ▼◀	Mazorra and Núñez (2003)
Phaseolus vulgaris (1)	Greenhouse; pots with garden soil and manure (2:1)	4 or 8 d; at the end of this period soil WC reached 24% or 19% (*control 31%*), plants were then rewatered and immediately measured	5×10^{-6} and 10^{-6} M EBL and HBL; leaf spraying (1×)	Flowering/flowering +5 d/flowering +5, 9 and 13 d	No. of nodules, nitrogenase activity ▲▲; root length, nodulated root FM, *trans*-zeatine riboside content in roots ▲▶; pod yield, no. of nodules ▶▶; no. of pods ○X; pod length ○○; ABA content in roots ◀○	Upreti and Murti (2004)
Arabidopsis thaliana (1)	GC; Petri plates with agar, then pots with sand + MS medium	12, 14, 36, 48, 60, 72, 80 or 84 h (*also 2 d after rewatering*)	10^{-6} M EBL; into the cultivation medium	NS (prior to the start of stress)/26 d/26 d + 0, 12, 14, 36, 48, 60, 72 or 84 h; also (plant survival) 31 d or 31 d + 6 h	Dehydrin transcript level ○▲○; *RD29A* transcript level ○○▲; *ERD10* transcript level ▶○▲; *RD22* transcript levels X▼▲; plant survival ▲	Kagale et al. (2007)

(continued)

Table 5.1 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment /start of the stress period / measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Brassica napus (1)	GC; Petri plates with agar, then pots with sand + MS medium	1, 3, 6, 9, 12, 14, 24, 36, 48 or 72 h (*also 2 d after rewatering*)	10^{-6} M EBL; into the cultivation medium	NS (prior to the start of stress)/17 d/17 d + 0, 3, 6, 9, 12, 14, 24, 36 or 72 h; also (plant survival) 20.5 or 21 d	*DREB*, *PIP1* transcript levels ▲▲▲; *CBF5* transcript level ▲▲○; *D22* transcript level ▶▲○; dehydrin homolog transcript level ▲▼○, *BTG-26* transcript level ▼▼▼; plant survival ▲	Kagale et al. (2007)
Pisum sativum (wt + 2MT)	Greenhouse; pots (14 cm) with composted fine pine bark and sand (8:3)	14 or 15 d. Leaf Ψ −1.50 MPa (*control −0.30 MPa*)	Mutants in the *PsDWF1* and *PsBRI1* genes; also 4×10^{-10} M EBL; leaf coating (4× in 1-d intervals)	Not applicable, also 35 d/21, 22 or 28 d/35 or 42 d	Ψ ▲○; WC ◎; stem diameter ◎◀; ABA content ◀○; plant height, leaf length and width ▼▼	Jäger et al. (2008)
Robinia pseudoacacia (1)	Field; fine loess-derived soil; rainout shelter when needed	10 d; at the end of this period soil FC reached 55% or 35% (*control 75%*), soil WC 12–13% or 7–8% (*control 17–18%*). Leaf WC 82 or 75% (*control 85%*), leaf Ψ −0.59 or −1.51 MPa (*control −0.60 MPa*)	8×10^{-7}, 6×10^{-7}, 4×10^{-7} and 2×10^{-7} M BL; root soaking (for 5 min), then leaf spraying (1×)	1 year/1 year + cca 2.5 months/1 year +2.5 months +10 d	Soluble saccharides and proline contents, POD, CAT and SOD activities ▶○; Ψ, WC, g_s, E, MDA content ◎○	Li et al. (2008)

Solanum lycopersicum (1)	Greenhouse, then GC; pots (11 cm) with sand, soil and peat (2:1:1) + Hgl solution; 1 plant/pot	3 or 5 d	10^{-6} and 10^{-8} M EBL; leaf spraying (1×)	35 d (V4)/38 d/38, 41 or 43 d	Shoot DM ▲▶; Car and proline contents ▲○, APX activity ▲▲; SOD activity ▲▶; shoot FM ▶▲; reduced Asc content, POD and CAT activities, activities of 4 POD and 4 APX isozymes ▶▶; oxidized Asc content ▶○; H_2O_2 and aldehyde contents ◀ ○; MDA content ▼○	Behnamnia et al. (2009a)
Solanum lycopersicum (1)	Greenhouse, then GC; pots with sand, soil and peat (2:1:1) + Hgl solution	3 or 5 d	10^{-6} and 10^{-8} M EBL; leaf spraying (1×)	35 d (V4)/38 d/38, 41 or 43 d	GR activity ▲▲; SOD activity, activities of 1 CAT, 4 APX and 4 POD isozymes ▲▶; CAT activity, proline content ▲ ○; APX and POD activities, soluble proteins content ▶▶; MDA content ○○; aldehyde content ◀○; H_2O_2 content ◀◀	Behnamnia et al. (2009b)
Brassica juncea (1)	Net-house; pots (25 cm) with sandy loam soil and manure (6:1)	7 d, plants were then rewatered and immediately measured. Leaf RWC 58 or 63% (*control 70%*)	10^{-8} M HBL; leaf spraying (1×)	30 d/8 or 15 d/60 d	Total plant FM and DM, plant height, root length, leaf area, RWC, g_S, ci, P_N, WUE, total Chl, N and proline contents, NR, POD, CAT and SOD activities ▲▲; CA activity ▶▲	Fariduddin et al. (2009)

(continued)

Table 5.1 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment /start of the stress period / measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Brassica napus (1)	GC; pots (12 × 12 cm) with sand, clay and peat (1:1:1)	3 or 4 d. Leaf RWC 88 or 83% (*control 92%*)	10^{-7} M EBL; leaf spraying (3× in 7-d intervals)	7 d/26 d/29 or 30 d	Leaf FM and DM, Na and K contents ▲▲; RWC, ca, reducing saccharides and proline contents ▲○; EL ▼○; MDA content ▼▼	Mousavi et al. (2009)
Solanum lycopersicum (2)	Greenhouse; pots (12 × 15 cm) with soil, then vermiculite + NS nutrient solution	1, 2, 3 or 4 d. Leaf RWC 50–59% or 36–48% (*control 80%*) depending on the genotype	10^{-6} M EBL; leaf spraying (1×)	V5/V5/V5 + 0, 1, 2, 3 or 4 d	RWC, CAT activity ▲○; ci, P_N, ABA content, SOD activity ►○; APX activity ○○; g_S, H_2O_2 and MDA contents ◄○	Yuan et al. (2010)
Zea mays (1)	Wire-house; pots (34 × 24 cm) with sandy loam soil; NPK fertiliser	13, 18 or 23 d or until harvest. Leaf RWC 66, 53 or 46% (*control 86, 71 or 65%*) depending on the measurement time	2×10^{-7} M BL; leaf spraying (1×)	Start of tasselling +6 d/ start of tasselling/start of tasselling +13, 18 or 23 d; also 108 d	Grain yield, no. of grains per ear, total plant biomass, shoot DM and FM, leaf area, no. of leaves, RWC, ci, P_N, WUE, SOD activity ▲▲, POD and CAT activities ▲►; WUEi, Chl *a* content ▲○, proline content Chl *b* and total Chl contents ►○; no. of grain rows per ear ○▲; ear length, 100 grain weight, harvest index, plant height, g_S, soluble proteins content ○○; MDA content ◄○	Anjum et al. (2011)

Glycine max (1)	GC, then greenhouse; pots (40 × 20 cm) with soil; 8 plants/pot	10 d (*also 2 d after rewatering*)	5×10^{-7} M EBL; seed soaking (for 24 h)	Seeds/12 d/22 or 24 d	E ▲▲; OJIP CF ▶○; P_N ●▲; respiration rate ●○	Janeczko et al. (2011a)
Triticum aestivum (1DS, 1DR)	Cage-house; pots (12 L) with sandy loam soil	10 d (*also 10 d after rewatering*). Leaf RWC 65–67% (*control 69–70%*)	2×10^{-6}, 10^{-6} and 5×10^{-7} M BL; leaf spraying (1×)	80 d/70 d (anthesis)/80 or 90 d	No. of spikes, no. of grains per spike, grain yield, 1000 grain weight, plant height, leaf area, leaf DM, RWC, g_S, E, P_N, Chl stability index. *Not possible to separate the data from the stressed and non-stressed BR-treated plants*	Dhayal et al. (2012)
Calendula officinalis (1)	Greenhouse; pots with NS substrate	14 d; at the end of this period soil WC reached 51% (*control 90%*)	2×10^{-5} M NS; leaf spraying (3× in 5-d intervals)	Start of flowering +1 d/ start of flowering / full flowering	CAT and SOD activities, total Car, lycopene, lutein contents in petals ▲▲; POD activity, flavoxanthine content in petals ▲○; luteoxanthine content in petals ●▲; MDA content in petals ▼○	Sedghi et al. (2012)
Cicer arietinum (1DS, 1DR)	Cage-house; pots with sandy loam soil	8 d	2×10^{-6}, 10^{-6} and 2×10^{-7} M BL; leaf spraying (1×)	Prior to flowering, or pod initiation stage/ flowering or pod formation stage/NS	No. of pods, no. of seeds per pod, seed yield per pod, seed weight per plant, harvest index, total plant biomass, plant height. *Not possible to separate the data from the stressed and non-stressed BR-treated plants*	Verma et al. (2012)

(continued)

Table 5.1 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment /start of the stress period / measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Carica papaya (2)	NS; pots (10 L) with sand, soil and manure (2:1:1)	Up to 15 d, at the end of this period soil WC reached 10% (*also up to 9 d after rewatering*). Leaf RWC 78% (*control 81%*)	2×10^{-7} M BIOBRAS-16; leaf spraying (5× in 1-d intervals)	70 d/75 d/76 to 99 d (1 to 2 d intervals)	Shoot DM, leaf area, RWC, F_v/F_m ⊙○○; Chl content ⊙◀○	Gomes et al. (2013)
Vigna radiata (1DS, 1DR)	Cage-house; pots with sandy loam soil; 5 plants/pot	10 d. Leaf WC 54 or 61% (*control 65 or 71%*) depending on the measurement time	2×10^{-6}, 10^{-6} and 2×10^{-7} M BL; leaf spraying? (1×)	25 d/25 d/35 or 45 d	No. of seeds per pod, seed yield per plant, seed weight, plant height, leaf area index, time to flowering, WC, g_s, E, P_N, leaf temperature, Chl stability index. *Not possible to separate the data from the stressed and non-stressed BR-treated plants*	Lal et al. (2013)
Helianthus annuus (3)	NS; bowls (15 L) with peat and perlite (4:1)	5 d	10^{-6} and 10^{-8} M HBL; leaf spraying (3× in 1-d intervals)	32 d/32 d (start of butonisation) / 37 d	Chl, proline and MDA contents ▲	Filová (2014)

Solanum lycopersicum (1)	Greenhouse, then GC; pots (11 cm) with sand, soil and peat (2:1:1) + Hgl solution	3 or 5 d. Leaf RWC 89 or 87% (*control 91%*)	10^{-6} and 10^{-8} M EBL; leaf spraying (1×)	V3/V3 + 3 d/V3 + 3, 6 or 8 d	Leaf FM, RWC, Chl *a*, total Chl, total and reducing soluble saccharides contents ▲▲; Chl b content, SOD activity ▲▶; leaf DM ▶▲; activities of 3 SOD isozymes ○○; ethylene content ▼▲; EL, lipoxygenase activity ▼▼	Behnamnia (2015)
Brachypodium dystachion (wt + 2MT)	Greenhouse; pots? With with NS substrate	Up to 12 d (*plant survival 7 d after rewatering*); soil WC reached 65, 40, 30 or 15% (*control 75%*). Leaf WC 24% (*at 15% soil WC*)	Mutants in the *BdBRI1* gene, also 10^{-6} M EBL; leaf spraying (1×)	Not applicable, also 14 d/21 d/33 d or NS	WC ▲; *ERD1*, *RD26*, *COR47* and *P5CS* transcript levels ▲▲; F_v/F_m ▶○; *DREB2A, RD22* and *RD29* transcript levels ○○; plant survival ▲	Feng et al. (2015)
Hordeum vulgare (wt + 5MT)	GC; pots (15 × 38 cm) with soil, Substral and sand (8:2:1); 10 plants/pot	16 d; at the end of this period 25% of max. soil water capacity was reached (*control 70%*). Small amounts of water added to balance unevent water loss from individual pots	Mutants in the *HvCPD*, *HvBRD*, *HvDIM* and *HVBRI1* genes	Not applicable/29 d/45 d (stressed plants) or 38 d (non-stressed plants)	28-homoCS content ▲▲; P_N ▲◀; JA content ▶▲; g_s, E ▶○; CS content ◆◀; EBL content ○▲; SA content ○▶; IAA, *trans*-zeatine and gibberellin GA4 contents ○○; gibberellin GA7 content ○▼; ABA content ◀▶	Gruszka et al. (2016)

(continued)

Table 5.1 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment /start of the stress period / measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Hordeum vulgare (wt + 2MT)	Open-air hall, then greenhouse; pots (15 × 40 cm) with soil, Substral and sand (8:2:1); 10 plants/pot	19 d (*time to flowering measured some time after rewatering*); at the end of this period 25% of max. soil water capacity was reached (*control 70%*). Small amounts of water added to balance unevent water loss from individual pots	Mutants in the *HvDWARF* gene	Not applicable / 23 d/42 d (stressed plants) or 37 d (non-stressed plants), also 53 or 66 d	P5CS activity ▲►; q_{NP}, *HSP70* transcript level ►○; OJIP CF parameters ✕; Car and kestose contents ○▲; q_P, NPQ, proline dehydrogenase activity ○►; F_0, IAA content ○○; F_0', F_s, Chl *a* content ○◄; Chl *b* content, RuBisCO activity, *trans*-zeatin content ○▼; glucose and fructose contents ◄▲; nystose content ◄; Φ_{PSII}, F_v, F_m, F_v/F_m, F_v'/F_m', OAT activity ◄○; F_m', F_v' ◄◄; *trans*-zeatin riboside and *cis*-zeatin contents, *HSP90* transcript level ◄▼; sucrose content ▼◄; g_S, E, ci, P_N ▼○; ETR, proline, ABA, total cytokinin, *cis*-zeatin riboside and IPA contents ▼▼; plant height ▼▼▼; time to flowering ○▲	Janeczko et al. (2016)

Arabidopsis thaliana (wt + 2MT)	GC; pots with soil; 1 plant/pot	9 d; at the end of this period soil WC reached 50 or 35% depending on the genotype	Mutants in the *AtCYP85A2* and *AtERA1* genes	Not applicable/NS/9 d after start of the stress period	Water loss ▼	Northey et al. (2016)
Brassica napus (wt + 2MT)	Greenhouse; pots (8 × 7.5 cm) with commercial soil mixture	1 or 3 d, also 12 d (*plant survival 7 d after rewatering*)	Plants overexpressing the *AtDWF4* gene	Not applicable/28 d/29 or 31 d; also 40 d	*RD20* and *RD22* transcript levels ▲▲; plant survival ▲	Sahni et al. (2016)
Lathyrus sativus (1)	GC; pots (24 × 26 cm) with loess soil and vermiculite (2:1); 10 plants/pot	2, 4, 5, 7, 10, 11 or 13 d. Leaf RWC 78, 68, 60, 42, 38, 40, 30 or 28% (*control 90%*)	2×10^{-7} M EBL; seed soaking (for 24 h), leaf spraying (3× in 1-d intervals) and combination	Seeds or 13 d/15 d/17, 19, 20, 22, 25, 26 or 28 d	Plant height, leaf area, RWC, g_s ◉○	Xiong et al. (2016)
Spinacia oleracea (wt + 2MT)	GC; pots with soil	10 d. Leaf RWC 45% (*control 78%*)	Plants overexpressing the *SoCYP85A1* gene	Not applicable/42 d/42 or 52 d	Root length, no. of roots, CS content ▲▲; RWC, proline content, POD, CAT and SOD activities, *APX*, *CAT*, *GST*, *SOD*, *LEA5* and *ADC1* transcript levels ▲○; *CED1*, *AMDC* and *ERD10C* transcript levels ◉○; BL content ◀○; ABA, H_2O_2 and MDA contents ▼○; water loss rate ▼	Duan et al. (2017)

(continued)

Table 5.1 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment /start of the stress period / measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Agrostis stolonifera (wt + 1MT)	GC; trays (30 × 20 cm) with sphagnum moss, vermiculite and perlite (3:2:1)	4, 8, 12 or 16 d (*also 4 or 8 d after rewatering, plant survival 10 d after rewatering*). Leaf RWC 98, 98, 68 or 50% (*control 99%*)	Plants overexpressing the *AtBAT1* gene	Not applicable/28 d/28, 32, 36, 40, 44, 48 or 52 d, plant survival also 54 d	RWC ▶▲○; plant survival ▲	Han et al. (2017)
Solanum lycopersicum (1DS, 1DR)	Net-house; pots (20 cm) with NS substrate; experiments repeated in 2 successive years	7 d (*plants then rewatered*), at the end of this period soil WC reached 16% (*control 54%*). Leaf RWC 53, 67 or 70% (*control 80, 86 or 94%*) depending on the measurement time	3×10^{-6} and 10^{-6} M EBL; leaf spraying (4× in 2-d intervals)	61 d/60 d/81, 101 or 121 d	Fruit yield, days to fruit set, lycopene content in fruits, no. of flower clusters, no. of leaves, RWC, SOD activity in leaves ▲; % of fruit set, fruit diameter ▶; H_2O_2 content in leaves ◀	Jangid and Dwivedi (2017)
Zea mays (wt + 1MT)	GC; pots (8 × 8 cm) with mixed Irish and Baltish peat; 1 plant/pot	3, 4 or 9 d (*plant survival 3 or 7 d after rewatering*); soil WC reached 45%, 30% or 20% (*control 70%*)	Mutant in the *ZmBRD1* gene	Not applicable/ emergence of the fourth leaf/emergence of the fourth leaf +0, 3, 4, 9, 12 or 16 d	RWC, E, g_S, P_N ▲○; WUE ▶○; ci ◆○; plant survival ▲	Castorina et al. (2018)

Hordeum vulgare (wt + 5MT)	GC; pots (15 × 40 cm) with soil, Substral and sand (8:2:1); 10 plants/pot	15 d; at the end of this period 25% of max. soil water capacity was reached (*control 70%*). Small amounts of water added to balance unevent water loss from individual pots	Mutants in the *HvCPD*, *HvBRD*, *HvDIM* and *HVBRI1* genes	Not applicable/29 d/44 d (stressed plants) or 38 d (non-stressed plants (V5)	Oxidized Asc content ▲ ○; β- and δ- tocopherols, α- and δ-tocotrienol contents ◉ ○; β-carotene contents ◉ ◄; oxidized Gln content ◧ ◄; reduced Gln and total Gln contents ▼ ◄; α-, γ-, and total tocopherols contents, reduced Asc content ▼▼	Gruszka et al. (2018)
Capsicum annuum var. *frutescens* (1)	Greenhouse; pots with soil, rice husk ash and manure (2:2:1)	5 d; 25% of pot water capacity (*control = full pot water capacity*). Leaf RWC 40% (*control 90%*)	10^{-6} M EBL and DHE; leaf spraying (1×)	56 d/57 d/62 d	ETR, Φ_{PSII}, F_v'/F_m' ▲▲; capsaicin content in fruits ▲ ►; fruit FM per plant, shoot FM and DM, RWC; g_S, E, ci, F_v/F_m, soluble saccharides and proline contents ▲ ○; P_N ▲ ◄; q_P ◨ ►; H_2O_2 content ▼ ○; EL ▼ ◄; MDA content ▼ ▼	Khamsuk et al. (2018)

Abbreviations: ABA abscisic acid, *APX* ascorbate peroxidase, *Asc* ascorbate, *BL* brassinolide, *CA* carbonic anhydrase, *ca* air CO_2 concentration, *Car* carotenoids, *CAT* catalase, *Chl* chlorophyll, *ci* intercellular CO_2 concentration, *CS* castasterone, *DHE* 7,8-dihydro-8α-20-hydroxyecdysone, *DM* dry mass, *DR* drought-resistant variety/genotype, *DS* drought-sensitive variety/genotype, *E* transpiration rate, *EBL* 24-epibrassinolide, *EL* electrolyte leakage (membrane permeability), *ETR* photosynthetic electron transport rate, F_0, F_m, F_v, *resp.* F_0', F_m', F_v' minimum, maximum or variable Chl fluorescence in the dark-, resp. light-adapted state, *FC* field capacity, *FM* fresh mass, F_s steady-state Chl fluorescence, F_v/F_m, *resp.* F_v'/F_m' maximum quantum yield of Photosystem II in the dark, resp. light-adapted state, *GC* growth chamber, *Gln* glutathione, *GR* glutathione reductase, g_S stomatal conductance, *HBL* 28-homobrassinolide, *Hgl* Hoagland, *IAA* indole-3-acetic acid (auxin), *IPA* isopentenyladenine, *JA* jasmonic acid, *MDA* malondialdehyde, MDHAR monodehydroascorbate reductase; *MS* Murashige-Skoog, *MT* mutant or transgenic plants, *NPQ* non-photochemical quenching of Chl fluorescence, *NR* nitrate reductase, *NS* not stated, *OAT* ornithine-6-aminotransferase, *OJIP CF* various parameters describing efficiency of primary photosynthetic processes derived from fast Chl fluorescence kinetics, *P5CS* pyrrolin-5-carboxylate synthase, P_N net photosynthetic rate, *POD* guaiacol-type peroxidase, q_P photochemical quenching of Chl fluorescence, *RuBisCO* ribulose-1,5-bisphosphate carboxylase/oxygenase, *RWC* relative water content, *SA* salicylic acid, *SOD* superoxide dismutase, *WC* water content, *wt* wild type, *WUE* water use efficiency, *WUEi* intrinsic water use efficiency, Ψ water potential, Ψ_π osmotic potential, Φ_{PSII} effective quantum yield of Photosystem II photochemistry

Table 5.2 Studies dealing with the effect of brassinosteroids (BRs) on plants subjected to drought stress simulated by restricted watering. Major aspects of the experimental setups (if mentioned in the respective study) are presented and the results are summarised using the same symbols and abbreviations defined in Table 5.1

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	Reference
Beta vulgaris (1)	NS; pots (9 × 4.5 cm) with sand, general nutrient solution; 5 plants/pot	NS; irrigation to maintain 55% saturation of soil water capacity (*control 90%*)	100 and 1000 mg/ha HBL; leaf spraying (1×)	V1/from the start of cultivation/ V1 + 4 weeks	Root DM ▲○; leaf DM, leaf length ●○	Schilling et al. (1991)
Beta vulgaris (1)	NS; pots (6 L) with sand, general nutrient solution; 2 plants/pot	NS; irrigation to maintain 40–50% or 25–30% of max. soil water capacity (*control 80%*)	100 and 1000 mg/ha HBL or 100 mg/ha EBL; leaf spraying (1×)	V6/from the start of cultivation/ V6 + 5 months	Total plant and root DM, sucrose yield in roots ▶○; leaf DM ●▶; sucrose content and acid invertase activity in roots ●○; sucrose synthetase activity in roots ●	Schilling et al. (1991)
Brassica juncea (1)	Field; clay loam soil	NS	1.2×10^{-6}, 8×10^{-7} and 4×10^{-7} M NS; leaf spraying (1× or 2×?)	Prior to flowering, during pod formation or both times/at the time of BR treatment/ NS	No. of siliques, no. of seeds per siliqua, seed mass, seed yield per plant, % of oil in seeds, straw, seed and oil yields, harvest index. *Not possible to separate the data from the stressed and non-stressed BR-treated plants*	Kumawat et al. (1997)

Triticum aestivum (2)	Field with rainout shelters, NPK fertiliser, experiments performed in 2 successive years (*each year measurement of only some parameters*)	NS; average 15% or 12% soil WC (*control 60–70%*) during cultivation period depending on the year. Leaf WC 52–57% of the non-stressed plants	10^{-9} M EBL; seed soaking (for 1 h)	Seeds/from the start of cultivation?/NS	Grain mass per plant, no. of tillers, soluble proteins content ▲▲; E ▶▼; P_N ◆○; flower initials productivity ◉◆; length of ears, no. of grains per ear ◉▲; plant height, WC ◉○; starch content ◉◀; 1000 grain weight ◀○	Prusakova et al. (2000)
Triticum aestivum (3)	Greenhouse; pots with soil; 20 plants/pot	NS; irrigation to maintain 37% soil FC	10^{-9} M EBL; leaf spraying (1×)	Start of flowering/40.DC (booting)/49.DC to 91.DC	Straw DM ▲; ear DM, grain and straw yields ◉	Hnilička et al. (2007)
Triticum aestivum (6)	Greenhouse; pots with soil	NS; irrigation to maintain 37% soil FC	10^{-9} M EBL; leaf spraying (1×)	61.DC (start of flowering)/40.DC (booting)/after harvest	Starch and net energy contents in grains. *Not possible to separate the data from the stressed and non-stressed BR-treated plants*	Hnilička et al. (2008a)
Triticum aestivum (6)	Greenhouse; pots with soil	NS; irrigation to maintain 37% soil FC	10^{-9} M EBL; leaf spraying (1×)	61.DC (start of flowering)/40.DC (booting)/NS	E, P_N. *Not possible to separate the data from the stressed and non-stressed BR-treated plants*	Hnilička et al. (2008b)
Triticum aestivum (3)	Greenhouse; pots with soil	NS; irrigation to maintain 37% soil FC	10^{-9} M EBL; leaf spraying (1×)	61.DC (start of flowering)/40.DC (booting)/49 to 91.DC	Grain and straw yields ▲	Hnilička et al. (2008c)

(continued)

Table 5.2 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	Reference
Glycine max (1)	Outdoor; pots (30 × 25 cm) with sandy loam soil; NPK fertiliser; polyethylene cover; 4 plants/pot	7 d or until harvest; irrigation to maintain 35% soil FC (*control 80%*). Leaf Ψ −2.30 MPa (*control −0.50 MPa*)	2×10^{-7} M BL; leaf spraying (1×)	R1/R3 (R1 + additional 7 d)/ R3 + 7 d or at harvest	Seed yield, total plant biomass, root DM, P_N, RuBisCO activity, ^{14}C in nodules and pods, soluble saccharides content, SOD activity ▲▲; Ψ, F_v/F_m, total Chl and proline contents, POD activity ▲○; shoot DM ▶▲; ^{14}C in leaves ▶◆; PEPC activity, ^{14}C in roots ●▲; ^{14}C in stem ●○; MDA content ▼○; EL ▼▼	Zhang et al. (2008)
Oryza sativa (1)	GC; pots (20 cm) with loam soil + Hgl solution	14 or 35 d; irrigation to maintain 50% soil FC. Leaf RWC 42% (*control 85%*), leaf Ψ −1.16 MPa (*control −0.41 MPa*)	10^{-8} M EBL and HBL; seed soaking (for 4 h) or leaf spraying (1×)	Seeds or 28 d (V4)/21 d (V4)/35 or 56 d	Total plant FM and DM, plant height, Ψ, Ψ_π, RWC, ci, P_N, P_N/ci, WUE, soluble phenols, anthocyanins and proline contents, APX, CAT and SOD activities ▲; Ψ_p, g_S, E, H_2O_2 and MDA contents; EL ▼	Farooq et al. (2009)
Triticum aestivum (6)	Greenhouse; pots with soil	NS; irrigation to maintain 37% soil FC	10^{-9} M EBL; leaf spraying (1×)	61.DC (start of flowering)/40.DC (booting)/at harvest	Total lipids and net energy contents in grains ▶; starch and soluble proteins contents in grains ●	Hnilička et al. (2009)

Oryza sativa (1)	GC; pots (24 × 20 cm) with soil	4, 8, 12 or 16 d; irrigation to maintain 50% soil FC. Leaf RWC 75, 74, 68 or 62% (*control 65, 60, 48 or 43%*), leaf Ψ −0.80, −0.88, −0.96 or −1.12 MPa (*control −0.50 MPa*)	10^{-8} M EBL; leaf spraying (1×)	28 d (V4)/28 d (V4)/32, 36, 40 or 44 d	Total plant FM and DM, plant height, Ψ, Ψ_π, E, P_N, WUE, anthocyanin and proline contents, CAT and SOD activities ▲; RWC, g_S, soluble phenols content, APX activity ▶; H_2O_2 content ◀; Ψ_p, MDA content, EL ▼	Farooq et al. (2010)
Brassica oleracea (*Botrytis* cultivar group) (1)	Greenhouse; pots (15 L) with garden substrate and sand (2:1) + knop-Benson solution	NS; irrigation to maintain 20% of soil full water capacity (*control 25%*)	10^{-9} M EBL; leaf spraying (1×)	V6–V7/from the start of cultivation?/ V6–V7 + 0, 8, 16, 23, 30, 37 or 43 d	E ▶►; P_N ○○	Hnilička et al. (2010)
Satureja hortensis var. *bakhtiarica* (1)	Greenhouse; pots (20 × 40 cm) with soil	NS; irrigation to maintain 2/3 or 1/3 of full soil FC (*control full soil FC*)	10^{-6}, 10^{-8} and 10^{-10} M HBL; mode of application NS	V3–V5/some time after BR treatment/ NS	Total plant biomass, oil yield in shoot ▲◆; root DM ▲►; stem diameter ▲◄; no. of sub-shoots ▶◄; % of essential oils in shoot ○○; plant height ○◄	Eskandari (2011)
Xanthoceras sorbifolia (1)	Outdoor, rainout shelter when necessary; pots (17 L) with loess soil; 1 plant/pot	20 d; irrigation to maintain 55% or 35% soil FC (*control 75%*), *i.e.*, 12–13% or 7–8% soil WC (*control 17–18%*). Leaf RWC 80 or 58% (*control 87%*)	8×10^{-7}, 6×10^{-7}, 4×10^{-7} and 2×10^{-7} M BL; root soaking (for 5 min), then leaf spraying (1×)	1 year/1 year + cca 2.5 months/1 year +2.5 months + 20 d	POD and CAT activities ▲▲; reduced Asc content, APX activity ▲▶; RWC, soluble proteins, soluble saccharides, proline and reduced Gln contents ▲○; SOD activity ▶○; MDA content, EL ▼○	Li and Feng (2011)

(continued)

Table 5.2 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	Reference
Capsicum annuum (1)	Greenhouse, then GC; pots (30 × 30 cm) with grass peat and perlite (8:2) + Enshi solution	1, 5 or 10 d (*also 1 or 5 d after rewatering*); without watering for 4 d, then irrigation to maintain 45% soil WC (*control 80%*)	2×10^{-8} M EBL; leaf spraying (2× in 5-d interval)	60 d (V20–25)/60 d (V20–25)/61, 65, 70, 71 or 75 d	E ▲▲▲; total plant FM ▲▲▶; P_N ▲▶ ▲; g_S ▲○▲; F_v/F_m, Φ_{PSII}, q_P, NPQ ▶▶ ○; ci ○○○; maximum P_N, LSP ▲ ▲; AQY ▲○; LCP ▼▼	Hu et al. (2013)
Vigna unguiculata (1)	Field; soil composed of clay, silt and sand (42:40:18)	NS; irrigation always after EVAP reached 120 mm (*control 60 mm*)	4×10^{-6} and 2×10^{-6} M NS; seed soaking or leaf spraying (2×)	Seeds or at V6 and the stage of bud formation/after pruning/harvest time	No. of pods per plant, 1000 seed weight, seed yield, harvest index ▲ ▲; no. of seeds per pod, total plant biomass ▶▶	Hashemi et al. (2015)
Zea mays (2)	Greenhouse; pots (30 × 35 cm) with clay loam soil and sand (2:1); NPK fertiliser; 2 plants/ pot	55 d; irrigation to maintain 75% or 50% soil FC (*control 100%*), *i.e.*, 11.6% or 7.7% soil WC (*control 15.5%*)	2×10^{-7} M EBL; leaf spraying (1×)	60 d (V6-V8)/30 d/85 d	Grain yield per plant, no. of grains per plant, root and shoot DM, plant height, leaf area, no. of leaves, ratios of reduced/oxidized Asc and Gln, membrane stability index ▲ ▲; reduced Asc and Gln contents, CAT and SOD activities ▲▶; glycine betaine, proline, oxidized Asc and Gln contents, APX activity ▲○; MDHAR, DHAR and GR activities ▶ ○; O_2^- and MDA contents, EL ▼○; H_2O_2 content ▼▼	Talaat et al. (2015)

Lactuca sativa (2)	GC; seedling trays with compost based on peat; 1 plant/pot	21 or 28 d; total quantity of irrigation water 28 mm (*control 39 mm*); efficient water capacity 60% (*control 75%*)	10^{-7}, 10^{-9} and 10^{-11} M AA; leaf spraying (1×)	15 d/from the start of cultivation/21 or 28 d	Root FM, root length ▶▶; shoot FM, plant height, stem diameter ▶○; shoot and root DM ◉○	Doležalová et al. (2016a)
Allium cepa (2)	Field; experiments repeated in 2 successive years	59 d or approx. 4 months; total quantity of irrigation water 190 or 335 mm (*control 225 or 390 mm*) depending on the year; efficient water capacity 50% (*control 70%*)	10^{-7}, 10^{-9} and 10^{-11} M AA; leaf spraying (1×)	45 d/from the start of cultivation/59 d or approx. 4 months	Bulb yield ▶○; plant height, bulb FM ◉▶; bulb DM, bulb height and thickness of neck, total Asc content in bulbs ◉○; bulb diameter ◀▶	Doležalová et al. (2016b)
Zea mays (2)	Greenhouse; pots (30 × 35 cm) with clay loam soil and sand (2:1); NPK fertiliser; 2 plants/pot	55 d; irrigation to maintain 75% or 50% soil FC (*control 100%*), *i.e.*, 12% or 8% soil WC (*control 16%*). Leaf RWC 48–55 or 40–45% (*control 65–68%*) depending on the genotype	2×10^{-7} M EBL; leaf spraying (1×)	60 d (V6-V8)/30 d/85 d	RWC ▲▲; RuBisCO activity ▲▶; total amino acids content ▲○; NR activity ▶▲; SAMDC and ODC activities ▶▶; soluble proteins, nitrate, flavonoids, soluble phenols, spermidine, spermine and ethylene contents, ADC activity ▶○; putrescine content ◆○; DAO activity ◀○; PAO and protease activities, content of carbonyl groups ▼○	Talaat and Shawky (2016)

(continued)

Table 5.2 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	Reference
Lathyrus sativus (1)	Outdoor, rainout shelter when necessary; pots (24 × 30 cm) with loess soil and vermiculite (2:1); NPK fertiliser; 5 plants/pot	60 d; without watering for NS time, then irrigation to maintain 50 or 35% soil FC (*control NS*)	2×10^{-7} M EBL; seed soaking (for 24 h), leaf spraying (1×) or into soil (2× in a 13-d interval) and all combinations	Seeds or 15 d or 37 d/40 d/100 d (maturity)	β-ODAP content in shoot ▶ ○; no. of seeds per plant, seed mass, harvest index, shoot and root DM, β-ODAP concentration in shoot, shoot and seed WUE (determined from yield) ◎○	Xiong et al. (2016)
Lathyrus sativus (1)	Outdoor, rainout shelter when necessary; pots (24 × 30 cm) with loess soil and vermiculite (2:1); 5 plants/pot	36, 52, 72 or 82 d; without watering for NS time, then irrigation to maintain 50 or 35% soil FC (*control NS*)	2×10^{-7} M EBL; into soil (5× in 5-d intervals)	40 d/18 d/54, 70, 90 or 100 d	Shoot WUE, β-ODAP concentration in seeds ▲ ▲; no. of pods, pod coat DM, no. of seeds and seed mass per plant, β-ODAP content in seeds, seed WUE ▶▲; pod DM, β-ODAP content in pods ▶▶; seed mass ◆▼; β-ODAP concentration in pod coats, β-ODAP amount in leaves ◎▲; root, shoot and leaf DM, β-ODAP concentration in pods and leaves, β-ODAP content in pod coats, ABA content in leaves ◎▶; total plant WUE ◎○	Xiong et al. (2016)

Carthamus tinctorius (3)	Field	NS; irrigation to maintain 120 or 160 mm EVAP (*control 80 mm*)	10^{-7} M NS; mode of application NS	NS/after flowering/NS	g_S, Chl *a/b* ratio, Car, soluble proteins and soluble saccharides contents. *Not possible to separate the data from the stressed and non-stressed BR-treated plants*	Zafari and Ebadi (2016)
Gossypium barbadense (1)	Field; clay loam soil; experiments repeated in 2 successive years	60, 90, 120 or 180 d; irrigation every 3 or 4 weeks, *i.e.*, 75 or 50% of control (*control irrigated every 2 weeks*)	10^{-6} and 10^{-7} M EBL; leaf spraying (5× in 1-d intervals)	40 d/from the start of cultivation/60, 90, 120 or 180 d	Seed yield and no. of open bolls per plant, fiber strength, total plant DM, plant height, leaf area, no. of leaves, soluble saccharides content ▲▲; fiber length, soluble phenols content, CAT and SOD activities ▶▶; total amino acids content ▶○; P content, POD activity ▶▲; Na content ▶◀; relative growth rate ✕◀; lint percentage, N and K contents ○○; net assimilation rate, proline content ◀◀; PPO activity ▼▼	Ahmed et al. (2017)
Solanum lycopersicum (1)	Greenhouse; pots (10 L) with soil, vermicompost and sand (3:2:1); NPK fertiliser	NS; irrigation to maintain 50% soil FC. Leaf RWC 49% (*control 72%*)	2×10^{-6} M BL; leaf spraying (2× in a 20-d interval)	25 d after transplanting/1 d after transplanting/60 d after transplanting	RWC, P_N, total Chl, soluble proteins and proline contents, NR activity ▲; CAT activity ▼	Sivakumar et al. (2017)

(continued)

Table 5.2 (continued)

Plant species (including number of analyzed genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	Reference
Phaseolus vulgaris (1)	Field; loam soil	NS; irrigation to maintain 90 or 120 mm EVAP (*control 60 mm*)	2×10^{-7} M EBL; leaf spraying (1×)	50% flowering/V4/ NS	Seed yield, proline content, APX, POD, CAT and SOD activities. *Not possible to separate the data from the stressed and non-stressed BR-treated plants*	Younesian et al. (2017a)
Phaseolus vulgaris (1)	Field; loam soil	NS; irrigation to maintain 90 or 120 mm EVAP (*control 60 mm*)	2×10^{-7} M EBL; leaf spraying (1×)	50% flowering/V4/ NS	POD activity ▲○; seed yield ▶►; CAT and SOD activities ▶○	Younesian et al. (2017b)
Festuca arundinacea (1)	Greenhouse; pots (18 × 20 cm) with soil and sand (3:1)	14 d; cessation of watering for some time?, then irrigation to maintain 50% or 25% soil FC (*control 75%*). Leaf RWC 70 or 40% (*control 90%*)	1.6×10^{-6} and 8×10^{-7} M BL; leaf spraying (3× in 5-d intervals)	66 d/63 d/77 d	Proline content ▲ ▲; WUE, Chl *b* content ▲►; Chl *a* content, POD, CAT and SOD activities ▲○; P_N ▶▲; RWC ▶○; WUE ◀ ►; g_S ◀ ◄; Chl *a*/*b* ratio ◀ ○; EL ▼○; E, MDA content ▼▼	Chen et al. (2018)

Calendula officinalis (1)	Field; soil composed of clay, silt and salt (39:30:31); ammonium nitrate fertiliser; experiments repeated in 2 successive years	20 d; irrigation always after EVAP reached 100 mm (*control 50 mm*). Leaf RWC 48–50% (*control 65%*)	10^{-7} and 10^{-8} M EBL; leaf spraying (1×)	50 d/60 d (flowering stage)/80 d	RWC, POD activity ▲▲; capitula yield, soluble proteins content ▲▶; PPO activity ▶▲; SOD activity ▶▼; % of essential oils and oil yield in petals ▶; CAT activity, EL ◀○; proline content ◀○; H_2O_2 and MDA contents ▼▼	Hemmati et al. (2018)

Additional abbreviations: AA 2α,3α,17β-trihydroxy-5α-androstan-6-one, *ADC* arginine decarboxylase, *AQY* apparent photosynthetic quantum yield, *^{14}C* ^{14}C partitioning, *DAO* diamine oxidase, *DC* Zadoks decimal code, *DHAR* dehydroascorbate reductase, *EVAP* evaporation, *LCP* light compensation point, *LSP* light saturation point, *ODAP* oxalyldiaminopropionic acid, *ODC* ornithine decarboxylase, *PAO* polyamine oxidase, *PEPC* phospho*enol*pyruvate carboxylase, *PPO* polyphenol oxidase, q_{NP} non-photochemical quenching of Chl fluorescence, *SAMDC* S-adenosylmethionine decarboxylase, Ψ_p pressure potential

Table 5.3 Studies dealing with the effect of brassinosteroids (BRs) on plants subjected to drought stress simulated by the cultivation of plants in the field experiencing only natural rainfall. Major aspects of the experimental setups (if mentioned in the respective study) are presented and the results are summarised using the same symbols and abbreviations defined in Tables 5.1 and 5.2

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Triticum aestivum (1DS, 1DR)	Field	7 d; only natural rainfall (*control = artificial irrigation*)	10^{-7} M and 2×10^{-8} M HBL; seed soaking or leaf spraying (1×, both leaf sides)	Seeds or 25 d/ Anthesis/Anthesis +7 d	No. of ears per row of plants, no. of grains per ear, grain yield, 1000 grain weight, harvest index ▲▲	Sairam (1994b)
Triticum aestivum (1)	Field	NS; only natural rainfall (*control = artificial irrigation*)	10^{-7} M and 2×10^{-8} M HBL; seed soaking (for 6 h)	Seeds/NS/at harvest	Total plant biomass, grain yield ▲▲; no. of ears per row of plants, no. of grains per ear, 1000 grain weight ▲○; harvest index ◉○	Sairam et al. (1996)
Glycine max (1DS, 1DR)	Field; brown soil	One year with lower rainfall (14 mm) during July (*control = another year with normal rainfall (71 mm) during July*)	5×10^{-7} M EBL; seed soaking (for 24 h) or 2×10^{-6} M EBL; leaf spraying (2×)	Seeds or V2 and flowering/ NS/4 months	BL content in seeds ▲; no. of pods per plant ▶►; no. of seeds per plant, seed weight per plant ▶▲, content of total phytoestrogens in seeds ▶; 1000 seed weight ◉○; Na, Mg, Cu, Fe, soluble proteins, glucose, fructose, stachinose + rafinose, maltose, sucrose contents in seeds ◉; Ca and K contents in seeds ◀; CS content in seeds ▼	Janeczko et al. (2011a)
Eleusine coracana (3)	Field	70 or 102 d?; only 450 mm rainfall, 31 rainy d during the whole 102-d period	2×10^{-7} M NS; leaf spraying (1×)	70 d (flower initiation)/from the start of cultivation?/70 or 102 d	Growth rate and relative growth rate ▶; no. of ears, grain yield, total plant DM, plant height, root length, no. of tillers ◉	Kumar et al. (2017)

Table 5.4 Studies dealing with the effect of brassinosteroids (BRs) on plants subjected to drought stress simulated by the application of polyethylene glycol (PEG) or some other osmolyte. Major aspects of the experimental setups (if mentioned in the respective study) are presented and the results are summarised using the same symbols and abbreviations defined in Tables 5.1 and 5.2

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/ start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical / physiological parameters were measured in leaves or shoots)	References
Triticum aestivum (1)	Laboratory; Petri plates with moist filter paper	2 or 4 d; PEG 6000, osmotic pressure −0.5 or −1 MPa	10^{-7} M and 2×10^{-8} M HBL; seed soaking (for 6 h)	Seeds/6 h/2 or 4 d	Soluble proteins content, α-amylase activity in grains ▲▲; plant height ▶▲; germination percentage ◎○	Sairam et al. (1996)
Sporobolus stapfianus (1)	Greenhouse, then GC; pots (15 cm) with sand, loam and peat (3:1:1) + Hgl solution	40 min; 0.4 to 4 M $CaCl_2$	2×10^{-6} M BL; into the cultivation medium (for 17 h)	NS/17 h after BR treatment/17 h + 40 min after BR treatment	Protoplasma water content in cells isolated from leaves ◎	Ghasempour et al. (1998)
Sporobolus stapfianus (1)	Greenhouse, then GC; pots with mixed sand, loam and peat + Hgl solution	50% PEG; measured 18 h after PEG removal	NS; leaf coating (1×); BR also present in PEG solution	NS/NS/18 h after start of the stress period	Leaf proteome X (*proteins were not identified nor truly quantified*)	Ghasempour et al. (1998)

(continued)

Table 5.4 (continued)

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/ start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical / physiological parameters were measured in leaves or shoots)	References
Zea mays (1DS, 1DR)	Incubator; vessels with modified MS medium	1 d; PEG 6000, osmotic pressure −0.5 MPa; measured 29 d after PEG removal	3×10^{-5} M NS; callus soaking (for 3 h?)	8 d/8 d + 3 h/38 d	Plant survival ▲; CAT activity ▶▶; POD activity ▶◀; Car content, APX and SOD activities ○▶; GR activity ○○; total Asc, H_2O_2 and MDA contents ◀○; EL ▼▼	Li and van Staden (1998a)
Zea mays (1DS, 1DR)	GC; pots with vermiculite + Hgl solution	1 or 2 d; PEG 6000, osmotic pressure −1 MPa. Leaf RWC 73–75%	8×10^{-5}, 4×10^{-5}, 2×10^{-5} and 2×10^{-6} M BL; seed soaking (for 17 h)	Seeds/7 d/7, 8 or 9 d	RWC ▲; plant height ◀; g_s ◀◀; EL ◀▼; E ▼◀	Li and van Staden (1998b)
Zea mays (1DS, 1DR)	GC; pots (9 cm) with vermiculite + Hgl solution; 10 plants/pot	1 or 2 d; PEG, osmotic pressure −1 MPa	2.6×10^{-5} M BL; seed soaking (for 14 h)	Seeds/17 h?/17 h? + 0, 1 or 2 d	CAT activity ▶▶; APX activity ▶◆; SOD activity ▶○; Car content ○▶; total Asc and MDA contents, GR and POD activities ○○; H_2O_2 content ◀○	Li et al. (1998)

Triticum aestivum (1DS, 1DR)	NS; pots (20 cm) with sand loamy soil; 10 plants/pot	1 or 2 d; 30% PEG 6000. Leaf RWC 70 or 54% (*control 84%*)	4×10^{-5}, 2×10^{-5} and 2×10^{-6} M BL; seed soaking (for 18 h)	Seeds/10 d/10, 11 or 12 d	Shoot and root DM and FM, plant height, leaf area, soluble saccharides and proline contents ▲; RWC ▲▲; root length, total Chl content ▶; E, EL ▼▼; leaf proteome ✕ (*proteins were not identified nor truly quantified*)	El-Khallal and Nafie (2000)
Zea mays (1)	NS; pots (20 cm) with sand loamy soil; 4 plants/pot	1, 2 or 3 d, also 1 or 2 d after plants were put into water; 30% PEG 6000, osmotic pressure −0.9 MPa	10^{-5} M BL; seed soaking (for 18 h)	Seeds/14 d/14, 15, 16, 17, 18 or 19 d	Total Chl, Car and reduced Asc contents ▲▲▲; CAT activity ▲▲○; APX activity ▲○▲; total, Mn- and Fe- SOD activities ▶▶▲; cu/Zn-SOD activity ▶▲○; POD activity ○○▲; H_2O_2 and MDA contents ▼▼▼; appearance of some new CAT and POD (but not APX and SOD) isozymes	El-Khallal (2002)

(continued)

Table 5.4 (continued)

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/ start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical / physiological parameters were measured in leaves or shoots)	References
Sorghum bicolor (2DS, 1DR)	Laboratory; Petri plates with moist filter paper	5 or 6 d (*germination percentage also 12, 24, 36 or 48 h*); 20% PEG 6000	3×10^{-6} and 2×10^{-6} M EBL and HBL; seed soaking, then into the cultivation medium (*solutions 2× replenished*)	Seeds/seeds/6 or 5 d, also (seed germination percentage) 12, 24, 36 or 48 h	Total plant FM and DM, plant height, germination percentage, soluble proteins content, CAT activity ▲; proline content ▶; APX and POD activities ▼	Vardhini and Rao (2003)
Oryza sativa (1DS, 1DR)	GC; plates with agar and MS medium	1 or 2 d; PEG 6000, osmotic pressure − 1 MPa. Leaf RWC 66 or 53% (*control 87%*)	2×10^{-7}, 2×10^{-8}, 2×10^{-9} and 2×10^{-10} M MH-5; into the cultivation medium	Seeds/15 d/15, 16 or 17 d	RWC ▲○; total plant FM and DM ▶►; plant height ▶○; stem diameter ▶◆; no. of roots ◉◄; germination percentage ▲	García et al. (2005)

Sorghum bicolor (1)	Laboratory; Petri plates with moist filter paper	5 or 6 d; (*germination percentage also 36 or 48 h*); 20% PEG 6000	3×10^{-6} and 2×10^{-6} M EBL and HBL; into the cultivation medium (for the whole stress period)	Seeds/seeds/5 or 6 d, also (seed germination percentage) 36 or 48 h	Total plant FM and DM, plant height; germination percentage; soluble proteins and proline contents, CAT activity ▲; POD activity ▼	Vardhini and Rao (2005)
Orobanche aegyptiaca (1), *Orobanche minor* (1), *Orobanche ramosa* (1)	Incubator; Petri plates with moist filter paper	7, 14, 21 or 28 d, measured only 10 d after PEG removal; PEG 8000, osmotic pressure −1 or −2 MPa	2×10^{-6} M BL; into the cultivation medium (for 10 d)	7 d/seeds/17, 24, 31 or 38 d	Germination percentage ▲▲	Song et al. (2006)
Sorghum bicolor (3DS, 1DR)	Laboratory; Petri plates with moist filter paper	6 d; 20% PEG	3×10^{-6} and 2×10^{-6} M EBL and HBL; seed soaking, then into the cultivation medium (*solutions 2× replenished*)	Seeds/seeds/6 d	GR and SOD activities ▲; protease, ribonuclease, PPO and IAA oxidase activities ▼	Vardhini et al. (2011)
Zea mays (1)	GC; trays with sand	8 or 12 h; 10% PEG	10^{-6} M NS; soaking the base of cut plants (for 4 h)	V2/V2 + 4 h/V2 + 12 or 16 h	ABA content ▶○; MDA and carbonyl groups contents ◀○; EL ▼○	Zhang et al. (2011)

(continued)

Table 5.4 (continued)

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/ start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical / physiological parameters were measured in leaves or shoots)	References
Chorispora bungeana (1)	GC; pots with soil and manure (6:1) + Hgl solution	3 d; 20% PEG 6000. Leaf WC 56% (*control 81%*)	10^{-7} M EBL; leaf spraying (3× in 2-h intervals)	NS (plant height cca 5 cm)/1 d after BR treatment/4 d after BR treatment	F_v/F_m, Chl *a* and reduced Asc contents, APX and CAT activities ▲▲; Φ_{PSII}, total Chl and reduced Gln contents, GR and SOD activities ▲○; WC, F_0, F_m, Chl *b* content ◙○; proline and MDA contents, EL ▼○	Li et al. (2012)

Raphanus sativus (1)	Laboratory; Petri plates with moist filter paper	1 d (*measured only 6 d after PEG removal*); 15% PEG, osmotic pressure −0.295 MPa	2×10^{-6}, 10^{-6} and 5×10^{-7} M EBL and HBL; seed soaking (for 24 h)	Seeds/seeds/7 d	Total plant FM, soluble proteins, DNA, RNA and proline contents, SOD activity ▲▲; total plant DM, plant height, APX and POD activities ▲▶; CAT activity ▶▲; germination percentage ▲○; ribonuclease activity ○○; MDA content ▼◀	Mahesh et al. (2013)
Arachis hypogaea (6)	Germination chamber; trays (70 × 30 cm) with sand	1 d; PEG 6000, osmotic pressure −0.5, −0.9 or −1.5 MPa; leaf RWC 70–80, 64–76 or 65–80% (*control 86–88%*) depending on the genotype	2×10^{-6} M BL; seed soaking (for 2 h)	Seeds/15 d/16 d	RWC ▶○; PPO activity ◆◆; CAT activity ▼▼; POD activity ◆▶	Savaliya et al. (2013)
Vigna radiata (1)	NS; Petri plates	8 or 1 d; PEG 6000, osmotic pressure −0.6 or -1.2 MPa	10^{-6}, 10^{-8} and 10^{-10} M HBL; into the cultivation medium	7 or 14 d/7 or 14 d/15 d	Soluble saccharides, N, K, P and proline contents ▲▲; germination percentage ▲▶	Alyemeni and Al-Quwaiz (2014)

(continued)

Table 5.4 (continued)

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/ start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical / physiological parameters were measured in leaves or shoots)	References
Cicer arietinum (1)	NS (Petri plates with water)	1 or 3 d; PEG 6000, osmotic pressure −0.2 or −0.4 MPa	2×10^{-6}, 10^{-6} and 5×10^{-7} M BL; seed soaking (for 2 h)	Seeds/2 h/1 or 3 d	Soluble saccharides content, POD activity ▶▶; proline content, CAT activity ◆▲	Gursude et al. (2014)
Salvia miltiorrhiza (1)	Greenhouse; pots (37 × 25 cm) with NS substrate	9 d; 20% PEG 6000	8×10^{-7}, 4×10^{-7} and 2×10^{-7} M NS; leaf spraying (1×)	Cca 3 months/Cca 3 months +2 d/Cca 3 months +11 d	Proline content ▲; POD, CAT and SOD activities ◆; MDA content ▼	Zhu et al. (2014)
Raphanus sativus (1)	NS; Petri plates with moist filter paper	1 d (*measured only 6 d after PEG removal*); 15% PEG 6000, osmotic pressure −0.285 MPa	2×10^{-6}, 10^{-6} and 5×10^{-7} M EBL and HBL; seed soaking (for 24 h)	Seeds/seeds/7 d	Soluble proteins, DNA, starch, reducing and non-reducing saccharides, and proline contents ▲▲; RNA content ▲▶	Balaraju et al. (2015)

Nicotiana benthamiana (1)	Greenhouse; NS	3 d; 16% PEG 6000. Leaf RWC 58% (*control* 92%)	10^{-7} M BL; leaf spraying (1×)	5–6 weeks/5–6 weeks + 1 d/5–6 weeks + 4 d	*AOX1* transcript level ▲▲; F_v/F_m ▶▲; SOD activity ▲○; RWC, reduced/oxidized Asc and Gln ratios, APX, GR and Gln peroxidase activities ▶○; alternative respiration efficiency ◉▲; CAT activity ◉○; O_2^- content, cell death ◀▶; NADPH oxidase activity, H_2O_2 and MDA contents, EL ◀○; NPQ ▼○	Deng et al. (2015)
Cajanus cajan (1)	Laboratory; Petri plates with moist filter paper	7 d (*germination percentage also 12, 24 or 36 h*); 20% PEG 6000; osmotic pressure −0.295 MPa	2×10^{-6}, 10^{-6} and 5×10^{-7} M EBL and HBL; seed soaking (for the whole stress period, after 4 d again added into the cultivation medium)	Seeds/seeds/7 d or 12, 24 or 36 h	Total plant FM and DM, plant height, germination percentage, glycine betaine, proline and reduced Asc contents, POD, CAT and SOD activities ▲▲; APX and GR activities ▲▶; H_2O_2 and MDA contents, EL ▼▼	Shahana et al. (2015)

(continued)

Table 5.4 (continued)

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/ start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical / physiological parameters were measured in leaves or shoots)	References
Vitis vinifera (1)	Greenhouse; containers (12 × 12 cm) with garden soil, vermiculite and humus (1:1:1), then black growth chambers (50 × 35 × 15 cm); hydropony + Hgl solution	3, 6, 9 or 12 d; 10% PEG 6000	4×10^{-7}, 2×10^{-7} and 10^{-7} M EBL; into the cultivation medium (for the whole stress period)	8 to 10 weeks (V8)/8–10 weeks (V8)/8–10 weeks (V8) + 0, 3, 6, 9 or 12 d	Stomatal length ▲; stomatal width, aperture and density ▶; total Chl and Chl *a* contents ▶; Chl *b* content, cell ultrastructure ◉; Φ_{PSII} ▲◆; F_v/F_m ▲○; NPQ ▶○; F_0 ▼○	Wang et al. (2015)

Cucumis sativus (1)	GC; hydropony? + Hgl solution	3 d; 16% PEG 6000	10^{-5}, 5×10^{-6}, 10^{-6}, 5×10^{-7} and 10^{-7} M BL; leaf spraying (1×)	V3/V3 + 12 h/V3 + 3.5 d?	*AOX* transcript level ▲▲; F_v/F_m, APX, Gln peroxidase, CAT and SOD activities, respiration rate, *ACS1*, *ACS2*, *ACS3*, *ACO1* and *ACO2* transcript levels ▲○; ethylene content, alternative respiration rate ▶▶; O_2^- content ▶; H_2O_2 content ◆▲; POD activity ○○; cell death ◀ ▶; NPQ, MDA content, EL ▼○	Wei et al. (2015)
Triticum aestivum (1DS, 1DR)	GC; trays with moist filter paper	3, 5 or 7 d; 5% mannitol	4×10^{-7} M EBL; seed soaking (for 3 h)	Seeds/seeds/3, 5 or 7 d	Shoot FM and DM ▲▲; 22 and 28 kDa dehydrins content ▶ ▶; 55 kDa dehydrin content ○▶; IAA and total cytokinins contents ▲; ABA and MDA contents ▼	Shakirova et al. (2016)

(continued)

Table 5.4 (continued)

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/ start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical / physiological parameters were measured in leaves or shoots)	References
Solanum tuberosum (wt + 1MT)	GC; glass bottles (7 × 9 cm) with agar and MS medium?	2, 6, 12, 24 or 48 h; 20% PEG 6000	Plants overexpressing the *StCPD* gene	Not applicable/V4 (28 d)/ V4 (28 d) + 0, 2, 6, 12, 24 or 48 h	Soluble proteins, soluble saccharides and proline contents, APX, GR, POD, CAT and SOD activities, *CPD* transcript level ▲▲; MDA content ▼▼	Zhou et al. (2016)
Solanum lycopersicum (1)	Laboratory; Petri plates with moist filter paper	15 d; PEG 6000; osmotic pressure −0.15 MPa	2×10^{-6} M BL; seed soaking (for 4 h)	Seeds/seeds/15 d	Plant height, root length, germination percentage; vigour and stress tolerance indices; soluble phenols content ▲; CAT activity ▼	Chandrasekaran et al. (2017)

Hordeum vulgare (1)	GC, then greenhouse; pots (5 L), hydropony + Hgl solution; 8 plants/pot	15 d; 8% PEG 6000	10^{-6}, 10^{-7} and 10^{-8} M BL; leaf spraying (3×, both leaf sides)	V1 + 7 d?/V1 + 7 d/ V1 + 22 d	P_N, total Chl, Chl *a* and Car contents, APX activity in leaves ▲▲; shoot DM, root FM, root length, ci, Chl *b* content, GR activity in leaves, SOD activity in roots ▲►; cell ultrastructure ▲O; shoot FM, root DM, plant height, g_S, E, POD activity in roots, SOD activity in leaves, APX and GR activities in roots ►►; POD activity in leaves ►◆; H_2O_2 content in leaves and roots, MDA contents in roots ◄◄; OH^- content in leaves ▼◄; MDA content in leaves ▼◆; OH^- content in roots ▼X	Gill et al. (2017)

(continued)

Table 5.4 (continued)

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/ start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical / physiological parameters were measured in leaves or shoots)	References
Triticum aestivum (1)	GC; black boxes (1 L), hydropony + Hgl solution	8 h; 20% PEG 6000	2×10^{-7} M EBL; leaf spraying (3× in 1-d intervals)	NS/3 d after BR treatment/NS (at the end of PEG treatment)	P_N, F_v/F_m, $OJIP_{PSII}$, total and initial RuBisCO activity, RuBisCO activation state, RuBisCO activase activity, *RCA A* and *RCA B* transcript and protein levels, POD, CAT and SOD activities ▲; total Chl content ▶	Zhao et al. (2017)
Solanum lycopersicum (wt + 1MT)	Greenhouse or GC?; pots with MS medium?	30 min; 250 mM sorbitol	Mutant in the *SlDWARF* gene	Not applicable/NS/30 min after start of the stress period	Ratio of stomatal aperture width and length ▲○	Lee et al. (2018)

Table 5.5 Studies dealing with the effect of brassinosteroids (BRs) on plants subjected to drought stress simulated by various less common methods. Major aspects of the experimental setups (if mentioned in the respective study) are presented and the results are summarised using the same symbols and abbreviations defined in Tables 5.1 and 5.2

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Oryza sativa (wt + 1MT)	GC, then incubator; Petri plates, hydropony + Yoshida solution	2, 4, 5, 6 or 12 h; air drying of leaf segments (*for F_v/F_m measurements*) or whole plants	Mutant in the *OsGSK1* gene	Not applicable/8 d/8 d + 0, 2, 4, 5, 6 or 12 h	*LIP5* and *DHN1* transcript levels ▲○; F_v/F_m ▶○; *SALT* transcript level ◉○	Koh et al. (2007)
Picea abies (1)	GC; transparent plastic vessels with moist filter paper	2 d (*measured 5, 12 or 19 d after return to the control conditions*); relative air humidity in testing vessels 58–62% (*control 90–92%*)	2×10^{-7} M AA; seed soaking (for 48 h)	Seeds/2 d/9, 16 or 23 d	Growth rate ○○; germination percentage ▶▶	Kuneš et al. (2016)
Pinus sylvestris (1)	GC; transparent plastic vessels with moist filter paper	2 d (*measured 5, 12 or 19* d *after return to the control conditions*); relative air humidity in testing vessels 58–62% (*control 90–92%*)	2×10^{-7} M AA; seed soaking (for 48 h)	Seeds/2 d/9, 16 or 23 d	Growth rate ▲○; germination percentage ▲◀	Kuneš et al. (2016)
Pseudotsuga menziesii (1)	GC; transparent plastic vessels with moist filter paper	2 d (*measured 5, 12 or 19 d after return to the control conditions*); relative air humidity in testing vessels 58–62% (*control 90–92%*)	2×10^{-7} M AA; seed soaking (for 48 h)	Seeds/2 d/9, 16 or 23 d	Growth rate ○○; germination percentage ○◀	Kuneš et al. (2016)

(continued)

Table 5.5 (continued)

Plant species (including number of analyzed varieties or genotypes)	Cultivation facility; type of cultivation substrate and containers	Length of the stress period and stress conditions; plant water status for the stressed and non-stressed plants	BR type, dosage and mode of application	Plant age (devel. stage) at the time of BR treatment/start of the stress period/ measurements	Analyzed parameters (if not otherwise stated, the biochemical/physiological parameters were measured in leaves or shoots)	References
Brassica juncea (1)	Outdoor; pots with black soil and manure	55 d?; type of soil from semiarid conditions	2×10^{-6}, 10^{-6} and 5×10^{-7} M BL; leaf spraying (4× in 5-d intervals)	35 d/from the start of cultivation?/55d	Leaf area, root diameter ▲; plant height, shoot FM, stem diameter ▶; total plant FM and DM, no. of leaves, root length ◉;	Latha and Vardhini (2016)
Vigna unguiculata (1)	Greenhouse; pots (10 × 15 cm) with sand and vermiculite (3:1) + Hgl solution	2 d; total removal of water from pots. Leaf Ψ −1.68 MPa (*control −0.65 MPa*)	10^{-7} and 5×10^{-8} M EBL; leaf spraying (3× in 6-d intervals)	6 d/18 d/20 d	WUE, P_N, F_v/F_m, Chl *b* content, SOD activity ▲▲; Ψ, P_N/ci, ETR, Φ_{PSII}, F_m, q_P, ▲►; total plant, stem and leaf DM, g_S, E, Car content, APX and POD activities ▲○; total Chl and Chl *a* contents ▶▲; CAT activity ▶○; root DM ◉○; ci, NPQ, relative excess energy at photosystem II, ETR/P_N ▼○; F_0 ▼◄; H_2O_2, and MDA contents, EL ▼○; O_2^- content ▼▼	Lima and Lobato (2017)
Solanum lycopersicum (wt + 1MT)	Greenhouse or GC?; pots with MS medium?	1, 2, 3, 4 or 5 h; detachement of leaves from plants and subsequent loss of water from these leaves	Mutant in the *SlDWARF* gene	Not applicable/ NS/28 d/28 d + 1, 2, 3, 4 or 5 h	Leaf FM ▼	Lee et al. (2018)

2.3 Drought-Induced Changes in the Content and Composition of Endogenous BRs

In fact, it seems that drought *per se* can change endogenous BR levels even without additional BR treatment or mutations in genes associated with BR biosynthesis or BR signalling. The first evidence of this phenomenon was presented by Jäger et al. (2008), who reported elevated castasterone (CS) levels in leaves of drought-stressed pea plants. Similar evidence was obtained in the study by Gruszka et al. (2016) with barley; in which plant exposure to drought again increased CS levels accompanied by inverse changes in the levels of 28-homoCS. Drought also resulted in the presence of detectable amounts of EBL which were not present in non-stressed plants. In contrast to these two studies, Duan et al. (2017) did not report any significant changes in the CS or brassinolide (BL) contents in leaves of drought-stressed spinach. Janeczko et al. (2011a) also presented some data on the CS and BL contents in soybean subjected to water shortage; in addition to drought, they also treated their plants with EBL and observed reduced CS amounts and elevated BL amounts. Tang et al. (2017) showed that the situation can be even more complex: they demonstrated the intraspecific variability in drought-induced changes in BR contents in *Setaria italica* leaves. A drought-induced elevation of the total BR content was observed in a drought-resistant genotype, while the situation was reversed for the sensitive one. In contrast, Tůmová et al. (2018) reported that the drought-resistant genotype of maize was characterised by a reduction of 28-norBL and 28-homoCS contents, while the sensitive genotype displayed an increased amount of 28-norCS and a reduced amount of 28-homodolichosterone. The CS, BL or typhasterol levels did not change with drought exposure. Further interesting evidence for the influence of drought on the endogenous BR content was presented by Liu et al. (2016), who showed that the content of total BRs in roots of trifoliate orange did not change with water shortage in the presence of mycorrhizal fungi, but without fungal colonisation it decreased. Haider et al. (2017) reported a non-significant elevation of the total BR levels for drought-stressed grapevine. Finally, Kumar et al. (2018) reported a drought-induced elevation of the amounts of BR-precursor campesterol in rice seedlings. Thus, although the information on this topic is slowly accumulating, it is very contradictory and no definite conclusions can be drawn at this time.

2.4 Drought-Induced Changes in the Expression of Genes Involved in BR Biosynthesis or Signalling

Similarly heterogeneous information can be found on the expression of genes involved in BR biosynthesis or BR signalling under drought conditions. A short search for studies containing transcriptomic or proteomic analyses and focused on drought stress revealed several papers that directly mention such BR-associated genes. Most of these analyses were performed at the transcriptional level; only

Oliver et al. (2011) reported altered amounts of one of the proteins participating in the late phase of BR biosynthesis, caused by drought exposure in the grass *Sporobolus stapfianus*. Two papers mentioning drought-induced changes in the expression of the late BR-biosynthesis genes on the transcript level reported the downregulation of such genes (Rivero et al. 2010; Janiak et al. 2018). Regarding genes that participate in the early phase of BR biosynthesis, Rivero et al. (2010) mentioned a drought-induced elevation of *DIM/DWF1* expression in tobacco, whereas Le et al. (2012) observed reduced expression of this gene in soybean and Tang et al. (2017) reported no changes for *Setaria italica*. Other early BR-biosynthesis genes mostly displayed an increase in expression after drought (Peleg et al. 2011; Cartagena et al. 2015; Bai et al. 2017; Tang et al. 2017; Janiak et al. 2018). Some authors also described changes in the expression of genes that participate in BR signalling (*e.g.*, Rivero et al. 2010; Peleg et al. 2011; Le et al. 2012; Dash et al. 2014; Shamloo-Dashtpagerdi et al. 2015; Haider et al. 2017; Tang et al. 2017; Badhan et al. 2018; Janiak et al. 2018). In this case, the situation is even more complex, abounding with conflicting reports.

Regardless, the mention of BR-associated genes directly in the text of some scientific paper is rather rare, considering the overwhelming number of transcriptomic or proteomic analyses focusing on drought. I have no doubt that, should someone perform a meta-analysis of the data available in gene expression databases using appropriate bioinformatics approaches, quite a large number of studies would be found reporting drought-induced changes in the expression of BR-associated genes. This can be perhaps regarded as one of the challenges for future BR/drought researchers. However, we must also consider that most genes proposed in such studies to be related to BR signalling were identified only on the basis of their orthology with known *Arabidopsis* (or rice) BR-signalling genes. Insufficient information on the *true* components of BR signalling (or BR biosynthesis) pathways in diverse plant species is currently one of the major problems of the whole BR research.

2.5 Role of BRs in the Plant Drought Response – Analysis at the Gene Expression Level

Thus far, our information on the association between BR-signalling and drought-signalling pathways is mostly indirect, based on reports of common sets of genes/proteins that are known to be regulated by some component of the BR-signalling pathway (particularly the BES1 transcription factor) as well as components of stress signalling pathways (particularly RD26 and WRKY46/54/70 transcription factors). Other currently available evidence connects the signalling pathway of BRs with ABA. These two phytohormones are commonly considered to have an antagonistic relationship, although the evidence is not completely unequivocal. It seems that the crosstalk between BRs and ABA occurs as early as the formation of BRI1/BAK2 receptor complexes and continues through the BR-signalling pathway. An excellent

summary of these subjects was recently published by Nolan et al. (2017); the reader specifically interested in this topic is thus referred to this review paper and the references therein.

Direct evidence for the regulation of gene expression by exogenously applied BRs or in BR-deficient or BR-insensitive plants subjected to some type of drought simulation is still rather rare. Thus far, only seven studies conducted with such plants have assessed the changes in transcript abundance in such plants (Tables 5.1, 5.2, 5.3, 5.4, and 5.5) and only for a few selected genes (usually those known to participate in the plant stress response). The results were rather ambiguous – the changes in transcript levels depended not only on the respective gene but also, *e.g.*, on the length or intensity of the stress treatment (Kagale et al. 2007; Sahni et al. 2016) or the analysed genotype (Janeczko et al. 2016). A whole-genome transcriptomic analysis has not yet been performed; this is another challenge for future BR/drought researchers. Moreover, because the stress-induced changes in transcript levels are frequently not reflected by the changes in the level of proteins (which is evident from diverse studies simultaneously analysing the proteome and transcriptome in drought-stressed plants), examination of the regulation of the plant drought response by BRs at the gene expression level should also switch its focus from transcripts to proteins. Papers by Ghasempour et al. (1998) and El-Khallal and Nafie (2000), which claim to perform proteomic analyses in drought-stressed BR-treated plants, cannot truly be viewed as such because the authors neither identified the respective proteins nor precisely quantified their changes.

2.6 Role of BRs in the Plant Drought Response – Analyses at the Morphological, Physiological and Biochemical Levels

Although some authors have examined the effects of BRs only on yield or biomass production, or plant morphology, the major physiological and biochemical aspects of the plant drought response were analysed, at least to some extent, in most cases, although it is evident that the measurement of some parameters is strongly preferred over others (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). The majority of such characteristics have been determined in leaves (or in whole shoots of very young seedlings). This is, of course, understandable for parameters describing photosynthesis or stomatal characteristics; however, information on the BR effect on other plant organs under drought conditions is sorely missing. Only a few authors have analysed some parameters directly associated with the yield and product quality of fruits, seeds or roots; others have measured parameters associated with the general plant drought response in roots, flower petals or seeds of BR-treated drought-stressed plants. Even the studies that assess simple morphological parameters of roots do not comprise even 20% of the available literature (Tables 5.1, 5.2, 5.3, 5.4, and 5.5).

At this point, I could start to enumerate groups of diverse characteristics that have been assessed thus far in studies examining the BR/drought relationship and to verbally describe the changes induced by BR treatment or mutations in BR-associated genes. This has been the usual routine of many previously published reviews that have examined BRs and plant stress, without any regard for diverse factors that could affect the described results. Instead, I have summarised these data in Tables 5.1, 5.2, 5.3, 5.4, and 5.5, simultaneously presenting the main information on the respective experimental setups of these studies. When examining the data on parameters that were evaluated in a greater number of BR/drought studies, I realised that, viewed as a whole, the results are in most cases rather ambiguous. Quite regularly, some authors described a BR-caused increase in some parameter under drought conditions, whereas others observed a reduction of the same parameter and still others reported no changes at all. Even in the same study, the results frequently differed, *e.g.*, between the examined genotypes of the respective species or the particular variants of BR treatment or drought simulation. We must also consider that many authors did not present the results of a statistical evaluation of their data, making it impossible to determine whether the reported results and conclusions at least *could be* valid (indeed, in some cases, a statistical analysis was never even performed!). Additionally, I strongly suspect that the currently available information on the BR/drought relationship is rather biased simply because the results of experiments in which BRs displayed absolutely no effect on drought-stressed plants were frequently discarded. We can hope that this situation improves in the future.

However, at least one rather definite conclusion on the BR effect on plants stressed by a water shortage *can* be drawn from the available data. Thus far, it seems that BRs applied to drought-stressed plants diminish various signs of cell damage and reduce the production of reactive oxygen species. How BRs induce such effects is, nevertheless, a question that remains still unanswered. This phenomenon could be due to their participation in active reduction of the plant water deficit (*e.g.*, by the regulation of stomatal function and transpiration efficiency but also by improving the size of the root system or diminishing the plant leaf area). Such plants would ultimately experience a reduced degree of drought stress. However, a substantial number of studies did not describe any BR-associated changes in plant water status, or they observed a higher transpiration rate in BR-treated drought-stressed plants compared with non-treated ones. BRs also seem to actively improve the photosynthetic efficiency, which could be associated with their regulation of stomatal behaviour as well as the direct regulation of photosynthetic processes at some level (Holá, 2011). However, an enhancement, decline or no change in photosynthetic parameters has been reported in the available BR/drought studies. More detailed information on the stomatal properties and individual parts of the photosynthetic processes would certainly be welcomed.

BRs probably also regulate the content of osmoprotective compounds such as proline, soluble saccharides or other compatible solutes. This phenomenon has been frequently (but not always) observed in BR-treated drought-stressed plants but also in the non-stressed ones; thus, it does not have to be *specifically* associated with

drought response. Another possibility is the occurrence of a BR-induced boost of the cellular antioxidant system; however, changes observed for such parameters are even more variable, and all three types of responses to BRs again have been described for both stressed and non-stressed plants. Moreover, only some antioxidant enzymes have been assessed more frequently (Tables 5.1, 5.2, 5.3, 5.4, and 5.5); more information is needed on the response of diverse types of non-enzymatic antioxidants together with other protective compounds to BR excess or deficiency.

The topic of BR crosstalk with other phytohormones currently seems to be rather popular among authors of various reviews; unfortunately, the available information on the interaction between BRs and other phytohormones is mostly based on indirect evidence and is not particularly conclusive. Very few studies have dealt with changes in the contents of other phytohormones in drought-stressed BR-treated plants of BR mutants and again, they are mutually contradictory. Other aspects of plant cell biology that could be potentially related to the response to water stress (*e.g.*, the mitochondrial alternative oxidation pathway, degradation of proteins, cell ultrastructure, plant anatomy, *etc.*) have been examined very rarely (Tables 5.1, 5.2, 5.3, 5.4, and 5.5), and thus care must be taken in the interpretation of the respective results.

3 BR/Drought Studies from Various Methodological Viewpoints

It is not particularly surprising that it is impossible to observe truly common trends for most parameters evaluated thus far, given the overall variability of the examined species and, particularly, the experimental designs. The following sections will examine diverse aspects of this variability and will attempt to highlight several shortcomings that can encountered in the available BR/drought studies.

3.1 Types of Drought Simulation and General Conditions of Plant Cultivation

Studies examining the effects of exogenously applied BRs or changes in the genes associated with BR biosynthesis or signalling in drought-stressed plants can be roughly divided into three major and two minor categories based on the method applied for drought simulation (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). The first major group consists of studies by researchers who simply ceased to water their experimental plants at some time point and allowed the cultivation substrate to gradually dry out (Table 5.1). This approach is similar to the drought situations that actually occur in nature. The main disadvantage of this techinque is, of course, the difficulty of guaranteeing the same level of soil water content for all plants. However, if both

the soil and plant water status are completely monitored throughout the drought period to ensure good interpretation of the obtained results (Verslues et al. 2006), and if large numbers of plants are evaluated to obtain statistically robust samples, this problem can be overcome. Unfortunately, the first condition has very rarely been met in the available BR/drought studies; most authors simply state the length of their drought simulation period and do not concern themselves with more detailed specifications (Table 5.1). The second condition also cannot be always accommodated due to the space constrictions of plant cultivation facilities. Additionally, for some parameters, it would be extremely difficult to analyse a truly large number of samples because of various technical issues.

Another option is to maintain some stable (suboptimum) level of the soil water content either from the start of plant cultivation (*e.g.*, by reduced watering) or by cessation of watering for some time and then replenishing the water in small amounts to ensure that all plants will experience the same diminished soil moisture (Table 5.2). This certainly allows for more standardised drought conditions and, from this perspective, it could be preferable to the approach mentioned in the previous paragraph. However, continuous replenishment of water "as necessary" means that such plants constantly undergo stress-recovery cycles, resulting in a very different physiological response. Similar situations do of course occur in nature, but this type of drought simulation tells an entirely different story from the first scenario and should be viewed in this context.

A minority of BR/drought studies has been performed with field-grown plants subjected to natural rainfall (in some place where it does not occur very frequently) and compared with artificially watered plants (Table 5.3). This approach is similar to restricted watering because such plants also usually receive some amount of water during the drought-simulating period, but the environmental variability is of course much greater. Such field experiments should be repeated during several seasons; to draw sound conclusions from only 1-year field experiments is inappropriate, and even 2 years are often not sufficient. Unfortunately, this has almost always been the case for studies examining BR/drought relationship in plants grown in field (or other outdoor) conditions (Tables 5.1, 5.2, and 5.3).

The third major category of studies simulated drought using polyethyleneglycol (PEG) or some other osmolyte (Table 5.4). In my opinion, the data obtained from such experiments cannot truly reflect what is happening in drought-stressed plants in nature. The commonly used application of PEG induces water (osmotic) stress very rapidly ("shock treatment"), thus evading the natural course of the plant drought response with its gradual changes and opportunity for plants to acclimate to such conditions. Genes that are activated by the exposure of plants to abrupt water stress are very different from these activated by the gradual imposition of a water deficit (Ambrosone et al. 2011, 2017). Additionally, the root system of plants (the development of lateral roots, the establishment of exodermis/endodermis) strongly depends on the cultivation medium (Redjala et al. 2011). The hydroponically grown plants that are usually utilised for PEG experiments (or plants grown on agar, which is the second type that can be encountered in these studies) thus have a very different root system compared with plants grown naturally in soil.

I should also mention several rather uncommon methods utilised by some authors to simulate drought conditions: these include air drying of plants/leaf segments, reduced relative air humidity in testing vessels or the total removal of water from pots with otherwise hydroponically grown plants. I have included these studies in Table 5.5; however, their informative value is, in my opinion, rather doubtful.

Although the cultivation of plants directly in the field has not been a particularly popular approach among scientists examining the effects of BRs on the plant drought response and has various disadvantages, it certainly corresponds best to real situations. Some authors have attempted to combine outdoor cultivation with more controlled conditions using various net-houses, wire-houses, cage-houses or rainout shelters and growing their plants in pots. However, most of the work was performed with pot-grown plants placed either in greenhouses or growth chambers (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). Each type of growing facility has its pros and cons (Poorter et al. 2012b). However, although many authors did not present the necessary information regarding the *precise* cultivation conditions used for their plants (particularly the relative air humidity for greenhouses or growth chambers, which should be imperative for drought-focused experiments!), it is evident from those that documented these parameters that the cultivation conditions often inadvertently included some other unfavourable environmental factor, such as, *e.g.*, low irradiation (frequently encountered in growth chamber experiments) or a nutrient shortage (particularly in long-term experiments without any fertilisation). An inadequate size of cultivation containers can be an additional and very important issue; if mentioned at all, it sometimes seems to be rather small for the final size and/or number of cultivated plants (Poorter et al. 2012a). Thus, even the "control", non-stressed plants could in fact be stressed by water shortage. Indeed, it is surprising how frequently we are presented with results obtained from plants under "non-stress" conditions that display relative water content (RWC) of their leaves in the 70–80% range and sometimes even lower (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). These values indicate the occurrence of at least mild or moderate water stress (Flexas and Medrano, 2016) and should be considered as a sign of something wrong with the plant cultivation and the whole experimental setup.

3.2 Drought Intensity and/or Length

The absence of any data on the actual drought intensity ascertained *both* by evaluation of the soil water content (or soil field capacity) and, even more importantly, the determination of the plant water status is a common deficiency of many BR/drought papers. The authors of studies simulating drought by cessation of watering or field studies usually state only the length of the drought period, but they do not present any moisture data on the cultivation substrate; the situation is, of course, better for the other two major categories of BR/drought studies (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). However, and much worse in my opinion, more than 60% papers on BR/drought do not present any information on the actual plant water status, not even

simple measurements of the leaf RWC (Tables 5.1, 5.2, 5.3, 5.4, and 5.5)! Thus, any interpretation of the obtained results in the context of drought intensity is, of course, very difficult because we do not know the extent of stress (if at all) that the experimental plants *truly* experienced due to the water shortage.

Based on the information that *is* available, studies simulating rather severe drought stress probably prevail over these that staged mild or moderate stress conditions. Several authors purposefully examined the effects of two different stress intensities during their evaluation of the possible role of BRs in the plant drought response (Tables 5.2 and 5.4). Curiously, although some of these studies reported a more marked effect of BR treatment on plants subjected to a greater drought intensity (*e.g.*, Sairam et al. 1996; Talaat et al. 2015; Chen et al. 2018), a dependence of BR-induced changes on the degree of water stress experienced by the respective plants is not obvious from most of these papers.

Such analyses should not be confused with another type that also deals, to some extent, with different drought intensities: examination of the effects of BRs at 2–3 different times (or, very rarely, at more time points). Such papers are more frequent than the type mentioned in the previous paragraph. However, the difference between time points is usually only 1 or 2 days, which does not allow for very different drought intensities. In case a longer time course was followed and the plant water status truly differed between several time points, it again did not seem to have a marked effect (*e.g.*, Singh et al. 1993; Anjum et al. 2011; Xiong et al. 2016). Interpretation of the results obtained from long-term experiments can, of course, be complicated by the advancing development of plants; this will be discussed in a subsequent section of this chapter.

Still another aspect of BR/drought studies that is loosely related to the intensity of the water shortage concerns the comparison of drought-sensitive and -resistant genotypes, which could differ in the degree of drought experienced. Of course, this would depend on the particular mechanism of their resistance to water scarcity (*i.e.*, drought avoidance, tolerance or escape, Fang and Xiong, 2015). Unfortunately, such genotype-comparing studies are not frequent and, with some exceptions, have usually been performed with plants subjected to PEG treatment, *i.e.*, under very unnatural conditions (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). Moreover, the exact causes of drought resistance displayed by the respective genotypes (and the conditions under which it was determined, which could be very different from the experimental conditions of the studies on the BR/drought relationship) were never stated. Data on the plant water status were also usually missing and when presented, the respective drought-resistant and -sensitive genotypes did not greatly differ in their leaf RWC under drought conditions, which would signify that drought avoidance was not the situation herein.

A surprisingly few authors analysed the role of BRs in the plant response not only to the period of drought simulation, but also after its end. Each natural exposure of plants to drought (particularly in moderate climate zones) is sooner or later followed by normal rainfall, during which the plants should be able to recover from the drought stress. This recovery ability is equally important for plant life as the ability to withstand a water shortage *per se*. However, it can be based on entirely

different mechanisms then those that participate in the plant drought response. BRs could certainly play a role in this process and some authors have indeed reported a mostly positive effect of these phytohormones on diverse parameters measured in plants recovering from drought stress. However, Xu et al. (1994a) observed no marked effect and Gomes et al. (2013) reported even a negative effect of exogenously applied BRs on plants rewatered after a period of an insufficient water supply. Regarding BR mutants or transgenics, the results are ambiguous concerning whether lower or higher amounts of BRs are more advantageous for the ability of plants to recover from water shortage (Feng et al. 2015; Sahni et al. 2016; Han et al. 2017).

3.3 Types of Control Plants

All experimental setups for studies analysing the BR/drought relationship should rightly contain two types of control plants. The first one would be represented by plants that are not treated with BRs (or, in case of BR mutants, the respective *wt* plants), whereas the second one should consist of non-stressed plants undergoing the same type of BR treatment(s) as the stressed ones. The first type of control is a matter of course in all available BR/drought studies, although it is not always entirely clear whether the authors simply did not subject their control plants to any treatment at all or whether they treated them with precisely the same solutions as those containing BRs but without any steroid. The second option is, of course, the correct one; using the first could bias the results because BRs must first be dissolved in some alcohol and even traces of such solvents in the treatment solutions can affect the values of diverse plant parameters and thus distort the correct interpretation of the obtained data. This phenomenon has been pointed out in a previous book on BRs (Janeczko, 2011).

However, it is the second type of control that is missing from almost one third of the papers focusing on the role of BRs in the plant drought response (Tables 5.1, 5.2, 5.3, 5.4, and 5.5; always presuming that in the remainder of these studies the control consisted of *truly* non-stressed plants). Its absence could lead to another possibility of incorrect conclusions. In the case that BRs truly act as *specific* regulators of the plant drought response, we should expect different trends in stressed and non-stressed BR-treated plants, *i.e.*, the BR effect should be evident or at least more pronounced in the stressed ones. The exclusion of non-stressed plants from the experiments does not allow the differentiation of the drought-specific action of these phytohormones from their more general role in plants.

In fact, based on the available data, the changes observed in BR-treated drought-stressed plants frequently seem to be very similar to the changes caused by BR-treatment alone (*i.e.*, in non-stressed plants). Of course, there are exceptions, but even so, a significantly greater number of the available BR/drought studies that contained such a control reported a very similar BR effect on stressed and non-stressed plants (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). Naturally, the picture is not

perfectly clear and the observed effects can depend on a particular parameter. The differences between BR effects on stressed and non-stressed plants are usually more evident for parameters informing us about the cell damage than for those characterising photosynthesis, plant water management, antioxidative or osmoprotective processes, and they are even less obvious for plant morphology or yield. This result is not surprising because the non-stressed plants should not experience any cell damage, and thus the effect of any compound applied to plants on the respective parameters must be only minimal.

A relatively small (but not inconsiderable) group of authors utilised as their non-stressed control the measurements performed at the start of the drought simulation period (*i.e.*, at time zero). This strategy applies mostly to studies simulating drought by cessation of watering or by the application of some osmolyte (Tables 5.1 and 5.4). Fortunately, the duration of the stress period was rather short in most of these papers and the development of plants thus should not be an additional factor further confusing interpretation of the results. However, in some cases, the length of time between measurements at time zero and at the end of the drought simulation was such that any eventual comparison of stressed and non-stressed plants had to be influenced by the advancing plant development.

This brings me to an additional aspect that should be considered when designing the appropriate controls for BR/drought studies. Drought-stressed plants slow or even stop their development, while non-stressed plants do not. Thus, when the measurements are performed at the same time points (which is, of course, very convenient), the plants cultivated under optimum conditions will probably be in a more advanced developmental stage than these subjected to water shortage. Naturally, the comparison of two such groups of plants is not precisely correct; stressed plants should always be compared with a *developmentally corresponding* control. Although the drought simulation period in the studies utilising PEG was so short that this probably did not have a marked impact (Table 5.4), this certainly does not apply to the other types of BR/drought studies. Evidently, this factor should be considered when interpreting the results of these studies, particularly in drought simulations that take a long time. Only one group of Polish authors (Gruszka et al. 2016, 2018; Janeczko et al. 2016) did in fact take care to use *proper* control plants of the same developmental stage as their drought-stressed ones.

3.4 Plant Development

Under natural conditions, drought can occur any time and thus can affect plants in various developmental stages. From a purely agronomical viewpoint, scarcity of water in the later phases of plant development (particularly during reproduction) is considered to be the most important factor; however, earlier drought also has a considerable impact on plant growth and biomass production. Such periods of water shortage affecting young plants will undoubtedly occur more frequently with the current changes in the global climate. For an ordinary scientist working in BR/

drought research this at least ensures that any analysis performed with young plants could have potential applications in agricultural practice (with some reservations about the PEG studies in which the measurements are usually performed with very young seedlings, Table 5.4). Such studies probably comprise the majority of all papers published on this topic. Unfortunately, less than 50% of the respective studies contain information on the precise developmental stage of the experimental plants: only the age of the plants (and sometimes not even this parameter) is usually mentioned (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). This is another shortcoming of the available BR/drought papers that could be easily remedied. Photographs of the experimental plants at several time points of the experiments (*e.g.*, at the start of drought, at the time of BR treatment, at the time of the measurements) could provide even more precise information on the state of the experimental plants.

In addition to work performed with young plants, some authors have also analysed BR action in plants subjected to water shortage during flowering (or immediately before) or even in later reproductive stages (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). However, there does not seem to be any specific trend regarding the effects of BRs on various parameters that would single out these studies from the rest. At this time, no study has purposefully investigated the potential differences between BR effects on plants subjected to drought in different developmental stages. Two exceptions can seemingly be found. Kumawat et al. (1997) stated that they applied water stress to mustard plants at the stages of preflowering, pod formation or at both developmental stages, but they did not then differentiate between these groups of plants when analysing their data on BR effects. Alyemeni and Al-Quwaiz (2014) subjected mungbean plants to PEG-induced osmotic stress at the age of 7 or 14 days but the stress intensity was different in each case (−0.6 MPa and −1.2 MPa), which of course makes any comparison impossible. Thus, we have no actual information on this topic that could be utilised for further theoretical or practical purposes.

Another aspect of plant development that is poorly understood in the context of the role of BRs in plant drought response is the developmental stage of individual organs utilised for the measurements. As already stated, most physiological and biochemical parameters were assessed in leaves. Unfortunately, it is often impossible to determine from the description of the experiments whether the respective leaves were already developed or still developing at the start of the drought period. This could in fact be an important factor affecting the role of BRs in the plant molecular/biochemical response to water shortage, because leaves that are still growing respond to drought in a very different manner compared with mature ones (Skirycz and Inzé, 2010). Again, we lack almost any information on the relationship among BRs, drought and the developmental stage of leaves. I attempted to do my best with my collection of BR/drought papers and estimate that approximately one third of the authors performed measurements in leaves that were not yet mature (or even visible) at the start of the respective drought simulation period. However, a large number of this information was derived from studies conducted with plants subjected to stress simulation from the start of the cultivation period, *i.e.*, as seeds. Only Gomes et al. (2013) purposefully determined the effects of BRs on the chlorophyll content in papaya leaves that were, at the start of the drought period, either

still developing or already mature. They observed a BR-induced reduction of chlorophyll levels in older leaves but no effect on younger ones.

In addition to the different drought response mechanisms in leaves that are just starting to develop compared with leaves that are partially or completely developed at the start of the drought period, BR treatment *per se* probably affects young, mature and senescing leaves in different ways. Some evidence for this hypothesis has already been obtained (Kočová et al. 2010; Janeczko et al. 2011b; Rothová et al. 2014). The time lapse between drought/BR treatment and measurement of the respective parameters must also be considered. This period has frequently been rather long, and the completely developed leaves could start to senesce (regardless of any plant drought exposure). BRs can modulate the process of senescence (Sağlam-Çağ 2007; Fedina et al. 2017), which could further distort conclusions regarding the relationship between BRs and the plant drought response based on such results.

3.5 Timing and Mode of BR Application

The application of BRs either prior to or simultaneously with the start of stress simulation rather strongly prevails (approximately two thirds of the relevant papers; analyses performed with BR mutants or transgenics cannot rightly be considered here). This is particularly evident for the studies that simulated drought by cessation of watering and the PEG-utilising studies (Tables 5.1 and 5.4). The application of BRs before the water shortage starts to act on the analysed plants could led to a similar issue to that mentioned in the section dealing with diverse types of control plants. The observed changes associated with BR treatments could probably have a more general character that is not drought-specific, because they would be mostly induced in plants not yet experiencing drought. The necessity of comparing the drought-stressed plants with the appropriate non-stressed control is even more essential with such timings of BR treatments. As practical indicators of whether BR application can improve plant drought resistance in plants that *potentially* encounter a water shortage, such studies are of course perfectly valid. However, to learn more about the mechanisms by which BRs *specifically* regulate the plant drought response, the plants should first be exposed to drought and then be treated with BRs only after exhibiting symptoms of mild/moderate/severe water stress (depending on the experimental purpose). This procedure has been frequently applied in plants subjected to drought simulated by restricted but continuous watering (Table 5.2); however, as already stated, these studies should be considered as continuous stress-recovery experiments.

The mode of the BR application could be another factor possibly affecting the action of BRs in drought-stressed plants. In the first two major categories of BR/drought experiments (drought simulation by cessation of watering or restricted watering), which mostly utilised longer time courses, leaf (or whole shoot) spraying was the usual method of choice. However, the scientists who performed short-term

analyses with osmolytes usually either soaked the seeds in BR solutions or directly added these phytohormones to the cultivation medium. Only a few papers directly compared seed soaking and leaf spraying with regard to their potentially different impacts (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). Xiong et al. (2016) also added BRs directly into the soil and tested various combinations of these three application modes. For most yield, morphological, physiological or biochemical parameters thus evaluated, the results were usually very similar. Only Farooq et al. (2009), who performed such a comparison in rice, reported that leaf spraying displayed a slightly more pronounced effect on drought-stressed plants than soaking of seeds in BR solutions. This result is understandable because the interval between BR application and the time of measurements was much shorter for the spraying treatment compared with the seed soaking. In cases of other long-term experiments that used seed soaking as a mode of BR application (Tables 5.1 and 5.2), the effect of BRs on drought-stressed plants was usually rather insignificant.

Of course, this could be caused by still another factor, *i.e.*, the already mentioned limitations of BR transport from the site of their application. It was rather interesting to examine the results presented by scientists who utilised leaf spraying as a mode of BR treatment but measured diverse biochemical parameters in other plant organs (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). As expected, no significant impact of this type of treatment on characteristics measured in organs distant from the site of BR application was observed in most of these studies. Some (very rare) exceptions for parameters assessed in roots could perhaps be explained by inadvertent contamination of the cultivation medium during spraying. However, Upreti and Murti (2004) observed an increased activity of nitrogenase in nodulated roots of French bean after spraying their plants with BRs, which was accompanied by an elevation of cytokinin amounts. Any eventual effects of exogenously applied BRs on parameters measured in organs other than those to which they were applied could thus be also explained by BR-induced changes in the amounts of other phytohormones (or other signalling molecules) and the movement of these long-distance signals through the plant body.

Some of the spraying treatments were performed repeatedly (with various lengths of the intervals between individual applications), whereas other authors sprayed their plants only once (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). This difference did not seem to affect the final impact of BRs on the respective drought-stressed plants. Similarly, the length of seed immersion in BR solutions could be as short as 1 h or as long as 2 days, but also in no way influenced the results (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). Unfortunately, most authors did not state whether the spraying treatment was performed on the adaxial or abaxial side, or both sides, of the leaves (this could affect BR penetration) and whether it was implemented for the whole plant foliage visible at the time of BR application or only to the leaf that was later used for the respective measurements. Information on the precise amount of BR solutions used for spraying treatments is also almost always missing.

In some cases, other modes of BR application were utilised, mostly in some tree seedlings: BRs were injected directly into plant stem (Rajasekaran and Blake 1999) or plant roots were soaked in BR solutions before the seedlings were re-planted (but

this was then followed by leaf spraying; Li et al. 2008, Li and Feng 2011). One group of authors even soaked the base of cut maize seedlings in BR solutions before they subjected them to PEG-induced stress (Zhang et al. 2011).

3.6 BR Type and Concentration

BL, EBL and HBL are commonly accepted as the most biologically active BRs. Among these, EBL has been by far the most popular one in studies with BR-treated drought-stressed plants (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). Several authors compared the effects of EBL and HBL during the same experiment, although mostly in PEG-stressed plants. In most cases, no particular differences between these two types of BRs were observed for most of the assessed parameters. However, some authors reported that EBL acted in a slightly more pronounced manner than HBL (Upreti and Murti, 2004; Farooq et al. 2009). Several synthetic analogues of BRs were also sometimes utilised (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). Curiously, CS (considered to be an end-product of the BR biosynthetic pathway in monocots; Kim et al. 2008) was never applied to any of the analysed monocot plants; perhaps this could inspire future researchers to include this BR in their experiments as well as EBL, BL or HBL.

Greater variability can be encountered with regard to the concentrations of BR solutions applied to the experimental plants (from 0.1 mM to 10 pM); however, solutions in the 1 μM to 1 nM range are in general the most utilised ones. The authors who simulated drought by the application of some osmolyte commonly tended to work with higher concentrations of BR solutions compared with the others (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). This difference could perhaps be related to the fact that they mostly utilised seed soaking as the main mode of BR treatment, and it is possible that higher BR concentrations would be necessary to allow better penetration of these phytohormones into seeds than in case of leaf spraying. Unfortunately, the reasons for the selection of the respective BR concentrations are almost always unexplained. The most we can usually learn (and even then only rarely) is that the authors made their choice based on *some* previous, usually unpublished, experiments. We do not know whether such experiments were performed with stressed or non-stressed plants, the parameter(s) of plant morphology, physiology, biochemistry, *etc.*, on which they based their decision, or even whether the experiments were conducted with the same plant species! It is more than likely that what has been identified as "the best" BR concentration under one set of conditions does not have to apply to another.

However, almost one half of the authors working with plants treated with exogenous BRs tested more than one concentration of the respective BR solution directly in the respective studies. This has been popular particularly with short-term PEG studies, but the BR solutions typically differed either within one concentration order or at best only between two consecutive orders; wider ranges of BR concentrations were evaluated rarely (Tables 5.1, 5.2, 5.3, 5.4, and 5.5). Based on some of the

presented results, it seems that higher BR concentrations in the μM to nM range have a more positive impact on drought-stressed plants. However, other authors who tested plants exposed to exogenous BRs applied within this same range, reported the reverse situation or did not observe any effect of the BR concentration on plant performance under drought conditions. Thus, similarly to other experimental aspects of BR/drought studies, the results are extremely variable and cannot serve as a basis for any definite conclusions.

4 Conclusions and Future Challenges

Considering the seriousness of the problems caused by drought for the global environment, agriculture, economics, politics and a human society as a whole, and because the application of BRs has been suggested to be an economically possible option for alleviation of the negative effects of drought in plants, we certainly need valid information on the precise mechanisms of action of these phytohormones under such conditions. However, a thorough examination of diverse studies dealing with the BR/drought relationship we have at our disposal led me to the conclusion that our current knowledge is at best limited only to the statement that treatment with BRs can (usually) mitigate the negative effects of this stress factor. In my opinion, we are still far from truly answering the question of *how* BRs reduce the negative effects of drought in stressed plants. It is unfortunate that the overwhelming majority of BR/drought studies published to date evaluated only a relatively small number of parameters. A truly complex study that would *simultaneously* assess diverse aspects of plant morphology, water management, photosynthesis, cell damage, various cell protective systems, phytohormones, cell wall properties, plant anatomy, at least some level of gene expression, *etc.*, will probably remain only wishful thinking for some time. To obtain a fully comprehensive picture of the BR role in plant protection against drought stress, we must routinely expand the list of evaluated parameters and focus more on such aspects of the plant drought response that have thus far been only lightly touched upon. Here, is a list of topics on which I think particular attention should be focused:

- Changes in the levels of endogenous BRs caused by water shortage (and/or diverse types of exogenous BR treatments). Analyses of the contents of individual BRs would be preferable over mere determination of the total BR content because they could help us to precisely ascertain at which stage of BR biosynthesis drought imposes the greatest effect. Such analyses could be accompanied by an evaluation of the expression of various BR-biosynthetic genes (and *vice versa*).
- Bioinformatics methods could be used for a meta-analysis of data from various gene expression databases, focusing on drought-stressed plants and BR-associated genes. This strategy would, of course, require a valid identification of these genes in non-model plant species, which is a problem that is pertinent to all current BR research.

- Better knowledge of BR metabolism and inactivation under both stress and non-stress conditions is sorely needed.
- The role of BRs in root development and functioning under drought conditions should be more focused on, because the root system is a major factor affecting plant behaviour during water shortage.
- BR-related changes in shoot anatomy are another subject that has been left almost untouched and that could also play an important role in the plant response to an insufficient water supply.
- Interactions among BRs and other phytohormones during the plant drought response: more direct evidence is needed to identify various relationships at the levels of phytohormone biosynthesis, metabolism, transport and signalling, among others.
- More thorough and frequently performed analyses of BR effects on the components of the plant cell protective system other than proline and the major antioxidant enzymes. Metabolomic analyses could be of a great help in this capacity.
- Whole-genome assessment of the role of BRs in the regulation of gene expression in drought-stressed plants, performed not only at the transcriptome but also the proteome levels. The possibility of BR-associated drought-induced changes in the regulation of gene expression by various modifications of chromatin structure should not be overlooked.
- The subject of the possible BR role(s) in plants/organs exposed to water shortage in different developmental stages deserves our attention and should be examined from diverse viewpoints.
- The evaluation of genotypic differences in the plant drought response with regard to the possible role of BRs in different mechanisms of plant drought resistance thus far also persists on the side-lines of BR/drought research.

Finally, I want to appeal to all scientists working in the field of the BR/drought relationship, either as primary researchers and authors of potential new papers or as reviewers or editors for academic journals: please, do not throw away "negative" results of your experiments and always provide (or demand) a *very thorough* description of all aspects of the experimental design for the respective studies. Without such information, the results of the experiments can be very easily interpreted incorrectly and the task of obtaining truly meaningful information on this topic is rendered almost impossible.

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Chapter 6
Brassinosteroids and Senescence

Serap Sağlam Çağ

Abstract Leaf senescence is a genetically controlled process which can cause nutrients to transport through the newly developed young parts from old organs. Senescence process is effected by developmental and environmental signals and ultimately it is reprogrammed metabolically. It has been known that senescence process was effected by plant hormones. The senescence includes changes of their photosynthetic apparatus. Yellowing of cotyledones and leaves is clear that chlorophyll breakdown has served as the primary parameter for the measurement of senescence. It has been known that ethylene, ABA and brassinosteroids promote senescence but auxins, cytokinins and gibberellins are retardants of senescence. However, the correlation between hormones is very effective in the senescence process. The part of investigations on senescence has been included external application of a substance before the onset of senescence are in plants. The findings of these applications are still being discussed. In this chapter, the effect of brassinosteroids on senescence is discussed.

Keywords Brassinosteroids · Senescence · Cotyledon · Plant Hormones · Auxin

1 Introduction

The active lives of plants begin with germination as a result of the process of taking up water of the seeds. The first steps of plants' life activities are division and breeding. Following this process, the plants develop by differentiation and eventually die due to reactions that cause morphological changes.

The plants have a genetically organized life cycle. According to this cycle, after completing the period of growth that we have defined as the vegetative phase, the plants bloom by passing the reproductive phase and then the process ends up with a

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dramatic death. Plant species lose some cells, tissues and organs while exhibiting a developmental process that is unique to them.

Leaf is the major organ of photosynthesis. Leaf development is a process that is affected by endogen signals and external factors, besides being genetically regulated (Van Lijsebettens and Clarke 1998). The leaf produces nutrients by photosynthesis until the end of maturation period. After this production phase, the existing compounds in leaves are transported to the young organs and tissues that will continue their lives to reuse. So, the leaf that suffers from nutrient loss dies (Hörtensteiner and Feller 2002; Buchanan-Wollaston et al. 2003a). Briefly, it is called "senescence" in this development process some cells, tissues, organs, even whole organisms, die appropriately for the purpose. Because senescence is a programmed process, it occurs without being dependent on the age of the tissue. However, most often, senescence is seen in the elderly organs of perennial plants. Senescence do not usually appear in meristematic tissues, but are observed in differentiated tissues and cells. During tissue development, some cells die due to senescence.

Enzymatic and biochemical changes take place in the cells of the part where the senescence occurs. Most of these changes include catabolic reactions. For example, pigment changes are a biochemical change which occurs during the senescence process. Xanthophylls and carotenoids are appeared by chlorophyll breakdown. Then, the proteins are then gradually broken down and converted into amino acids. DNA and RNA are broken down. New crops resulting from demolition move towards the regions where the plant's growth activation is to be used in the next season or for future generations. The transport of these nutrients to the newly-emerging flower and fruit in the plant leads to lack of nutrients in vegetative organs. Therefore, it has been known for many years that generative organ formation causes the death of the plant (Molisch 1928).

The process of senescence is also regulated by hormones. Brassinosteroids with a steroidal structure play an important role in the mechanisms of biochemical events occurring during the senescence process (Clouse and Sasse 1998; Khripach et al. 2000; He et al. 2001; Rao et al. 2002; Srivastava 2002; Nemhauser and Chory 2004).

It has been reported that brassinosteroids promote senescence in the cutted cotyledon of cucumber seedlings (Zhao et al. 1990); eBL is also caused by senescence in the leaves of bean seedlings (He et al. 1996) and cutted leaves of *Arabidopsis* plant (He et al. 2001); *Xanthium* and *Rumex* explants were found to accelerate senescence (Mandava et al. 1981).

Senescence are delayed in most of the BR mutants, and life is prolonged. BR mutants remain green even after 100 days, even new flowers can be formed, while the start of senescence of wild-type *Arabidopsis* corresponds to about 60 days later (Choe et al. 1999). In addition to these findings, chloroplast senescence was also delayed in BR-deficient *Arabidopsis* mutants (Li et al. 1996). Sağlam-Çağ (2007) also found that 24-eBL application at high concentration (10 μM) in wheat leaf segments accelerated senescence. Despite all these findings, Srivastava (2002) reported that there is a relationship between the delay of the senescence and the BRs.

The molecular mechanism of BR's effect on senescence is still unclear. Any new information on genetic and biochemical studies on BRs will certainly help to establish mechanisms for future challenges in the agricultural field.

2 Significance of Senescence

People, even in prehistoric times, have given great importance to agriculture. The public has benefited from the wild plants growing in the natural environment and has made efforts to cultivate these plants specially. After many years of research, scientists have revealed that what controls life span is actually one of the basic biological questions. Plants have life-forms that are quite different in life time (Thomas 2003). Annual and biennial plants complete life cycles in a year or 2 years, while some clonal plants can live more than 10,000 years. The life span of the plant is genetically controlled. Senescence is actually a programmed cell death. It has been found that there is a link between the beginning of leaf senescence and whole plant senescence and the generative period with the genomes of monocarpic plants.

Although all plant senescence seen in *Arabidopsis* is controlled by generative organs, there is a weak correlation between the formation of generative organs and the beginning of leaf senescence (Noodén and Penney 2001). For further information about definition of senescence, it is a degenerative process that occurs at a certain time even under favorable growth conditions, which is genetically controlled and affected by environmental factors. However, it is also possible to delay the senescence. The plants grown in natural environment are exposed some times to environmental stress conditions that may adversely affect growth, metabolism and developmental effects in some periods. The number and quality of seeds, fruit maturation are important in agriculture because they are effected by senescence process.

When examined from this perspective, it is possible to keep the plant in vegetative period by delaying the senescence which will occur early by being affected by environmental factors and thus to prolong the life of the plant and increase the number of products. This information is very important in terms of agriculture and cultivation.

Except the natural process, unsuitable climate conditions (abiotic stress) cause premature senescence in plant, resulting in an average reduction of 50% in plant productiveness. Although senescence that occurs in the whole plant is disadvantageous in terms of agriculture, senescence which occurs in organs and tissues creates an advantageous situation for plant development. During leaf senescence, the senescence-related genes are described (Buchanan-Wollaston et al. 2003b; Zhang et al. 2018).

The transpiration slows down in the trees which shed leaves in autumn. This is an advanced form adaptation in which the plant gets advantageous to survive winter. Leaf fall provides the added soil of the food sources and fragmentation products necessary for the growth of plants. In addition, during the senescence, nutrients are transported from elderly organs to young organs and this gives an advantage in terms of developing new tissues and organs.

When examined cellularity, loss of chlorophyll and damage to cellular structures in the senescing tissue are the consequences of cell death. During the formation of vascular tissues in plant, senescence has great importance at the cell level. If global climate changes and changing environmental conditions are taken into account, the growth of the products by changing the senescence programs of plants, the cultiva-

tion and development of plants that can be better adapted to their environment, will contribute to agricultural products in the future.

3 Senescence Regulation

The first observations on the senescence were made by Hildebrand (1882) and Molisch (1928) in the second half of the nineteenth century and the first half of the twentieth century. Senescence is a biochemical process that ends with death, genetically programmed in the life process of plants.

Senescence is an extremely important process that gives an advantage to plants in the plant life process. There are four types of senescence: whole senescence, shoot senescence, simultaneous or synchronous senescence and sequential leaf senescence. Senescence plays an important role in the formation of certain tissues of the seedlings (e.g. xylogenesis) that enter into the growing process, beginning starting from seed germination.

Annual or perennial plants also undergo senescence during the period when their development shows generative activity. These phases affect the life of the plant in a positive way and facilitate. It is known that senescence, which occurs in the vegetative period of the plant, causes physiological, anatomical and morphological changes (Cutter 1979; Mencuccini and Munné-Bosch 2017). These changes have an important role in plant development. Senescence syndrome is not a process that occurs alone. The initiation of the catabolic reactions that occur during the senescence process takes place within a certain program in the cells. Correlation between the systems involved in this program can be achieved by intercellular communication (signalling). During senescence, many changes occur at the level of cells, organs and organisms.

There is a decrease in the volume of a tissue in which senescence has occurred. The earliest structural change during the senescence is the loss of membrane-selective permeability due to molecular breakdown. Cell membranes are a component required for cell integrity and provide signal transduction through phosphatidylinositol derivatives from membrane lipids; they play a crucial role in cell destruction. During the senescence, which occurs naturally in time or in environmental stress in the early stages, the membrane leakage increases and the permeability property is impaired. In this process, membrane lipids undergo molecular changes due to de-esterification, and as a result the membrane leakage begins (Troncoso-Ponce et al. 2013).

Pectinase, one of the peripheric enzymes of cell wall, helps to deteriorate the wall structure, supports the loosening of the wall and softens the tissue by breaking down the cell wall. Cellular membranes do not deteriorate simultaneously during senescence. During degradation, the macromolecules are catabolized and, through the production of energy, the products of catabolism are re-released into the growing parts of the plant, where they are metabolized. These changes do not occur at the same time in all cells, but are in accord with the timing of senescence (Matile 1992).

The toxicity of reactive oxygen species is determined by various enzymatic and non-enzymatic protective antioxidant defences. These antioxidant enzymes are primary antioxidant enzymes of superoxide dismutase (SOD), catalase, peroxidase (POD) and ascorbate-glutathione cycle enzymes. Oxidative stress increases during plant senescence, whereas antioxidant protection decreases (Buchanan-Wollaston et al. 2003a; Zimmermann and Zentgraf 2005).

Chloroplasts are the probable main target of increased oxidative stress during senescence (Munné-Bosch and Alegre 2002). Therefore, the balance between the development of antioxidant systems against increased reactive oxygen species during the regulation of leaf senescence is very important.

Some complex macromolecules cause changes in the appearance of plant organs after they are broken down. The first visual indicator of the senescence observed in leaf is the colour change that occurs with the decrease in the amount of chlorophyll. Because, as a result of the breakdown of the chlorophyll molecule giving the green colour of the chloroplast, tissue loses its characteristic green colour. Due to the fact that the contents of the chloroplast in tissue is higher, other pigments are masked. When chlorophyll is broken down in the senescing leaves has occurred, the yellow pigments becomes visible and yellow colour formation is observed. Yellowing begins from the leaf veins and continues outward. If the speed of photosynthesis falls below a certain initial level, this causes the senescence. It is predicted that the photosynthetic fall acts as a signal. The focus is on the possibility that the concentration of sugar, the major product of photosynthesis, may be the basis for signal deliver. The significant researches have been done in this subject. As a matter of fact, one of the changes is that starch which constitutes the content of certain tissues is transported as a result of hydrolysis, by turning into sugar. Changes in the gene expression occur during senescence. Transcriptome of *Arabidopsis* leaf cells in which senescence has occurred contains 2491 unique genes (Guo et al. 2004).

It is emphasized that the eukaryotic translation initiation factor, 5a (EIF5A) isoform, may be an important step in controlling the onset of senescence (Wang et al. 2001, 2003; Thompson et al. 2004). In transgenetic plants, leaf senescence is inhibited, by being suppressed activation of EIF5A. Fruit has also gained importance with the delay of the senescence process in this way, in terms of agriculture. This clearly shows the effects of EIF5A on senescence (Wang et al. 2003, 2005).

As the analytical methods develop, we will increase our knowledge about the senescence and the relationship with brassinosteroids.

4 The Mechanism of Regulation of Senescence by Brassinosteroids

Senescence is the last stage of plant development. However, senescence has occurred in a programmed manner at the cellular, tissue and organ grade, during the development of plants. Xylogenesis is also programmed cell death at the cellular and tissue

grade. It is known that IAA plays a role in this process (Altman and Wareing 1975; Even-Chen et al. 1978; Cutter 1979). Similarly, BRs promote xylem differentiation during vascular development. BRs promote xylem formation, whereas they suppress phloem differentiation. Thus, brassinosteroids play a crucial role in vascular development.

Experiments conducted in this subject have shown that the ratio of phloem/xylem in vascular systems of *det2* mutants is impaired. In non-synthesized BR mutants, a decrease in the number of vascular bundles has been detected (Savaldi-Goldstein and Chory 2006). In addition, auxin and BRs are interrelated in the senescence process, which is programmed cell death, such as vascular differentiation (Nemhauser and Chory 2004; Savaldi-Goldstein and Chory 2006; Bajguz and Hayat 2009). On the other hand, senescence occurs earlier in seedlings developing under biotic and abiotic stress conditions. For example, nitrogen, sodium, magnesium, potassium, phosphorus, chlorine, manganese and copper deficiency accelerate leaf senescence (Thomas and Stoddart 1980; Çağ et al. 2004). However, Çağ et al. (2004) have investigated senescence in cutted rocket cotyledons in case of zinc deficiency and they have found that senescence is delayed (Figs. 6.1, 6.2 and 6.3).

It is known that Zn provides IAA stabilization (Takaki and Kushizaki 1970; Bertoša et al. 2008) and this result (Çağ et al. 2004) is explained by correlating between Zn and the IAA. They think there may be a relationship between auxin-binding receptors (ABP) and BRs and senescence. Because in their studies, researchers have determined that auxin have influenced at the speed of senescence process (Kaplan-Dalyan and Sağlam-Çağ 2013; Sağlam-Çağ and Okatan 2014; Çıngıl-Barış and Sağlam-Çağ 2016). Sağlam-Çağ and Okatan (2014), in their study, has applied C^{14}IAA to apical tip. They prevented C^{14}-IAA from reaching the cotyledons, by destroying living cell in stem and they have found that senescence doesn't occur in these cotyledons according to control group.

On the other hand, it has also been reported that application of 24-epiBL against Zn-induced oxidative stress has a curative effect (Ramakrishna and Rao 2012). There is a correlation between exogenous application of brassinolide method and morphogenesis, plant development and senescence periods. It has been found that

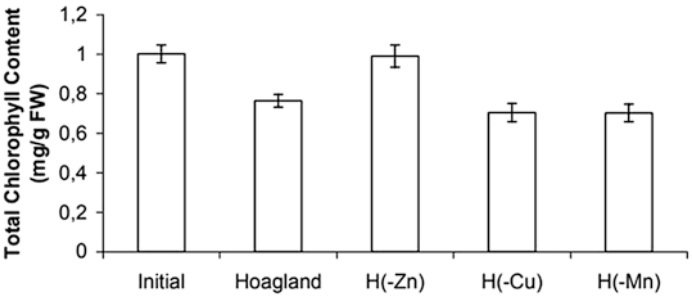


Fig. 6.1 Senescence delay in the absence of zinc. Chlorophyll amounts of the cotyledons before and after incubation in different solutions. Bars represent the standard errors

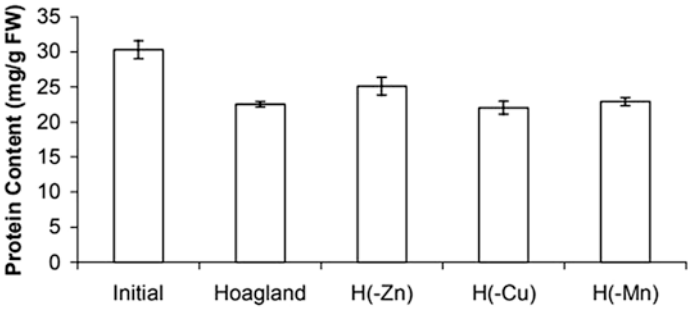


Fig. 6.2 Senescence delay in the absence of zinc. Nitrogen amounts of the cotyledons before and after incubations in different solutions of the micronutrients tested. Bars represent the standard errors

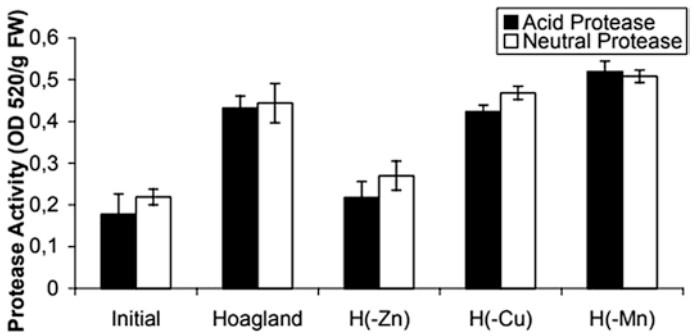


Fig. 6.3 Senescence delay in the absence of zinc. Protease activities of the cotyledons before and after incubation. Bars represent the standard errors

BR stimulates senescence in *Xanthium* and *Rumex* explants (Mandava et al. 1981), in cutted cotyledons of cucumber seedlings (Zhao et al. 1990) and in cutted leaves of *Arabidopsis* plant (He et al. 2001). It has been shown that in BR-deficient *Arabidopsis* mutants, chloroplast senescence has also been delayed (Li et al. 1996). Senescence is controlled by various environment factors (exogen), anatomical and morphological age of plant, reproductive phase of plant and endogen factors such as hormones (Buchanan-Wollaston 1997; He et al. 2001; Çıngıl-Barış and Sağlam-Çağ 2016). In particular, some plant hormones and growth regulators influence this process. Growth regulators such as ethylene, abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and strigolactone (SL) promote senescence, whereas auxin, cytokinin (CK) and gibberellins powerfully delay senescence (Jibran et al. 2013). So, ethylene accelerates senescence (McGoodwin 2008). There are interrelated connections between the molecules in signal transmission pathway that become active during senescence. In order to reveal the relationship these relationship during

senescence, the hormones affecting the process are widely applied to plants in an exogenic.

Different concentration of eBL is exogenously applied to wheat leaf segments. As a result of this application, it has been determined that eBL accelerates and delays senescence process with measurement of various analysis such as peroxidase and protease activity, protein amount and chlorophyll content (Sağlam-Çağ 2007). It has been observed that eBL accelerates senescence especially at high concentration (10 μM) and delays it at low concentration (0.001 μM).

Çıngıl-Barış and Sağlam-Çağ (2016) carry out a study showing that eBL works synergistically with auxin. In this study, researchers benefited used the whole plant. So, they examined the effect of eBL on cotyledon senescence that occurred in cotyledons of *Glycine max* L. seedlings. For this purpose, different concentrations of eBL and 2,3,5-triiodobenzoic acid (TIBA) solutions an inhibitor of the transport of an auxin were sprayed to seedlings. At the end of the experiment, the eBL (in particular 10^{-9} M) stimulates senescence, and in the case of co-administration with TIBA, it has been detected that it delays the senescence in the presence of chemical analyses (Figs. 6.4, 6.5, 6.6, and 6.7).

We reported that eBL does not act alone on senescence without auxin in whole plant experiments, differently from cutted organs. Further we have found that senescence accelerated in the presence of auxin. Indeed, in a previous study on this subject, researchers have also applied 2,3,5-triiodobenzoic acid (TIBA) to the sunflower (*Helianthus annuus* L.) seedlings grown in vertical and horizontal positions and that treat the same seedlings with 10^{-9} M and 10^{-11} M eBL (Kaplan-Dalyan and Sağlam-Çağ 2013).

It has been noted that eBL (especially 10^{-9} M) accelerates senescence in both horizontal and vertical plants without TIBA application (Figs. 6.8, 6.9, 6.10, and 6.11), whereas when TIBA is applied, senescence which normally occurs early in the lower cotyledons of plants in the horizontal position (Sağlam and Okatan 1990), is significantly delayed by eBL application (Kaplan-Dalyan and Sağlam-Çağ 2013).

It has been determined that brassinosteroids stimulate senescence only in the presence of auxin at the end of the experiment. It is stated that when BL is used with IAA, there is a dramatic increase in ethylene. The dramatic increase in ethylene

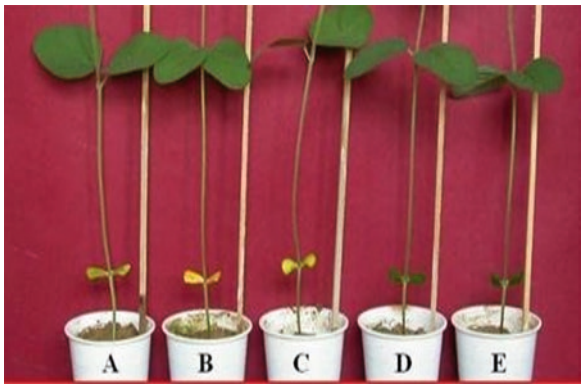


Fig. 6.4 The senescence ratios of the cotyledons of the soybean seedlings in the presence of eBL and/or TIBA
A: Control
B: 10^{-9} M eBL
C: 10^{-11} M eBL
D: 10^{-9} M eBL + TIBA
E: 10^{-11} M eBL + TIBA

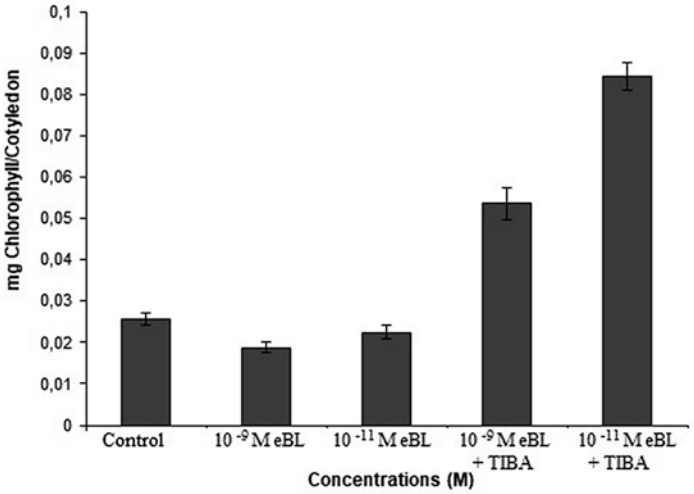


Fig. 6.5 Comparison of the chlorophyll amounts in cotyledons of the harvested seedlings treated with 10^{-9} M eBL, 10^{-11} M eBL, 10^{-9} M eBL + TIBA and 10^{-11} M eBL + TIBA and the average green space of the control plants' cotyledons when they reach to 50%. Bars represent the standard errors

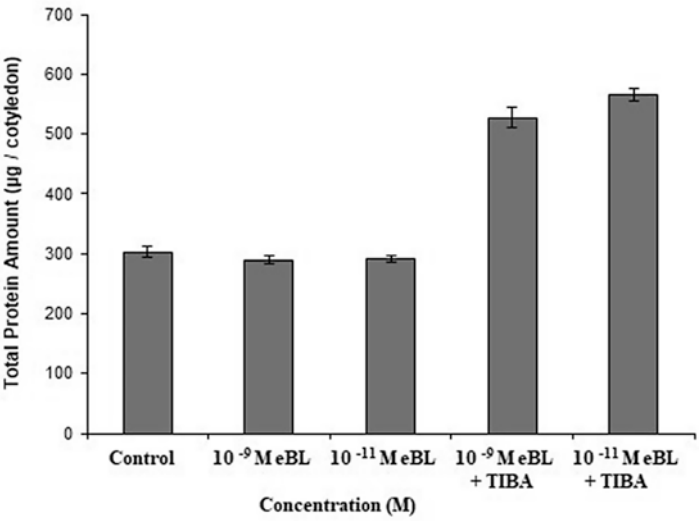


Fig. 6.6 Comparison of the total protein amounts in cotyledons of the harvested seedlings treated with 10^{-9} M eBL, 10^{-11} M eBL, 10^{-9} M eBL + TIBA and 10^{-11} M eBL + TIBA and the average green space of the control plants' cotyledons when they reach to 50%. Bars represent the standard errors

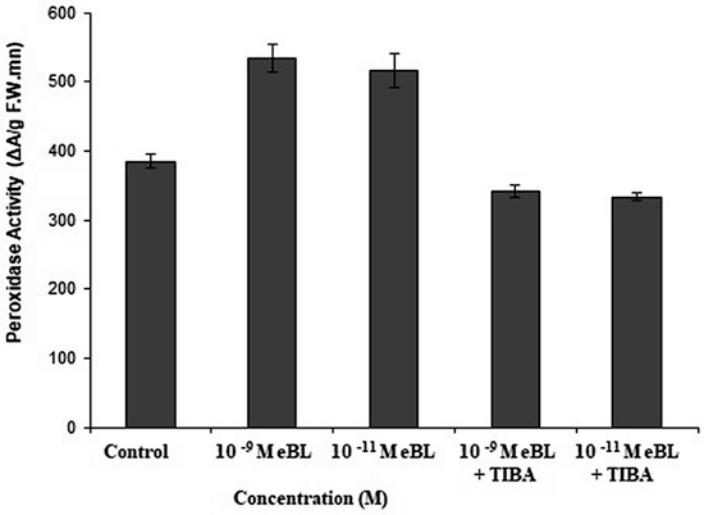
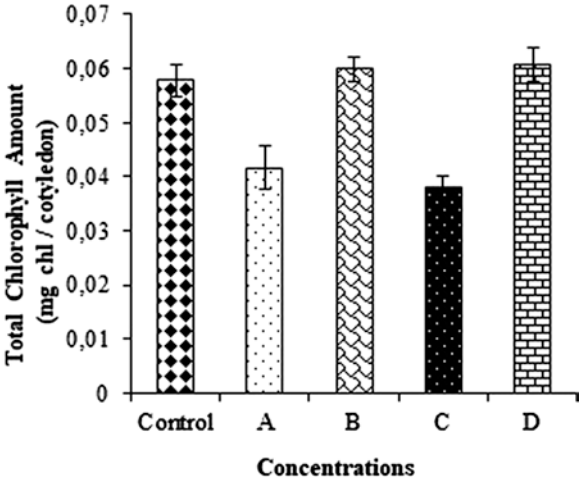


Fig. 6.7 Comparison of the peroxidase activity in cotyledons of the harvested seedlings treated with 10^{-9} M eBL, 10^{-11} M eBL, 10^{-9} M eBL + TIBA and 10^{-11} M eBL + TIBA and the average green space of the control plants' cotyledons when they reach to 50%. Bars represent the standard errors

Fig. 6.8 Total chlorophyll amounts of the cotyledons of vertically placed seedlings
Control
A: 10^{-11} M eBL
B: 10^{-11} M eBL + TIBA
C: 10^{-9} M eBL
D: 10^{-9} M eBL + TIBA
Bars represent the standard errors



production is thought to be caused by the combined application of these hormones. As is known, ethylene production increases during senescence.

It has been seen that when auxin (10^{-5} M) and BL (10^{-7} M) are applied exogenously to *Zea mays* L., ethylene production increases dramatically (Yun et al. 2009). When these hormones are applied simultaneously, the increase in ethylene level is greater than the sum of the effects of each. This positive correlation has been

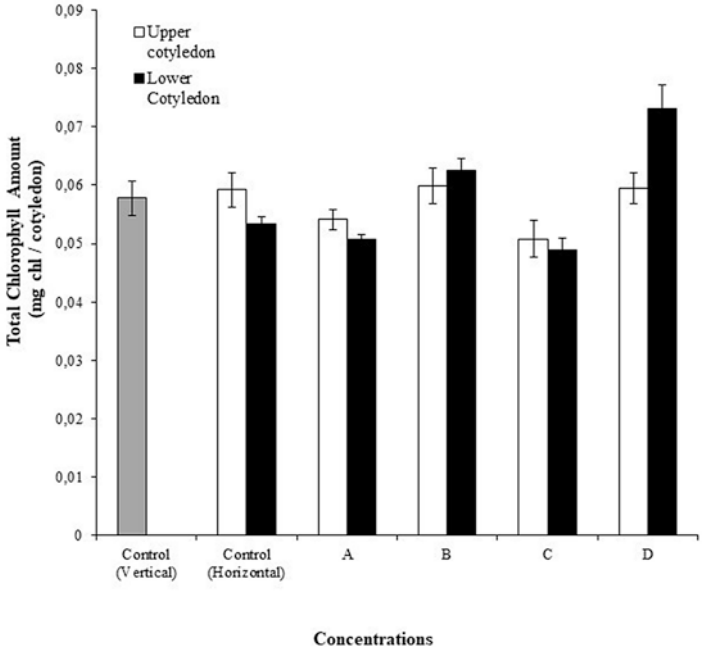


Fig. 6.9 Total chlorophyll amounts of the cotyledons of horizontally placed seedlings
Control
A: 10^{-11} M eBL
B: 10^{-11} M eBL + TIBA
C: 10^{-9} M eBL
D: 10^{-9} M eBL + TIBA
Bars represent the standard errors

recorded during gene expression and change of ACC synthase activity. Due to the fact that BL promotes ethylene biosynthesis, it is thought that it needs IAA to increase the elongation in the roots. For this reason, it is suggested that BL effects both ethylene production (in early phase) and by inducing auxin. Interestingly, a group of researchers (Choe et al. 1999) has stated that the majority of BR mutants present a prolonged life span and delayed senescence. BR mutants remain green even after 100 days, or even create new flowers, while a wild-type *Arabidopsis* plant becomes senescence after approximately 60 days.

Leaf senescence and cotyledon senescence can be delayed by application of cytokinin (Gan and Amasino 1997; Brault and Maldiney 1999). He et al. (1996) have found that eBL accelerates senescence, within this period, peroxidase (POD) activity increases, whereas, superoxide dismutase (SOD) and catalase (CAT) activities decrease, and that there is a marked increase in malondialdehyde levels. They have been said that BRs make this through "activated oxygen". Sağlam-Çağ (2007) has also found that chlorophyll and protein content decrease with 24-eBL application to wheat leaf segments and that the POD activity increases and senescence accelerates accordingly.

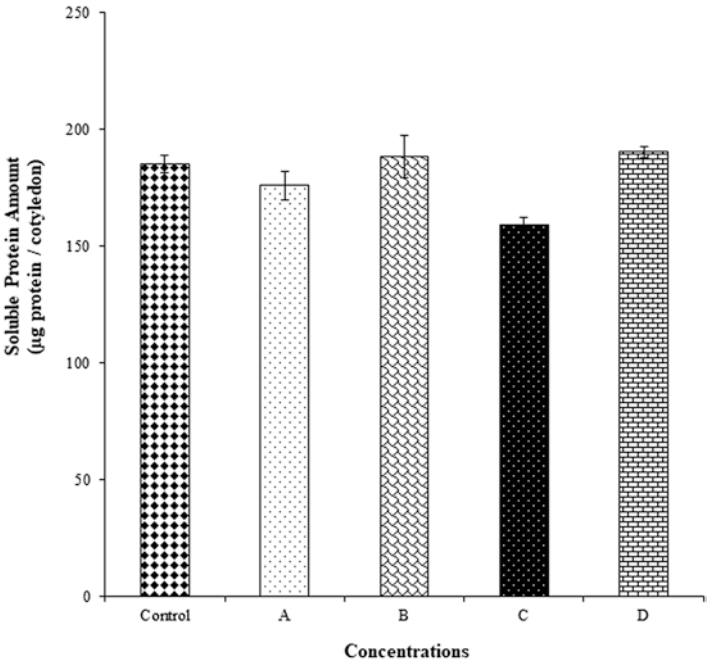


Fig. 6.10 Total protein amounts of the cotyledons of vertically placed seedlings
Control
A: 10^{-11} M eBL
B: 10^{-11} M eBL + TIBA
C: 10^{-9} M eBL
D: 10^{-9} M eBL + TIBA
Bars represent the standard errors

Despite all this information, Srivastava (2002) suggests that BRs are concerned with the delay of the senescence. The molecular mechanism of BR effect on senescence is still unknown.

5 Conclusion

In this chapter, I try to emphasize the relationship between brassinosteroids and senescence occurring in plants from various aspects. Growth conditions also change due to changes in the physical and chemical components occurring in the living environment of plant. Variable factors in the developmental environment affect the beginning and progression of plant senescence.

BRs are a steroid hormone that regulates plant growth and development. BRs are exogenously applied to plants in nanomolar or micromolar concentrations. Brassinosteroids interact with auxins, cytokinins, and gibberellins via connective

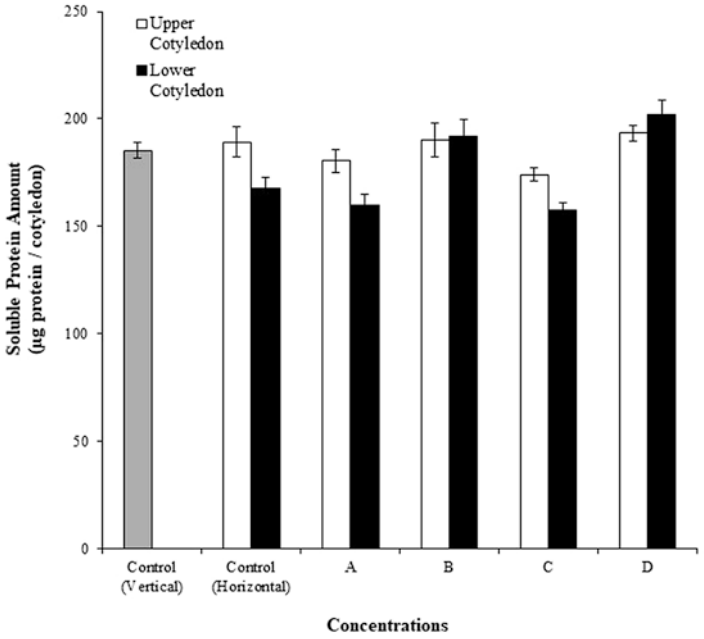


Fig. 6.11 Total protein amounts of the cotyledons of horizontally placed seedlings
Control
A: 10^{-11} M eBL
B: 10^{-11} M eBL + TIBA
C: 10^{-9} M eBL
D: 10^{-9} M eBL + TIBA
Bars represent the standard errors

pathways. Brassinosteroids are exogenously applied to developing young tissues by spraying or incubation. So, it effects on growth, development, cell division, cell elongation by controlling biochemical reactions. Exogenously applied brassinolide influences the senescence process. These practices accelerate or delay senescence depending on concentration. During senescence, there is a signal exchange between gene expression and hormones (Divi et al. 2010). The mechanism of action of BRs is illuminated at the molecular level by researchers. But how the hundreds of gene expressions are regulated is not certainly understood yet.

High concentrations of BR stimulate the production of ethylene, like the same auxin. Thus, it is likely that the incentive effect of BR on senescence is via the ethylene pathway. As a matter of fact, BRs are already able to stimulate senescence in high concentrations. In this case, BRs and auxin play a synergistic role in the senescence process. There are studies related to this subject.

All global changes and radiation threatening environment will dramatically have an impact on plant growth (McCarthy et al. 2001). Ongoing researches will demonstrate the connection integrity of senescence with brassinosteroids in the near future.

Thanks to this information, we can say that increasing the leaf and fruit with the delay of senescence is important in agriculture and cultivation.

It can be ensured that the yield increase is due to the transfer of the monomers of the plants which are promoted early to senescence with the brassinolide applied at high concentration to the storage organs. Thus, the maximum benefit is obtained from the substances produced by the plant.

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Chapter 7
Brassinosteroid Mediated Regulation of Photosynthesis in Plants

Husna Siddiqui, Fareen Sami, Mohammad Faizan, Ahmad Faraz, and Shamsul Hayat

Abstract Brassinosteroids (BRs) are sterol derivatives with multiple hydroxyl groups occurring universally in plants. Photosynthesis is the process which acts as base for the growth of the plant. BRs promote the activation as well as synthesis of enzymes responsible for the formation of chlorophyll. BRs regulate different components of photosynthetic machinery like photochemistry, stomatal conductance and enzymes of Calvin cycle. BRs promote photosynthetic carbon fixation by altering the functioning of stomata. The BR-mediated regulation of various photosynthetic components operates constitutively to promote net photosynthetic rate and ultimately, the growth and development of the plants. Thus, the role of BRs in regulating photosynthesis becomes an important area of research. The present chapter summarizes the BR-mediated changes in photosynthesis and its associated components under normal and stress conditions.

Keywords Brassinosteroids · Primary photochemistry · Carbohydrate synthesis · Net photosynthetic rate · Abiotic stress

1 Introduction

Phytohormones are naturally occurring organic compounds that affect different physiological processes at a very low concentration. They are easily transported across the plant body (Went and Thimann 1937). Brassinosteroids (BRs) are sterol derivatives having multiple hydroxyl groups, structurally quite similar to the animal steroid. BRs are found throughout the plant kingdom and in all parts of the plant. BRs cannot be transported over long distance within plant body. Various physiological and morphological processes are regulated by BRs. BRs regulate photosynthesis, the key process which acts as a base for the growth of the plant.

During photosynthetic process, chlorophyll captures solar energy to synthesise carbohydrates and to liberate O_2 (Pan et al. 2012). Chloroplast, acts as a seat for

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light and dark reactions (Ashraf and Harris 2013). Photosystem II (PSII) and photosystem I (PSI) operate sequentially in thylakoid membrane and take part in the reduction of $NADP^+$ to NADPH. Non-cyclic electron transport is the only pathway on Earth's atmosphere through which oxygen is generated.

Despite having abundant data related to regulation of photosynthetic processes by BRs, the exact mechanism underlying their effect remains unclear. Nevertheless, theories have been proposed to explicate the probable mechanism of BR-mediated photosynthesis regulation. Like, BRs might activate or induce enzymes involved in chlorophyll biosynthesis or might surmount the stomatal limitations thus escalating the CO_2 entry into the leaf and its availability for photosynthetic enzymes, resulting in elevated photosynthetic carbon fixing efficiency (Holá et al. 2010).

2 BR Receptor and Signalling

Clouse et al. (1996) in an experiment on *Arabidopsis* identified *brassinosteroid-insensitive 1* (*BRI1*) as an essential element of BR signalling. The binding of BL to *BRI1* is highly specific (Kinoshita et al. 2005). *BRI1* is a lecuine-rich repeat-receptor serine/threonine kinase located in cell membrane. *BRI1* possess 25 LRRs. The island domain (chain of amino acids) flanks between LRRs 21 and 22. Island domain along with LRR22 forms the minimal structure required for BR adherence (Kinoshita et al. 2005). As soon as the BR binds to *BRI1*, it elicits the interface of *BRI1* with BAK1 thus, proving *BRI1* as the receptor for BRs.

Signalling of BRs involve phosphorylation and dephosphorylations at different steps in this pathway. BL binding to the receptor *BRI1* phosphorylation occur at several sites. Binding of BL leads to the release of BRI1-kinase inhibitor1 (BKI1) along with *BRI1*activation. *BRI1* along with *BRI1*-associated receptor kinase1 (BAK1) protein phosphorylates BSK protein (Wang and Chory 2006). BSK protein phosphorylation activates BRI1-supressor1 (BSU1). Dephosphorylation of BIN2 (brassinosteroid insensitive-2) kinase by activated BSU1 results in disintegration of proteosome organization (Peng et al. 2008; Kim et al. 2009). To generate BR response the degradation of BIN 2 is necessary because BIN2 represses the BR-mediated expression of genes. Unavailability of BRs, trigger the entry of BIN2 into the nucleus hence phosphorylating BRI1-EMS-suppressor1 (BES1) and brassinozole-resistant1 (BZR1) proteins. Phosphorylation of BES1 and BZR1 makes these proteins incompetent for binding to DNA and blocks the transcription (Li and Nam 2002; Vert and Chory 2006). Although, binding of BRs blocks the phosphorylation of BES1 and BZR1 proteins. These proteins bind with DNA to express various genes. BES1 and BZR1 play an essential role in BR biosynthetic pathway by controlling negative feedback regulation by enhancing BR induced gene expression and repressing BR biosynthesis, respectively (He et al. 2005; Yin et al. 2005).

3 BR-Mediated Regulation of Photosynthetic Components

BRs play regulatory role during photosynthetic processes (Siddiqui et al. 2018a). It regulates various components of photosynthesis like photosystem machinery, stomatal conductance, stomatal movement, calvin cycle enzymes and sugar accumulation. There is a large pool of literature concerned with the BR-mediated changes in photosynthesis, and Tables 7.1, 7.2, 7.3, 7.4, 7.5 and 7.6 summarizes these studies in presence/absence of stress conditions.

3.1 Effect of Brassinosteroids on Photosynthesis in Plants

3.1.1 Photosystem Machinery

Measurement of chlorophyll fluorescence parameters could be used to study different photochemical reactions inside the leaf thylakoid membrane. Bhatia and Kaur (1997) determined activity of Hill reaction in chloroplast to assess electron transport during photosynthesis, where BR application promoted it (Verma et al. 2011). Reaction centre ejects an electron to Q_A via primary acceptor, pheophytin. Transfer of active electron to the subsequent carrier is essential for the uptake of another electron by Q_A from P_{680}. The state in which this transfer of electron does not occur is regarded as 'closed' and it results in reduction of PSII quantum efficiency. The dip in fluorescence signal following an early ascend is known as 'quenching' and determines the open PSII reaction centres (Krause and Weis 1991). Quenching could be photochemical (qP) or non-photochemical (NPQ). The transport of electrons leads to reduction of NADP and generation of ATP which are utilized during calvin cycle for sugar synthesis (Baker and Oxborough 2004). The amount of light captured by chlorophyll that could be utilized in photochemistry is known as PSII quantum efficiency (ϕPSII) and F_v/F_m is the maximum quantum efficiency achieved when all PSII centres are open. Electron transport rate (ETR) directly depends on ϕPSII and gives a clue of photosynthetic rate in general. BRs regulate the primary photochemical reactions (F_v/F_m, ϕPSII, qP, NPQ and ETR) in plants. BRs promote photochemical quenching, PSII efficiency and ETR, but decreases NPQ to prevent loss of energy as heat. Thereby, increasing the generation of assimilatory powers for sugar synthesis which eventually marks the enhancement of growth and metabolism (Yu et al. 2004; Berger et al. 2004; Xia et al. 2006; Ogweno et al. 2008; Shahbaz et al. 2008; Jiang et al. 2012; Hu et al. 2013; Lima and Lobato 2017; Siddiqui et al. 2018b). The relationship between BRs and genes in regulating the process of photosynthesis could be established by studying the BR-deficient mutants or BR-treated plants (Oh et al. 2011; Bai et al. 2012). An altered BR response in *Arabidopsis* mutant demonstrate reduction in PSII efficiency, smaller PSII complex, thylakoid enlargement and inhibition of CO_2 evolution (Krumova et al. 2013). Similarly, genes related with photosynthesis were down-regulated in *Arabidopsis* mutant

Table 7.1 Effect of exogenous application of brassinosteroids (BRs) on photosynthetic pigments

Species	BR selected	Concentration	Mode of application	Time of BR treatment	Sampling stage	Photosynthetic pigments	Author
Vigna radiata	HBL	10^{-10}, 10^{-8}, 10^{-6} M	Foliar spray	14 DAS	15 and 21 DAS	Chl content	Alyemeni and Al-Quwaiz (2016)
Pistacia vera	HBL	10^{-10}, 10^{-8}, 10^{-6} M	Foliar spray	Six leaves stage	After 6 weeks of application	Chl a, Chl b	Farazi et al. (2015)
Vigna radiata	EBL	0.5 µM, 1 µM, 1.5 µM, 2.0 µM	Foliar spray	3 days consecutively	10 and 15 days old seedlings	Chl a, Chl b, total chl and Car content	Asha and Lingakumar (2015)
Prunus persica	BL	50 and 100 ppm	Foliar spray	Tree stage	Tree stage	Chl a, Chl b, total chl	Gabr et al. (2011)
Vigna radiata	BL	0.10, 0.25, 0.50 ppm	Foliar spray	Pre-flowering and Flowering stage	10 days interval from 30–70 DAS	Chl a, Chl b, total chl	Maity and Bera (2009)
Lycopersicon esculentum	EBL	10^{-8}, 10^{-6} M	Foliar spray	Stage of 3 fully developed leaves	3 or 5 days after BR treatment	Chl a, Chl b, Car content	Behnamnia et al. (2009)
Glycine max	EBL	10^{-9}, 10^{-7}, 10^{-5} M	Foliar spray	5 DAS	End of BR treatment	Chl and Car content	Cevahir et al. (2008)
Brassica oleracea	EBL	10^{-9} M, 10^{-7} M, 10^{-5} M	Cotyledon soaking (3 days)	8 DAS	End of BR treatment	Chl content	Çağ et al. (2007)
Phaseolus vulgaris, Hordeum vulgare	NS	5 µM	Seed soaking (6 h)	Seeds	2, 4, 6 and 8 days after germination	Chl content	Ali and Abdel-Fattah (2006)

Table 7.2 Effect of exogenous application of brassinosteroids (BRs) on different components of photosynthesis

Species	BR selected	Concentration	Mode of application	Time of BR treatment	Sampling stage	Photosynthetic parameters studied	Author
Wolffia arrhiza	EBL	10^{-12}, 10^{-11}, 10^{-10}, 10^{-9},10^{-8}, 10^{-7}, 10^{-6} M	Incubation with BRs in nutrient solution for 7 days	NS	End of BR treatment	Chl a, Chl b, Car content	Bajguz and Asami (2005)
Brassica napus	EBL	10^{-7} M	Incubation with BRs in nutrient solution for 14 days	3 DAS	End of BR treatment	Chl and Car content	Janeczko et al. (2005)
Phaseolus aureus	BL	0.1 ng/L, 10 ng/L, 1 µg/L, 100 µg/L	Incubation in BRs in nutrient solution	4 DAS	End of BR treatment	Chl content	Abdullahi et al. (2002)
Lycopersicon esculentum	Synthetic BRs	0.01 mg/L	Leaf segment soaking (24 h)	15 DAS	End of BR treatment	Chloroplast structure	Sam et al. (2001)
Chlorella vulgaris	BL, EBL, HBL, castasterone, homocastastreone, 24-epicastasterone	10^{-12}, 10^{-8} M	Incubation with BRs in nutrient solution for 24 and 36 h	Cell culture	End of BR treatment	Chl a, Chl b	Bajguz and Czerpak (1998)
Vigna radiata	HBL	0.1 mg/L, 0.5 mg/L	Foliar spray	15 and 30 DAS	22, 36 and 50 DAS	Chl a, chl b content, chl a/b, Hill reaction activity	Bhatia and Kaur (1997)
Cucumis sativus	BL	10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} ppm	Cotyledon soaking (12 h)	Cotyledonary stage	End of BR treatment	Chl content	He et al. (1991)
Cucumis sativus	BL	10^{-7}, 10^{-6}, 10^{-5} M	Seed soaking	Seeds	12 DAS	Chl content	Katsumi (1991)
Hordeum vulgare	EBL	10^{-8} M	Leaf segment soaking (2 h)	10 DAS	End of BR treatment	Chloroplast structure	Kulaeva et al. (1991)

(continued)

Table 7.2 (continued)

Species	BR selected	Concentration	Mode of application	Time of BR treatment	Sampling stage	Photosynthetic parameters studied	Author
Brassica juncea	EBL, HBL	10^{-8} M	Foliar spray	25–30 DAS	45 and 60 DAS	Chl content, F_v/F_m, $\phi PSII$, qP, NPQ, ETR, P_N, G_s, C_i, E, CA activity, Potassium and magnesium content	Siddiqui et al. (2018b)
Vigna unguiculata	EBL	50 and 100 nM	Foliar spray	6-day old seedling with 6 days interval till 18th day stage	20th day stage	F_v/F_m, $\phi PSII$, qP, NPQ, ETR, P_N, G_s, C_i, E, ETR/P_N, WUE, P_N/C_i	Lima and Lobato (2017)
Vigna radiata	HBL	10^{-8} M, 10^{-6} M,	Foliar spray	29 DAS	45 DAS	P_N, G_s, C_i, CA activity	Yusuf et al. (2014)
Satureja khuzestanica	HBL	10^{-10}, 10^{-8}, 10^{-6} M	Foliar spray	14, 40 and 80 days after planting	140th day	Chl a, chl b content, P_N, total sugars, reducing and non-reducing sugars, starch	Eskandari and Eskandari (2013)
Capsicum annum	EBL	0.01 mg/L	Foliar spray	20–25 leaf stage (2 months old)	1, 5, 10, 11 and 15 days after treatment	P_N, C_i, E, G_s, F_v/F_m, $\phi PSII$, qP, NPQ	Hu et al. (2013)
Cucumis sativus	EBL	0.1 μM	Foliar spray	4th leaf stage	3, 24, 72, 120, 168 h after BR treatment	Total chl content, $\phi PSII$, qP, rubisco activity, V_{cmax}, J_{max}, SPS, AI activity, total soluble sugar, sucrose, hexose, starch, sucrose synthase transcript	Jiang et al. (2012)

Cucumis sativus	EBL	0.1 μM	Foliar spray	After 3 days pe-culture	9 and 10 days of treatment	Chl a, chl b content, chl a + b, chl a/b ratio, F_v/F_m, $\phi PSII$, qP, NPQ, P_N, G_s, C_i, E	Yuan et al. (2012)
Lycopersicon esculentum	HBL, EBL	10^{-10}, 10^{-8}, 10^{-6} M	Foliar spray	44 DAS	45 DAS	Chl content, P_N, G_s, C_i, CA activity	Hayat et al. (2011)
Pelargonium graveolens	EBL	0.5×10^{-6} M, 10^{-6} M, 3×10^{-6} M	Foliar spray	30, 50 and 70 days after transplant	120 days after transpant	Chl a, chl b content, P_N	Swamy and Rao (2009)
Vicia faba	EBL	2×10^{-9} M, 2×10^{-8} M	Seed soaking (24 h)	Seeds	6–30 DAS	F_v/F_m, qP, NPQ, ETR, P_N	Piñol and Simón (2009)
Lycopersicum esculentum	EBL	0.01 mg/L, 0.1 mg/L, 1 mg/L	Foliar spray	42 DAS	54 DAS	F_v/F_m, $\phi PSII$, qP, NPQ, P_N, C_i, G_s, V_{cmax}, J_{max}	Ogweno et al. (2008)
Triticum aestivum	EBL	0.0125 mg/L, 0.025 mg/L, 0.0375 mg/L	Foliar spray	43 DAS	88 DAS	Chl a, chl b, chl a/b ratio, F_v/F_m, P_N, C_i, G_s	Shahbaz et al. (2008)
Brassica juncea	HBL	10^{-8} M	Foliar spray	30 DAS	60 DAS	Chl content, P_N, G_s, C_i, CA activity	Fariduddin et al. (2009)
Vigna radiata	HBL	10^{-8} M, 10^{-6} M	Seed soaking and foliar spray alone and in combination	Seeds and leaves after 15 days of sowing	30 and 50 DAS	Chl content, P_N, G_s, carboxylation efficiency, CA activity	Fariduddin et al. (2008)
Triticum aestivum	EBL	0.052×10^{-6} M, 0.104×10^{-6} M, 0.156×10^{-6} M	Seed soaking followed by BR treatment via hydroponic solution for 45 days	Seeds	End of BR treatment	Chl a content, F_v/F_m, P_N, G_s, C_i	Ali et al. (2008a)

(continued)

Table 7.2 (continued)

Species	BR selected	Concentration	Mode of application	Time of BR treatment	Sampling stage	Photosynthetic parameters studied	Author
Brassica juncea	HBL	10^{-8} M	Foliar spray	30 DAS	40 DAS	Chl content, P_N, CA activity	Alam et al. (2007)
Brassica juncea	HBL	10^{-8} M	Foliar spray	30 DAS	60 DAS	Chl content, P_N, CA activity	Hayat et al. (2007)
Triticum aestivum	EBL	0.0125 mg/L, 0.025 mg/L	Foliar spray	56 DAS	83 DAS	Chl a, chl b content, P_N, G_s, C_i	Qayyum et al. (2007)
Cicer arietinum	HBL	10^{-10}, 10^{-8} M	Seed soaking (4 and 8 h)	Seeds	60, 90 and 120 DAS	Chl a, chl b content, CA activity	Ali et al. (2007)
Lycopersicon esculentum	HBL	10^{-8} M, 10^{-7} M, 10^{-6} M 10^{-5} M	Root soaking (15, 30 and 45 min)	20 DAS	50 and 80 DAS	Chl content, CA activity	Ali et al. (2006)
Vigna radiata	HBL	10^{-10}, 10^{-8}, 10^{-6} M	Foliar spray	15 DAS	30 and 50 DAS	Chl content, CA activity, P_N, G_s, C_i	Fariduddin et al. (2006)
Lycopersicon esculentum	EBL	10^{-6} M, 10^{-5} M, 2×10^{-5} M	Foliar spray	28 DAS	42–44 DAS	P_N, G_s, C_i	Singh and Shono (2005)
Vigna radiata	HBL	10^{-10}, 10^{-8}, 10^{-6} M	Foliar spray	25 DAS	35 and 45 DAS	P_N, Chl content, CA	Fariduddin et al. (2004)
Cucumis sativus	EBL	0.01 mg/L, 0.1 mg/L, 1 mg/L	Foliar spray	3rd fully developed leaf stage	3,6,9,24,26 h and 3, 5, 7 days after BR treatment	Chl content, F_v/F_m, $\phi PSII$, qP, P_N, C_i, V_{cmax}, J_{max}, initial and total activity of rubisco, rubisco content	Yu et al. (2004)

Lycopersicon esculentum	ComCat	2 mg/L	Dipping in BR solution	28 DAS	4 days after BR treatment	Chl content, F_v/F_m, $\phi PSII$, qP, NPQ, ETR, P_N, carboxylation efficiency, G_s, CA activity, Rubisco small sub-unit expression	Berger et al. (2004)
Vigna radiata	HBL	10^{-8}, 10^{-6}, 10^{-4} M	Seed soaking (4, 8, 12 h)	Seeds	30, 40, 50 DAS	Chl content, CA activity, P_N, G_s, C_i	Fariduddin et al. (2003)
Brassica juncea	HBL	10^{-8} M	Foliar spray	30 DAS	60 DAS	Chl a, chl b, chl a/b ratio, CA activity	Hayat et al. (2001)
Triticum aestivum, Leucosinapis alba	BL	10^{-6} M	Foliar spray	10 DAS	12–20 DAS	Chl a, chl b, Car content, chl a/b ratio, P_N, Rubisco content and activity	Braun and Wild (1984)

Table 7.3 Effect of exogenous application of brassinosteroids (BRs) on different components of photosynthesis in the presence of salinity stress

Species	BR selected	Concentration	Mode of application	Time of BR treatment	Sampling stage	Salinity stress effects on photosynthetic parameters	BR-mediated effects	Author
Lactuca sativa	EBL	0, 1, 2, and 3 μM	Seed soaking and foliar spray	Seeds and 31 DAS	End of BR treatment	Reduces chl content and G_s	Restores chl content and G_s	Siddiqui et al. (2018c)
Brassica juncea	HBL	10^{-10}, 10^{-8}, 10^{-6} M	Foliar spray	15, 30 and 45 DAS	60 DAS	Reduces chl, P_N, CA activity	Restores chl, P_N, CA activity	Alyemeni et al. (2013)
Oryza sativa	EBL	10^{-11}, 10^{-9}, 10^{-7} M	Seed soaking (8 h)	Seeds	12 DAS	Reduces chl a, chl b content	Restores chl content	Sharma et al. (2013)
Brassica juncea	EBL	10^{-8} M	Foliar spray	25 DAS	45 DAS	Reduces chl, P_N, G_s, Ci, WUE, CA activity	Restores chl, P_N, G_s, Ci, WUE, CA activity	Ekinci et al. (2012)
Triticum aestivum	EBL	0.052×10^{-6} M, 0.104×10^{-6} M, 0.156×10^{-6} M	Seed soaking followed by BR treatment via hydroponic solution for 45 days	Seeds	End of BR treatment	Reduces Chl a content, $F_v/F_{m,}$ P_N, G_s, C_i	Restores chl a content, $F_v/F_{m,}$ P_N, G_s, C_i	Ali et al. (2008a)
Brassica juncea	EBL	10^{-6} M	Foliar spray	15 DAS	30 DAS	Reduces Chl and car content, P_N, G_s, C_i, CA activity	Reduces Chl and car content, P_N, G_s, C_i, CA activity	Ali et al. (2008b)
Triticum aestivum	EBL	0.0125 mg/L, 0.025 mg/L, 0.0375 mg/L	Foliar spray	43 DAS	88 DAS	Reduces Chl a, chl b, chl a/b ratio, F_v/F_m, P_N, C_i, G_s	Restores chl a, chl b, chl a/b ratio, F_v/F_m, P_N, C_i, G_s	Shahbaz et al. (2008)
Brassica juncea	HBL	10^{-10}, 10^{-8}, 10^{-6} M	Through soil	14 DAS	60 DAS	Reduces chl, P_N, CA activity	Restores chl, P_N, CA activity	Hayat et al. (2007)

Table 7.4 Effect of exogenous application of brassinosteroids (BRs) on different components of photosynthesis in the presence of drought stress

Species	BR selected	Concentration	Mode of application	Time of BR treatment	Sampling stage	Drought stress effects on photosynthetic parameters	BR-mediated effects	Author
Capsicum annum	EBL	0.01 mg/L	Foliar spray	20–25 leaf stage (2 months old)	1, 5, 10, 11 and 15 days after treatment	Reduces P_N, C_i, E, G_s, F_v/F_m, $\phi PSII$, qP	Restores P_N, C_i, E, G_s, F_v/F_m, $\phi PSII$, qP	Hu et al. (2013)
Chorispora bungeana	EBL	0.1 μM	Foliar spray	5 cm tall plants	End of drought treatment	Reduces chl a, chl b and chl a + b content, F_m	Restores chl a, chl b and chl a + b content, F_m	Li et al. (2012)
						F_v/F_m, Φ_{PSII}	F_v/F_m, Φ_{PSII}	
Lycopersicon esculentum	EBL	1 μM	Foliar spray	Five true leaves stage	1, 2 and 3 days after water cessation	Reduces P_N, G_s, Ci	Restores P_N, G_s, C_i	Yuan et al. (2010)
Oryza sativa	EBL, HBL	10^{-8} M	Seed soaking (2 days) and foliar spray	Seeds, 28 DAS	35 DAS	Reduces P_N, G_s, Ci	Restores P_N, G_s, C_i	Farooq et al. (2009)
Glycine max	BL	0.01 mg/L	Foliar spray	Beginning of Bloom	14 days after treatment	Reduces chl content, F_v/F_m, P_N, rubisco and phosphoenol pyruvate carboxylase activity	Restores Reduces chl content, F_v/F_m, P_N, rubisco and phosphoenol pyruvate carboxylase activity	Zhang et al. (2008)
Triticum aestivum	HBL	0.1 and 1 ppm	Foliar spray	30 and 32 DAS	Anthesis stage	Reduces chl content and P_N	Restores chl content and P_N	Sairam (1994a)
Triticum aestivum	HBL	0.01 and 0.05 ppm	Seed soaking (6 h) and foliar spray	Seeds and 25 DAS	Anthesis stage	Reduces chl content and P_N	Restores chl content and P_N	Sairam (1994b)

Table 7.5 Effect of exogenous application of brassinosteroids (BRs) on different components of photosynthesis in the presence of thermal stress

Species	BR selected	Concentration	Mode of application	Time of BR treatment	Sampling stage	Thermal stress	Thermal stress effects on photosynthetic parameters	BR-mediated effects	Author
Secale cereale	EBL	0.25 mg dm^{-3}	Foliar spray	3-leaf stage	End of cold treatment	Cold	Reduces PSII efficiency	Restores PSII efficiency	Pociecha et al. (2016, 2017)
Oryza sativa	EBL	10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M	Foliar spray	35 DAS	0, 1, 3, 5 and 7 days after heat stress	Heat	Reduces chl a, chl b, chl a + b and car content P_N, C_i, E, G_s, F_v/F_m, $\phi PSII$, qP and soluble sugar content. Increases NPQ	Restores chl a, chl b, chl a + b and car content P_N, C_i, E, G_s, F_v/F_m, $\phi PSII$, qP and soluble sugar content. Reduces NPQ	Thussagunpanit et al. (2015)
Solanum melongena	EBL	0.05, 0.1, 0.2, and 0.4 μM	Foliar spray	4 or 5 true leaf stage	After 8 days of cold treatment	Cold	Reduces chl a, chl b, chl a + b and car content P_N, C_i, E, G_s, F_v/F_m, F_v/F_0, $\phi PSII$, Qp. Increases NPQ	Restores chl a, chl b, chl a + b and car content P_N, C_i, E, G_s, F_v/F_m, F_v/F_0, $\phi PSII$, Qp. Reduces NPQ	Wu et al. (2014)
Zea mays	EBL	0.1, 1.0 and 10 μM	Foliar spray	10 DAS	7, 14 and 21 days after BR treatment	Cold	Reduces total chlorophyll content	Restores total chlorophyll content	Singh et al. (2012)
Cucumis sativus	HBL	10^{-8} and 10^{-6} M	Foliar spray	30 DAS	10 days after BR treatment	Cold	Reduces chl content, F_v/F_m P_N, C_i, E, G_s, E and WUE	Restores chl content, F_v/F_m P_N, C_i, E, G_s, E and WUE	Fariduddin et al. (2011)

Lycopersicum esculentum	EBL	0.01 mg/L, 0.1 mg/L, 1 mg/L	Foliar spray	Before heat stress	0, 4, 8, 12 days after heat treatment	*Heat*	*Reduces* F_v/F_m, $\phi PSII$, qP, P_N, C_i, G_s, V_{cmax}, J_{max}	*Restores* F_v/F_m, $\phi PSII$, qP, P_N, C_i, G_s, V_{cmax}, J_{max}	Ogweno et al. (2008)
Brassica napus	EBL	0.05 and 1 μM	Injection into the apoplast of cotyledons or primary leaves	17 DAS	End of cold treatment	Cold	Chl a, chl b and Car degradation	Prevents Chl a, chl b and Car degradation	Janeczko et al. (2007)
Lycopersicon esculentum	EBL	1, 10, and 20 μM	Foliar spray	28 DAS	42–44 DAS	Heat	Reduces P_N, G_s, C_i	Restore P_N, G_s, C_i	Singh and Shono (2005)

Table 7.6 Effect of exogenous application of brassinosteroids (BRs) on different components of photosynthesis in the presence of heavy metal stress

Species	BR selected	Concentration	Mode of application	Time of BR treatment	Sampling stage	Heavy metal	Heavy metal stress effects on photosynthetic parameters	BR-mediated effects	Author
Brassica juncea	EBL	10^{-8} M	Foliar spray	21 and 22 DAS	30 DAS	Selenium	Reduces chl content, P_N, G_s, Ci, CA activity	Restores chl content, P_N, G_s, Ci, CA activity	Naz et al. (2015)
Trigonella foneu-graecum	HBL	0.5, 1 μM	Foliar spray	10 and 20 DAS	30 DAS	Lead	Reduces chl content and photosynthetic rate	Restores chl content and photosynthetic rate	Swamy et al. (2014)
Raphanus sativus	EBL	10^{-11}, 10^{-9}, 10^{-7} M	Seed soaking (8 h)	Seeds	7th day	Mercury	Reduces chl a, chl b, total chl and car content	Restores chl a, chl b, total chl and car content	Kapoor et al. (2014)
Vigna radiata	HBL	10^{-8} and 10^{-6} M	Foliar spray	29 DAS	45 DAS	Nickel	Reduces chl content, $F_v/F_{m,}$ P_N, G_s, Ci, E CA activity	Restores chl content, F_v/F_m, P_N, G_s, Ci, E, CA activity	Yusuf et al. (2014)
Cucumis sativus	EBL	0.01 μM	Foliar spray	30 DAS	10 days after BR treatment	Copper	Reduces chl content, F_v/F_m and P_N	Restores chl content, F_v/F_m and P_N	Fariduddin et al. (2013)
Raphanus sativus	EBL	10^{-9} M	Seed treatment	Seeds	7th Day	Chromium	Reduces chl a, chl b and car content, F_v/F_m	Restores chl a, chl b and car content, F_v/F_m	Choudhary et al. (2012)
Lycopersicon esculentum	HBL, EBL	10^{-8} M	Foliar spray	59 DAS	60 and 90 DAS	Cadmium	Reduces chl, P_N, G_s, Ci, WUE, E, CA activity	Restores chl content, P_N, G_s, Ci, WUE, E, CA activity	Hayat et al. (2010)
Brassica juncea	HBL	10^{-9}, 10^{-8} and 10^{-6} M	Seed soaking (8 h)	Seeds	30 DAS	Copper	Reduces chl, P_N, G_s, Ci, CA activity	Restores chl content, P_N, G_s, Ci, CA activity	Fariduddin et al. (2009)

Raphanus sativus	EBL	10^{-6}, 2×10^{-6} M	Seed soaking (24 h)	Seeds	20 DAS	Cadmium	Reduces Car content, P_N, G_s and CA activity	Restores Car content, P_N, G_s and CA activity	Anuradha and Rao (2009)
Vigna radiata	HBL, EBL	10^{-8} M	Foliar spray	14 DAS	21 DAS	Aluminium	Reduces chl, P_N, G_s, Ci, CA activity	Restores chl content, P_N, G_s, Ci, CA activity	Ali et al. (2008c)
Brassica juncea	HBL	10^{-8} M	Foliar spray	30 DAS	40 DAS	Nickel	Reduces Chl content, P_N, CA activity	Restores Chl content, P_N, CA activity	Alam et al. (2007)
Brassica napus	EBL	10^{-7} M	Incubation with BRs in nutrient solution for 14 days	3 DAS	End of BR treatment	Cadmium	Reduces Chl and Car content	Restores Chl and Car content	Janeczko et al. (2005)

resulting in undersized plants, retarded photosynthetic process and disturbed the PSII assemblage (Kim et al. 2012).

3.1.2 Photosynthetic Pigments

As it is an established fact that chlorophyll is the primary pigment present in plants that absorbs light and utilizes it to synthesize photosynthates. Hence, estimating its content in a leaf could predict the photosynthetic rate (Dalio et al. 2011). BR-mediated increase in chlorophyll (chl) content has been reported in various plants (Bajguz and Czerpak 1998; Gabr et al. 2011; Farazi et al. 2015). The level of Chl increases along with carotenoid (car) content upon BR application (Bajguz and Asami 2005; Janeczko et al. 2005, 2007; Cevahir et al. 2008; Behnamnia et al. 2009; Asha and Lingakumar 2015). BRs increased the chlorophyll content in *Zea mays*, *Vigna radiata*, *Cucumis sativus, Phaseolus aureus, Brassica juncea*, *Cicer arietinum*, *Vicia faba*, *Triticum aesitivum* and *Pelargonium graneoleus* (Braun and Wild 1984; Katsumi 1991; He et al. 1991; Hayat et al. 2001; Abdullahi et al. 2002; Fariduddin et al. 2003, 2004, 2006; Ali et al. 2007; Piñol and Simón 2009; Swamy and Rao 2009; Maity and Bera 2009; Yuan et al. 2012; Alyemeni and Al-Quwaiz 2016). 24-epibrassinolode (EBL) application enhances chlorophyll content in different plant species (Ali and Abdel-Fattah 2006; Çağ et al. 2007). BR application also improved the chloroplast structure (Kulaeva et al. 1991; Sam et al. 2001). This BR-mediated increase in chl content could be a result of enhancement in the activation as well as synthesis of enzymes responsible for the formation of chlorophyll (Behnamnia et al. 2009). Moreover, the increase in chlorophyll content has been correlated with rise in magnesium content in leaves of *Brassica juncea* upon BR treatment (Siddiqui et al. 2018b).

3.1.3 Stomatal Activity

The effect of BRs on stomatal conductance (G_s) is an important aspect to be considered because stomata act as doors for carbon dioxide to enter into the cell. The data related to BR-mediated changes in G_s shows a mixed response. In some studies, there was no significant change in G_s upon BR treatment (Qayyum et al. 2007; Ali et al. 2008a; Shahbaz et al. 2008; Ogweno et al. 2008) whereas in some, G_s increased significantly (Singh and Shono 2005; Fariduddin et al. 2006, 2008, 2009). Yusuf et al. (2014) studied the effect of two different concentrations of 28-homobrassinolide (HBL) on G_s in *Vigna radiata*, both the concentrations were found to enhance the G_s when compared to the normal water sprayed plants. However, the effect was more pronounced when treated with the lower concentration. Likewise, two analogues of BRs were selected (HBL and EBL) having similar concentration to assess the stomatal conductance and change in stomatal pore. Both the analogues succeeded in bringing an elevation in G_s and widening of stomatal pore, but EBL proved to deliver better results over HBL. It was suggested that the rise in the leaf potassium

content promoted the widening of stomatal pore because it is the potassium concentration in and around the guard cell which determines the movement of guard cells. It maintains wider stomatal aperture by adjusting the solute potential of guard cells (Smith and Stewart 1990; Siddiqui et al. 2018b). In many studies, the enhancement in photosynthetic rate was related to the increase in G_s. Conversely, in other studies there was no effect of G_s found on photosynthetic rate. Hence, this fluctuation in the observation might depend on plant species, the analogue selected or on the concentration of a BR analogue for the experiment.

3.1.4 Calvin Cycle and Carbohydrate Metabolism

Rubisco, regulated by rubisco activase is an important C_3 or calvin cycle enzyme that catalyzes the ribulose bisphosphate (RuBP) carboxylation and is also the most abundant protein present on earth. BRs enhance rubisco content, carboxylation rate and RuBP regeneration (Braun and Wild 1984; Portis 1992; Yu et al. 2004; Xia et al. 2009). BRs were found to increase the rubisco activity and various other C_3 cycle enzymes, this effect might be a result of up-regulation of a particular gene that encoded these enzymes. BRs up-regulate different genes encoding various enzymes of calvin cycle such as rubisco large and small sub-unit (*rbcL*; *rbcS*), glycerate P 3- kinase, triose-P isomerase, fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase and ribulose-5-phosphate kinase (Berger et al. 2004; Jiang et al. 2012; Li et al. 2016).

Carbonic anhydrase (CA) catalyses the bicarbonate (HCO_3^-) and carbon-di-oxide inter-conversion which is reversible in nature and shares a close alliance with rubisco in C_3 plants (Sültemeyer et al. 1993; Badger and Price 1994). CA activity in leaves of different plants such as *Brassica juncea, Lycopersicon esculentum, Vigna radiata* and *Cicer arietinum* increased with application of HBL (Hayat et al. 2001; Fariduddin et al. 2003, 2006; Ali et al. 2006; Alam et al. 2007). The increase in CA activity would increase the CO_2 availability around rubisco thus, affecting the carboxylation efficiency. The increase in calvin cycle activity results in enhanced sugar synthesis that is further utilized for growth and metabolism (Siddiqui et al. 2018b).

3.1.5 Net Photosynthetic Rate

Net photosynthetic rate (P_N) could be defined as the net rate of CO_2 uptake per unit area of leaf. Braun and Wild (1984) were amongst the first researchers to evaluate this parameter in presence of BRs. The impact of BRs on P_N has been widely studied worldwide. Bajguz and Czerpak (1998) analyzed the effects of different BRs (brassinoloide (BL), HBL, EBL, castasterone, homocastasterone and 24-epicastasterone) on P_N in *Chlorella vulgaris*. BL was found to be the most active whereas, homocastasterone the least. BL was followed by EBL and HBL, 24-epicastasterone and proved better than homocastasterone in terms of its effects. A group of researchers at Department of Botany, Aligarh Muslim University, India

has studied extensively on PN and reported the positive effect of BRs irrespective of the concentration, mode of application or analogue selected (Siddiqui et al. 2018a). Besides this similar results were also reported by other workers (Singh and Shono 2005; Fariduddin et al. 2003, 2006, 2009; Xia et al. 2006; Hayat et al. 2007; Alam et al. 2007; Qayyum et al. 2007; Farooq et al. 2009; Siddiqui et al. 2018b). HBL application promotes P_N, chl content along with total sugar content (Eskandari and Eskandari 2013). All the different modes of BR application (seed soaking, root dipping or foliar spray) proved effective in enhancing photosynthetic rate. This effect could be attributed to BR-mediated rise in internal CO_2 concentration (C_i) and G_s. The BR-mediated increase in chlorophyll content along with stomatal conductance, CO_2 assimilation, rubisco and CA activity act constitutively to promote P_N (Hayat et al. 2011; Gruszka 2013) which could be confirmed by a rise in sugar level upon BR treatment (Siddiqui et al. 2018b).

Hence, it could be concluded that BRs regulate photosynthesis at various levels under normal conditions. BRs promote PSII efficiency and electron transport rate resulting in enhanced production of NADP and ATP that are utilized during calvin cycle and other processes. Alongside, it also promotes the C_3 cycle enzyme activity and the accumulation of sugars and ultimately, elevating the photosynthetic efficiency of the plants.

4 BR-Mediated Regulation of Photosynthesis Under Stress

Any external factor negatively influencing the plant growth and productivity thereby, making the conditions difficult for survival of crop is termed as a condition of stress (Rhodes et al. 2002). The regulatory effect of BRs on photosynthesis prompts to analyze the BR-mediated regulation of photosynthesis under stress conditions.

4.1 Salinity Stress

Soil is considered to be saline if it possesses electrical conductivity of 4 dS m^{-1} or even higher. Areas lying in the arid or semi-arid zones are the mostly affected by salinity and limiting the crop biomass and productivity (Flowers 2004; Koca et al. 2007). Salt stress results in the increase in toxic Na^+ concentration leading to disintegration of chlorophyll molecule thereby, reducing the chl content in plants (Yang et al. 2011). Salt stress promotes the synthesis of chl degrading enzymes (Reddy and Vora 1986). Salt stress reduced chlorophyll content in different plants like *Helianthus annuus, Triticum aestivum, Cicer arietinum, Brassica juncea*, *Ricinus communis* (Ashraf and Sultana 2000; Arfan et al. 2007; Ali et al. 2007, 2008b; Pinheiro et al. 2008; Perveen et al. 2010). Conversely, BR application restores the pigment loss (Anuradha and Rao 2003; Sharma et al. 2013). In *Lactuca sativa* all the concentrations of the BR used alleviatd the toxic effects of NaCl (Ekinci et al. 2012). Plants

given a sole HBL treatment without any stress grossed the highest values for P_N. Similarly, BR treatment in saline stressed *Brassica juncea* and wheat (Hayat et al. 2007; Ali et al. 2008a; Alyemeni et al. 2013; Siddiqui et al. 2018c) resulted in the reversal of destructive effects of salinity on P_N and its related attributes like G_s, transpiration rate (E) and water use efficiency (WUE). Dubey (2005) proposed that BR-mediated improvement in photosynthesis to be a result of change in either the stomatal factors or the non-stomatal ones. Likewise, EBL application mitigated the inhibitory effects of salinity on photosynthesis and related parameters in two wheat cultivars (Shahbaz et al. 2008).

4.2 Drought Stress

Drought stress is the condition where plant suffers scarcity of water to such an extent that situation gets hostile for survival of plant (Zhu 2001). Stress leads to elevation in generation of ROS (Sofo et al. 2005). Carotenoids, preventing photooxidative damage of chlorophyll decreases during stress but gets restored upon BR application. HBL restores the values for relative water content (RWC), chl and P_N in *Triticum aestivum* during drought (Sairam 1994 a, b). Similarly, P_N, G_s, C_i suffered reduction in their values in presence of water stress, however, follow-up treatment with EBL alleviated the toxicity of drought stress (Yuan et al. 2012). A dip in various photosynthetic parameters was observed in *Oryza sativa, Capsicum annum* and *Glycine max* subjected to drought stress, however, BR application proved useful in mitigating the harmful effects generated by drought stress (Zhang et al. 2008; Farooq et al. 2010; Li et al. 2012; Hu et al. 2013).

4.3 Thermal Stress

4.3.1 Heat Stress

In the present scenario, the danger of high temperature stress to crops is increasing day by day due to increase in global warming throughout the world (Hopkins 1995). Heat stress disrupts the integrity of plasma membrane and increases its permeability resulting in water loss, disturbance of leaf water potential and photosynthesis (Berry and Bjorkman 1980; Simões-Araújo et al. 2003; Zhang et al. 2005). BR induces thermotolerance in plants by protecting the degradation of chlorophyll and maintains its level (Singh and Shono 2005). The decrease in G_s, net CO_2 assimilation rate, E and P_N owing to heat stress got restored upon BR application (Singh and Shono 2005; Thussagunpanit et al. 2015). Abscisic acid (ABA) also known as stress hormone, increases in the presence of heat stress, indicating the necessity of ABA synthesis for tolerance against heat stress (Maestri et al. 2002). It could be attained by the activation of heat shock proteins (Pareek et al. 1998). BR promotes the

synthesis of ABA (Bajguz 2009) which will help to increase the heat tolerance. The P_N, G_s, C_i, PSII efficiency and qP decreased in the presence of heat stress, however, application of BR reversed the effects of stress (Ogweno et al. 2008).

4.3.2 Low Temperature Stress

The exposure of plant to low temperature disturbs the electron transport and carbon dioxide supply required in carbon reduction cycle which ultimately disturbs photosynthesis. Alongside, it also increases lipid peroxidation leading to water imbalance (Allen and Ort 2001). Hamada (1986) identified the potent role of BRs in presence of chilling stress. Cold treatment is capable of reducing chlorophyll content however; BR treatment prevents chlorophyll loss by inducing the enzymes responsible for the formation of chlorophyll (Wise and Naylor 1987; Hayat et al. 2007).

EBL increases chlorophyll content along with sugar contents both in the presence/absence of cold stress (Singh et al. 2012). Chilling stress reduced chlorophyll content, *Fv/Fm*, P_N and G_s, Ci, WUE, and *E* but upon giving HBL treatment all these parameters got restored (Fariduddin et al. 2011). PSII also suffers a loss during low temperature, however, BR application helps in reviving the plant metabolism and alleviated the inhibitory effects of low temperature (Wu et al. 2014). Thus, it could be concluded that EBL is capable of mitigating toxicity generated by low temperature via photosynthesis regulation. Janeczko et al. (2007) proposed that BR possess defensive properties against photosynthetic pigment degradation and membrane leakage caused by chilling conditions. In *Secale cereale* (winter resistant cultivar) EBL application increases photosynthetic efficiency and rubisco activity but decreases the total carbohydrate level under low temperature stress (Pociecha et al. 2016, 2017).

4.4 Heavy metal stress

The metals which possess a density above 5 g cm^{-3} are termed as heavy metal (Weast 1984). BRs prevent the heavy metal accumulation in plant parts moreover, it also curtail the toxicity symptoms generated by heavy metals (Bajguz and Hayat 2009).

Cadmium (Cd) is a severe toxic metal that easily accumulates and get translocated in plant parts, impeding the process of chlorophyll biosynthesis, perturbs cell water balance, promotes closing of stomata and ultimately retards the photosynthetic rate (Poschenrieder et al. 1989; Barceló and Poschenrieder 1990; Sheoran et al. 1990; Chugh et al. 1992; Singh and Tewari 2003). Cd accumulation in leaf severely inhibits the activity of protochlorophyllide reductase (enzyme involved in chlorophyll biosynthesis) probably by blocking the reductase protein at sulphydryl position (Ernst 1980; Stobart et al. 1985). Furthermore, it promotes chlorophyllase (chlorophyll degrading enzyme) activity (Reddy and Vora 1986). Thereby, reducing

the total chlorophyll content and retarding the photosynthetic processes (Vassilev and Yordanov 1997; Rady 2011). Degradation of chlorophyll and decrease in rubisco activity leads to photosynthesis reduction in the presence of stress (Adak and Gupta 1999; Pandey et al. 2001). Cadmium reduces chlorophyll content, relative water content and P_N though HBL application mitigates the toxicity symptoms (Hayat et al. 2007). Cd-mediated closing of stomata reduces the partial pressure of CO_2 in the sub-stomatal chamber thereby, reducing the G_s, *Ci* and *E* which constitutively disturbs processes leading to a decline in photosynthetic rate (Barceló and Poschenrieder 1990). PSII efficiency, SPAD chlorophyll and P_N declined severely in *Vigna radiata* seedlings upon exposure to cadmium stress but when given a follow-up treatment of BR, the damage was partially restored (Hayat et al. 2010). Low CO_2, decrease in SPAD chlorophyll and CA activity are additional factors contributing in lower P_N rate (Hayat et al. 2012).

Radish plants suffered a decline of about 48% in P_N exposed to Cd stress over the control plants. Closure of stomata appeared as a factor responsible for reduced photosynthetic rate amidst high level of stress. Treating the seeds with EBL promotes P_N and alleviates the toxicity generated by Cd. EBL was found capable of promoting chlorophyll and P_N even in the presence of cadmium stress (Anuradha and Rao 2009). Exposure to Cd marked the reduction in photosystem II active RC and electron transport rate (about 21% and 17%, respectively). On the whole, activity of oxygen evolving complex got reduced by 19% whereas, heat dissipation increased by 15%. When seedlings were cultured on medium with EBL in absence of Cd, stimulation of most of the photochemical reactions was observed however, the increase was minimal in comparison to the ones grown with Cd and EBL enriched medium. The reason could be a change in specific energy and photosynthetic electron transport. EBL protected the activity of O_2 evolving complex and energy loss in the presence of Cd. Hence, it confirmed the protective role of EBL on primary photochemistry of plants against Cd stress (Janeczko et al. 2005). A sharp decline in P_N, G_s, C_i, E and WUE observed in *Lycopersicon esculentum* due to cadmium, however, these decrease was partially reversed by the application of BR (Hayat et al. 2010).

Nickel (Ni) is one of the micronutrients essential for normal growth of plants. However, when present above a certain limit it starts acting as a toxic metal and induces injuries at cellular level and hampers the normal execution of different metabolic pathways, and in severe cases it might lead to death of the plant. Various anthropogenic activities like sewage sludge, metal waste disposal, pesticide/fertilizer use and combustion of fuels are few examples that serve as a source of nickel to plants (Khan et al. 2008; Zhang et al. 2010). Nickel dislocates Mg ion that serve as an integral part of pyroll ring of chlorophyll molecule, moreover, it also disrupts the electron transport rate thus, affecting the photosynthesis (Mohanty et al. 1989; Chen et al. 2009). It was observed that the level of chlorophyll, PSII efficiency and P_N in the plants treated with Ni was reduced, however, upon treating with HBL the damage was partially overcome (Alam et al. 2007; Yusuf et al. 2014).

Copper (Cu) is found in close association with fertilizers, fungicides and pesticides that are applied to soil and excess of Cu proves hazardous for survival of

plants (Chen et al. 2000). Cu is found in two ionic states i.e. Cu^{+} and Cu^{2+} and act as an indispensable component of regulatory protein composition, active participant of Kreb's cycle, electron transport during photosynthesis, generating stress response, hormonal signalling and cell wall metabolism (Marschner 1995; Raven et al. 1999). Enzymes like superoxide dismutase, cytochrome C oxidase, amino oxidase, and polyphenol oxidase possess Cu ions associated to them as a cofactor. Beyond the tolerable limit, Cu acts as a lethal metal and inhibits photosynthesis in plants (Kupper et al. 2009). It induces the generation of free radicals (Halliwell and Gutteridge 1984) that hinders with normal functioning of cell, damages cell organelles and inhibits metabolic reactions (Wolff et al. 1986).

Photosynthetic parameters like P_N, G_s, C_i, WUE, and E got reduced significantly in *Cucumis sativus* when exposed to Cu. However, degradation in leaf gas exchange parameters was overcome by EBL. Decrease in F_v/F_m ratio due to Cu stress was also surmounted by EBL application (Fariduddin et al. 2013). Similarly, HBL neutralized the harmful effect of Cu and restored the photosynthetic parameters (G_s, C_i, WUE, P_N, E) in *Brassica juncea* (Fariduddin et al. 2009).

4.4.1 Other Heavy Metals

Chromium (Cr) is extensively used in textile, plating and alloy industries (Avudainayagam et al. 2003) and its ample use in various anthropogenic activities leads to contamination of environment (Zayed and Terry 2003). Cr stress reduces chlorophyll and carotenoid content, and also disturbs the PSII assembly leading to decline in PSII efficiency; however, EBL alleviates the toxicity generated by Cr (Choudhary et al. 2012).

Aluminium (Al) is another heavy metal that generates toxicity in plants and limits the growth and development of plant. High solubility at a lower pH generates toxicity symptoms that are more pronounced in acidic soils (Mossor-Pietraszewska 2001). Al stress decreases the G_s, C_i RWC, WUE, chlorophyll content, CA activity and ultimately, P_N, however, BRs mitigates the toxicity generated by Al (Ali et al. 2008c). Similarly, lead (Pb) also proves to be a toxic heavy metal and reduces the chlorophyll content and P_N in *Trigonella foneu-graecum*, however, upon treating with BRs, the toxicity symptoms could be surmounted (Swamy et al. 2014). Mercury (Hg), famous for the occurrence of fatal minimata disease due to its toxicity and when enters the plant through foliar application, it destroys the photosynthetic pigments but EBL application reduced the toxic effects of Hg (Kapoor et al. 2014). EBL also protects the photosynthetic apparatus of *Brassica juncea* from selenium toxicity (Naz et al. 2015).

5 A Novel Mechanism Elucidating BR-Mediated Regulation of Photosynthesis

In simple terms, the synthesis of carbohydrates using solar energy by plants is termed as photosynthesis. It is a highly regulated process. Different components act together to facilitate this process, a possible mechanism underlying the BR-mediated regulation of photosynthesis has been discussed in the section below:

5.1 Primary Photochemistry

BR application increases the light absorbing chlorophyll content (Fariduddin et al. 2000; Swamy and Rao 2009) which consequently, increases the light absorbing capacity. This energy captured by chlorophyll is used to split water to oxygen for the release of electron in PSII and this is the only step on the earth's atmosphere where generation of oxygen takes place (Gururani et al. 2012; Tikkanen and Aro 2014). There is sequential electron transport (PSII to PSI) where, NADP is reduced to NADPH (reducing power) meanwhile, synthesis of ATP also occurs during the electron transport (Nellaepalli et al. 2014). Reactive oxygen species (ROS) generation is a side product of these reactions in PSII, and this ROS generation further increases under the stress (Takahashi and Badger 2011; Noctor et al. 2014). ROS destabilizes D1 protein that participates in PSII repair mechanism (Nishiyama et al. 2011; Nath et al. 2013). BRs possess the ability of promoting the stabilization of D1 protein and antioxidant enzymes (superoxide dismutase, peroxidase and catalase) activities thus, enhances the PSII efficiency and photosynthetic CO_2 fixation under normal conditions and also protects the photosynthetic machinery in presence of stress (Oh et al. 2010; Yuan et al. 2012; Siddiqui et al. 2018c; Fig. 7.1). Moreover, ROS generated by PSII that leads to photoinhibition of PSII and PSI is also reduced by BR application (Siddiqui et al. 2018b; Fig. 7.1).

NADPH formed is directed towards calvin cycle where oxidation of NADPH to NADP takes place in presence of carbon dioxide and calvin cycle enzymes, to produce photosynthates (Fig. 7.1). After getting oxidized, NADP is again readily available to accept electron in PSI. Hence, BR-mediated increase in PSII efficiency, ETR and ultimately, the NADPH production that increases NADPH availability for calvin pathway leading to sugar synthesis.

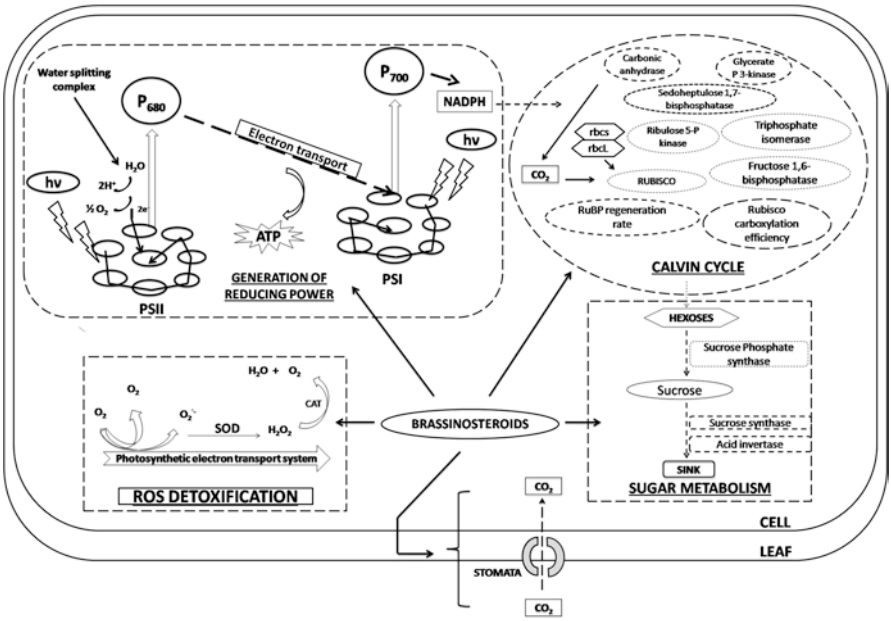

Fig. 7.1 BR mediated regulation of photosynthesis

5.2 Carbohydrate Synthesis

BRs induces calvin cycle enzyme (rubisco, glycerate P 3- kinase, triose-P isomerase, fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase and ribulose-5-phosphate kinase) encoding genes consequently, increasing the synthesis of sugars (Jiang et al. 2012; Li et al. 2016). In a BR biosynthetic mutant, d^{im} carboxylation efficiency of rubisco ($V_{c,max}$) and ribulose bisphosphate (RuBP) regeneration rate (J_{max}) decreases and conversely, over-expression of a BR biosynthetic gene, *Dwarf* encoding *CYP85A1*, considerably promoted the $V_{c,max}$ and J_{max} (Li et al. 2016). Thus, indicating the potential of BRs in the regulation of $V_{c,max}$ and $J_{max.}$

BRs promote the activity of rubisco and sucrose-P-synthase (SPS; sucrose transporting enzyme) in *Lolium perenne* L. prompting the BR-mediated control of dark reaction (Pociecha et al. 2016, 2017). BRs treatment promotes the synthesis of sucrose, soluble sugars and starch as a consequence of increase in the activities of sucrose synthase (SS), SPS, and acid invertase (Yu et al. 2004; Fig. 7.1). BRs increase the CA activity which catalyses the inter-conversion of HCO^{3-} to CO_2 for the RuBP carboxylase and CO_2 to HCO_3^- for phosphoenol pyruvate carboxylase (Moroney et al. 2001; Yusuf et al. 2014). G_s and stomatal aperture is increased by BRs (Hayat et al. 2011; Siddiqui et al. 2018b) is suggested the chance of allowing more CO_2 entry is directly proportional to the number of open stomata (Serna et al. 2012).

6 Conclusions

After summarizing the data concerning the potential of BRs in enhancing the photosynthesis, it could be suggested that BRs promote both light and dark reactions. It enhances primary photochemical reactions, photosynthetic pigments and also increases the stomatal functioning to promote the CO_2 entry into the cells. The enhanced activity of enzymes of calvin cycle along with carbonic anhydrase act together to increase the production of more photosynthates. Moreover, the enzymes involved in source to sink partitioning of photosynthates are also activated by BRs. Thus, the BR-mediated rise in photosynthetic efficiency enhances the overall growth and development of plants (Fig. 7.1).

Despite of abundant research on BR-mediated regulation of photosynthetic attributes there is still gap in the research and a detailed study related to the effect of BR on chloroplast development, PSI functioning as most of the research is confined to PSII functioning only, is needed. Besides these, the effects of BR on photosynthetic efficiency of C_4 and CAM plants could also be established. Application of molecular techniques like transcriptomics and proteomics could be used to understand the BR-signalling and BR-regulated processes more clearly.

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Chapter 8
Genetic and Molecular Bases of Brassinosteroid Metabolism and Interactions with Other Phytohormones

Damian Gruszka

Abstract Brassinosteroids (BRs) regulate diverse physiological processes during plant life cycle. Recent years have witnessed a significant progress in elucidating various aspects of BR biosynthesis and signaling, which was achieved through genetic, biochemical and physiological analyses of mutants isolated in model and crop species. Mechanisms of BR biosynthesis and signal transduction are interconnected with pathways of biosynthesis and signaling of other phytohormones. These interactions form a complicated network of dependencies and enable a coordinated regulation of the various physiological processes. It was also reported that components of the BR signaling pathway, playing roles of both positive or negative regulators of the process, are involved in mechanisms of plant response to various stimuli and stress conditions. This fine-tuning of plant physiological reactions to various stimuli allows a balance between growth rate and stress response to be achieved. The process of identification of new components of the BR signalosome is still ongoing, and functional analysis of the new components broadens the view of the complicated network of hormonal interactions. The chapter presents genetic and molecular aspects of the BR biosynthesis and signaling and interactions with other phytohormones, which mediate physiological processes in plants.

Keywords Brassinosteroid biosynthesis · Brassinosteroid signaling · Genetic regulation · Hormonal interactions · Metabolism · Mutants

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1 Introduction

Intensive genetic, biochemical and physiological studies, which have been conducted for almost three decades in various laboratories all over the world led to identification of enzymes involved in the brassinosteroid (BR) biosynthesis and signaling pathways. These processes were elucidated to the greatest degree in the model plant species *Arabidopsis thaliana,* whereas our knowledge about their progress in other species, including crops is rather limited (Vriet et al. 2012; Zhang et al. 2014a; Corvalan and Choe 2017). BRs are a class of polyhydroxylated steroid phytohormones and their biosynthesis is a part of the broader process – biosynthesis of sterols (Altmann 1998; Clouse and Sasse 1998). Sterol biosynthesis pathway splits into two branches, the first leads to biosynthesis of sitosterol and stigmasterol, which constitute crucial components of cellular membranes, whereas the second pathway leads to the BR biosynthesis (Lindsey et al. 2003; Schaller 2003).

Majority of the Arabidopsis genes encoding enzymes catalyzing various steps of the sterol and BR biosynthesis processes have been identified and their molecular functions were characterized together with phenotypic description of the identified mutants (Vriet et al. 2012). In the following years some of the homologous genes involved in the BR biosynthesis have also been identified in other plant species, including crops: *Gossypium hirsutum* (cotton) (Luo et al. 2007), *Zea mays* (maize) (Hartwig et al. 2011; Makarevitch et al. 2012), *Pisum sativum* (pea) (Nomura et al. 2004, 2007; Jager et al. 2007), *Oryza sativa* (rice) (Hong et al. 2002; Mori et al. 2002; Hong et al. 2003; Sakamoto et al. 2006), *Solanum lycopersicum* (tomato) (Bishop et al. 1999; Nomura et al. 2005; Lisso et al. 2006) and *Hordeum vulgare* (barley) (Gruszka et al. 2011a; Dockter et al. 2014; Gruszka et al. 2016a). Functional analyses of the genes allowed for phenotypic characterization of the identified mutants, which show various degree of growth reduction.

The molecular mechanisms of the BR perception and signal transduction from the transmembrane receptor complex through a complicated cascade of phosphorylation and dephosphorylation, up to BR-regulated gene expression have been studied intensively for the last two decades, which now renders BR signaling the best characterized molecular relay in plants (Kim and Wang 2010; Gruszka 2013; Li et al. 2016; Vukasinonic and Russinova 2018). Numerous components of the BR signaling have been identified in Arabidopsis, which was achieved through mutant identification via chemical mutagenesis, activation tagging, T-DNA insertional mutagenesis, gene overexpression and RNAi-mediated gene silencing, as well as genetic analysis of the identified single and multiple mutants (Vriet et al. 2012). The extensive studies on the BR perception and signaling in Arabidopsis allowed mutants in the homologous genes to be identified in other species: rice (Yamamuro et al. 2000; Morinaka et al. 2006; Nakamura et al. 2006; Bai et al. 2007; Koh et al. 2007; Li et al. 2009; Tanaka et al. 2009; Zhang et al. 2009a), barley (Chono et al. 2003; Gruszka et al. 2011b; Dockter et al. 2014), pea (Nomura et al. 1997, 1999, 2003; Ferguson et al. 2005) and tomato (Koka et al. 2000; Montoya et al. 2002).

It is becoming evident that the BR-dependent regulation of the broad range of morphogenetic and physiological processes is feasible through a complicated network of interactions of the components mediating the BR biosynthesis and signaling pathways with factors regulating metabolism of other phytohormones. This intricate crosstalk enables maintenance of the inter-hormonal homeostasis, but also allows an efficient reaction of plant physiology to constantly changing environmental conditions.

2 Genetic Regulation of the BR Biosynthesis

The BR biosynthetic pathway was initially described biochemically using cultured cells of *Catharanthus roseus* (Fujioka and Yokota 2003). As mentioned above, the BR biosynthesis is a part of the sterol biosynthesis pathway (Lindsey et al. 2003; Schaller 2003). Later on, the process of BR biosynthesis has been described to the greatest extent in Arabidopsis through physiological, genetic and biochemical approaches conducted on BR-deficient mutants (Fujioka and Yokota 2003; Bishop 2007; Ohnishi et al. 2012; Vriet et al. 2012). Interestingly, several enzymes mediating the BR biosynthesis have a broad substrate specificity, therefore they catalyze conversions of various intermediates at multiple steps in the pathway (Dockter et al. 2014). The first intermediate, which is specific for the BR biosynthesis pathway is episterol. This compound is converted by the Δ^7-sterol-C5-desaturase encoded by the *STE1/DWF7/BUL1* gene to 5-dehydroepisterol (Choe et al. 1999a). This intermediate is a substrate for the $\Delta^{5,7}$-sterol-Δ^7-reductase encoded by the *DWF5* gene and is converted to 24-methylenecholesterol (Choe et al. 2000; Schaller 2003). The latter is converted by the Δ^5-sterol-Δ^{24}-reductase encoded by the *DIM/DWF1* gene in a two-step reaction to campesterol (Choe et al. 1999b; Dockter et al. 2014). A recent study showed that the DWF1 enzyme has both isomerase and reductase activities catalyzing various reactions in the BR biosynthetic pathway (Youn et al. 2018). At the stage of campesterol synthesis the linear BR biosynthesis pathway splits into several sub-pathways. It is known that the BR biosynthesis is composed of three sub-pathways: the C-22 oxidation pathway, the late C-6 oxidation pathway and the early C-6 oxidation pathway. These sub-pathways are interconnected at various enzymatic steps, which constitutes an intricate network of reactions (Fujioka et al. 2002). Moreover, several enzymes e.g. CPD (C-23α-hydroxylase/C-3 dehydrogenase), DET2 (5α-reductase), DWF4 (C-22 hydroxylase), ROT3 and CYP90D1 (C-23 hydroxylases), as well as BR6ox1 and BR6ox2 (C6-oxidases) have broad substrate specificity, therefore they catalyze multiple reactions in the pathway (Ohnishi et al. 2006, 2012; Dockter et al. 2014) (Fig. 8.1). In Arabidopsis the final product of the BR biosynthesis is brassinolide, which is produced by conversion from castasterone (Shimada et al. 2001; Kim et al. 2005a). Both castasterone and brassinolide are active forms of BR, however castasterone shows only about 10% of the activity of brassinolide (Kinoshita et al. 2005). In monocots castasterone seems to be the final product of the BR biosynthesis (Kim et al. 2008). However, it has

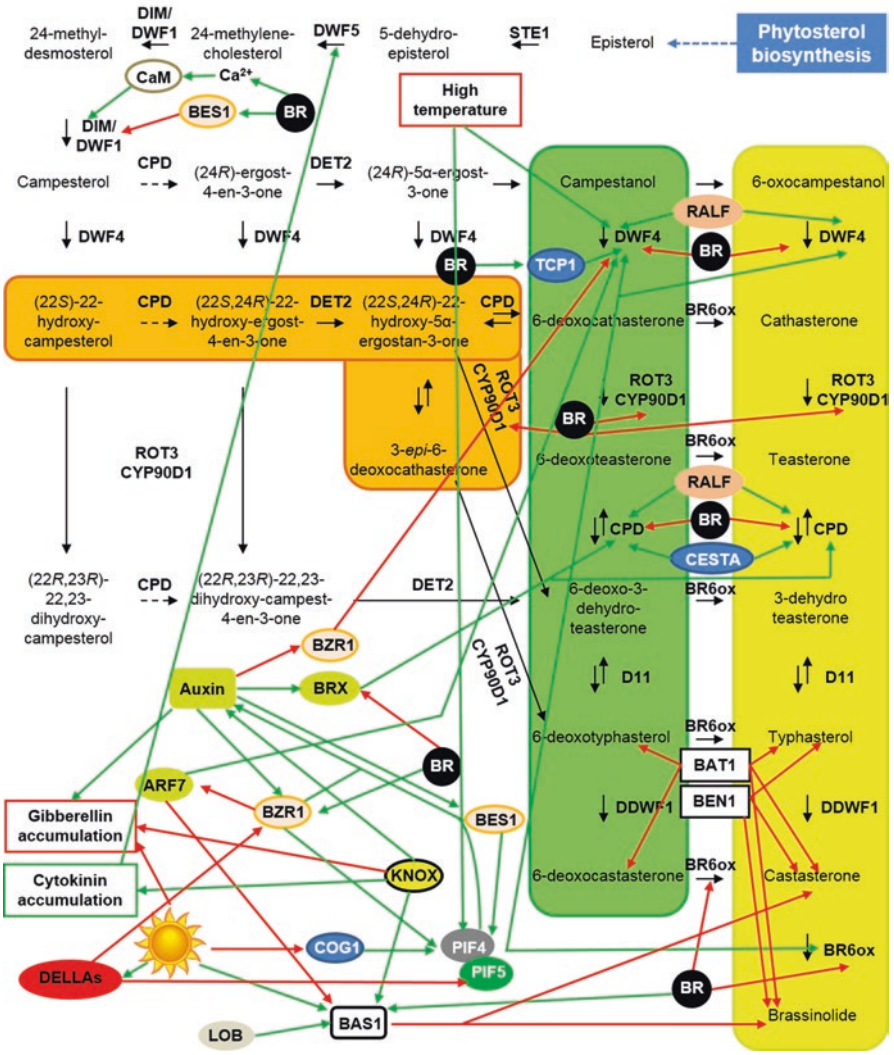

Fig. 8.1 The BR biosynthetic pathway and mechanisms regulating the BR accumulation. The C-22 oxidation pathway is highlighted in orange, the late C-6 oxidation pathway is marked in green, and the early C-6 oxidation pathway is marked in yellow. Names of the enzymes catalyzing different reaction steps are indicated next to the black arrows. Dashed lines indicate that a multifunctional enzyme catalyzes more than one enzymatic reaction. The scheme presents network of molecular interactions, which regulate the BR metabolism in a crosstalk with other phytohormones and in reaction to environmental cues. Details are given in the text. Green arrows denote a stimulating effect, whereas red arrows represent a negative, suppressive influence

been recently reported that in barley an accumulation of 24-epibrassinolide (another biologically active form of BR) was stimulated by drought, however at relatively low concentrations (Gruszka et al. 2016b).

In Arabidopsis, mutants defective in the BR biosynthesis were identified mainly based on abnormalities in skotomorphogenesis (etiolation test) and various degree of plant growth reduction (Kauschmann et al. 1996; Li et al. 1996; Szekeres et al. 1996; Clouse et al. 1996; Schaller 2003; Du et al. 2017). Mutations identified in the *STE1/DWF7/BUL1* gene in *A. thaliana* caused dwarf phenotype of mutants, whose height did not exceed 14% of the WT plants. Moreover, the mutants showed reduced fertility, prolonged lifespan, dark-green and wavy leaves and disturbances in localization of tracheary elements (Husselstein et al. 1999; Choe et al. 1999a). The dwarf phenotype of the mutants was caused by a defect in cell elogation, which was associated with abnormalities in formation of a spatial structure of cortical microtubules (Catterou et al. 2001a, 2001b). The mutant phenotype was caused by nonsense mutations localized in the first and third exons of the *STE1/DWF7/BUL1* gene, which rendered the encoded enzyme nonfunctional (Choe et al. 1999a).

Similar phenotypes were observed in Arabidopsis mutants, which carried alterations in the *DWF5* gene sequence. The mutants showed also abnormalities in seed development and germination. The identified mutantions included changes at the splicing sites (alleles *dwf5-2* and *dwf5-6*), nonsense substitutions (alleles *dwf5-3* and *dwf5-5*), a change of a highly conserved amino acid residue (*dwf5-4*) and a 1-bp deletion affecting transcript stability in the *dwf5-1* mutant (Choe et al. 2000).

Mutations identified in the *DWF1/DIM* gene in *A. thaliana*, also resulted in dwarf phenotype, the mutant plants showed reduced fertility, changes in leaf morfology, prolonged lifespan and abnormalities in etiolation. The dwarf phenotype was associated with a reduced cell elongation, which was caused by decreased expression level of the genes encoding tubulins and enzymes involved in modification of the cell wall structure. Several of the mutations identified in the *DWF1/DIM* gene are nonsense mutations (*dwf1-1*, *dwf1-2*, *dwf1-3*, *dwf1-4*, *dwf1-5*, *dwf1-9*), four mutations led to changes of highly conserved amino acid residues (*dwf1-7*, *dwf1-8*, *dwf1-10*, *dwf1-11*), one mutation (*dwf1-6*) was caused by an insertion of the Ac/Ds element (Takahashi et al. 1995; Klahre et al. 1998; Choe et al. 1999b).

Mutation in the *CPD* gene in *A. thaliana* was induced by insertional mutagenesis (T-DNA insertion) in the first exon of the gene (Szekeres et al. 1996; Fujioka and Yokota 2003). The *cpd* mutant showed de-etiolation during growth in darkness, which was associated with induction of expression of the genes encoding polypeptides involved in photosynthesis: RuBisCO and chlorophyll binding proteins. The mutant showed significant reduction of cell elongation, abnormalities in differentiation of tracheary elements, defects in leaf morphogenesis and sterility (Szekeres et al. 1996). Recently identified allele, *cpd91*, harbors a T-DNA insertion within the fifth intron of the gene and causes a severe dwarf phenotype of the mutant plants (Du et al. 2017).

A series of alleles in the *DET2* gene in Arabidopsis was isolated through chemical mutagenesis. Two alleles (*det2-1* and *det2-6*) contained missense mutation (E204K), four of the identified alleles (*det2-3*, *det2-4*, *det2-7* and *det2-8*) carried

various deletions distributed in different parts of the gene, whereas in two alleles (*det2-2* and *det2-5*) nonsense mutations were identified in different parts of the gene (Li et al. 1996). These mutations led to phenotypic feature, which proved to be specific for BR mutants – de-etiolation during growth in the dark, which was associated with a decrease in hypocotyl length, cotyledon development and expansion, initiation of leaf development and anthocyanin accumulation. During development under normal light conditions, the mutants showed reduced plant growth, dark green leaves, prolonged lifespan, decreased apical dominance and fertility reduction (Chory et al. 1991; Noguchi et al. 1999).

Genetic analysis of the Arabidopsis *DWF4* gene, led to identification of mutants, which showed typical BR-related phenotypic features, resulting from the defect in cell elongation. The mutant plants showed de-etiolated phenotype during growth in the dark, delayed flowering and senescence, shorter siliques and lack of fertility. Two of the alleles were obtained through T-DNA insertion, whereas the other alleles contained a 9-bp deletion (*dwf4-2*) leading to a change in sequence of the encoded polypeptide, and a nonsense substitution (*dwf4-3*) leading to formation of a truncated version of the polypeptide, devoid of crucial functional domains (Azpiroz et al. 1998; Choe et al. 1998; Vriet et al. 2012). In the recently identified alleles *dwf4-96* and *dwf4-44* the T-DNA insertions were identified within the seventh intron of the gene. The insertions led to various alterations in plant phonotype (Du et al. 2017).

A *knockout* mutation of the *CYP90D* gene by the T-DNA (*cyp90d1*) insertion did not cause any significant phenotypic effects. Similarly, transgenic plants in which an antisense *CYP90D* construct was expressed did not lead to any changes in plant phenotype. The BR-related phenotypic effect (severe dwarf phenotype) was only reported upon induction of double mutations in both the *CYP90D* and *ROT3* genes. Therefore, it is suggested that the genes play functions in the BR synthesis redundantly (Kim et al. 2005b). A further confirmation of the redundant function of the genes is their parallel participation in the alternative BR biosynthesis pathway, proceeding through 22-oxo-BR intermediates (Ohnishi et al. 2006). Proteins encoded by the *CYP90D* and *ROT3* genes show the highest level of sequence similarity among the P450 cytochrome family, what suggests that both these genes derive from a common ancestral sequence. Biochemical function of the CYP90D enzyme was validated with use of the BR biosynthesis intermediates. Moreover, it was reported that the *CYP90D* gene expression is attenuated by the exogenous BR treatment, which is a typical feature of genes encoding BR biosynthetic enzymes (Bancos et al. 2002; Goda et al. 2002; Kim et al. 2005b).

The *DWF11* gene, which encodes the CYP724B1 enzyme producing 6-deoxotyphasterol and typhasterol during the late and early C-6 oxidation pathway, respectively was identified only in rice. Function of the CYP724B1 enzyme was determined based on BR intermediates application experiments. Several mutations of the gene were also identified, which included 1-bp deletion in the second exon (*d11-1*), 1-bp insertion in the seventh exon (*d11-2*), a substitution of highly conserved amino acid (Thr>Ile) in the fourth exon (*d11-3*) and substitution in the third intron, leading to perturbation in splicing (*d11-4*). In the *d11-1*, *d11-2* and

d11-4 alleles, the identified mutations caused premature stop codon occurrence. The mutant plants showed erect stature, shortened internodes and reduced grain size (Tanabe et al. 2005).Various mutations which were identified in the *ROT3* gene in Arabidopsis, including 1-kbp deletion (*rot3-1*), substitution of a highly conserved glycine (*rot3-2*) or T-DNA insertion in the promoter region of the gene (*rot3-3*) entailed a defect in the elongation growth, which was particularly apparent during leaf development. A specific feature of these mutants was normal skotomorphogenesis (growth in the dark), whereas the above-mentioned dark-grown BR-deficient mutants showed de-etiolation during growth in the dark (Tsuge et al. 1996; Kim et al. 1998).

Function of the CYP92A6 enzyme which catalyzes production of 6-deoxocastasterone and castasterone during the late and early C-6 oxidation pathway, respectively has been described in pea (*Pisum sativum*). The enzyme is encoded by the *DDWF1* gene, and the encoded enzyme interacts with the GTP-binding polypeptide Pra2. The expression of both enzymes is inhibited by light, on the other hand their expression is stimulated in the dark. Interaction between these two enzymes may constitute one of mechanisms of the molecular transition between the processes of etiolation and de-etiolation (Clouse 2001; Kang et al. 2001).

A *knockout* mutation caused by a T-DNA insertion in the Arabidopsis *BR6ox1* gene did not evoke any significant phenotypic effects. Similarly, the same type of mutation induced in the paralogous gene *BR6ox2* did not result in any change in plant stature either (Shimada et al. 2001; Kim et al. 2005a). This suggests that in Arabidopsis both genes play redundant functions in the C-6 oxidation and during growth and development (Shimada et al. 2001; Castle et al. 2005; Nomura and Bishop 2006). Isolation of the double mutant *br6ox1br6ox2* resulted in a dwarf phenotype (Kwon et al. 2005; Nomura et al. 2005). Both genes most probably originated in a duplication event, as in their close vicinity in the Arabidopsis genome transposable elements were localized, whose recombination could result in the duplication (Castle et al. 2005). In the genome of tomato two homologous genes *LeBR6ox1* and *LeBR6ox3* were identified (Nomura et al. 2005). A *knockout* mutation in the *LeBR6ox1* gene resulted in dwarf phenotype (Bishop et al. 1999). This phenotype is caused by the fact that the *LeBR6ox3* gene is expressed exclusively in fruits (Nomura et al. 2005), which results in a lack of redundancy of these genes in the vegetative tissues (Kim et al. 2004; Montoya et al. 2005). In contrast to Arabidopsis and tomato, in the pea genome two genes which encode C6-oxidases were identified. Both enzymes synthesize castasterone, however none of them can produce brassinolide (Jager et al. 2007). Contrary to genomes of the above-mentioned dicot species (Arabidopsis, tomato and pea), it has been reported that rice and maize genomes contain one copy of the gene encoding BR-6-oxidase (Nelson et al. 2004; Kim et al. 2008; Makarevitch et al. 2012). In rice, deletions identified in the *OsDWARF* gene led to profound impairment of the encoded polypeptide and consequently to a severe dwarf phenotype (Hong et al. 2002; Mori et al. 2002). Similarly, in the maize homologous *ZmBrd1* gene a single-nucleotide substitution introducing a premature stop codon caused a profound truncation of the protein, which resulted in severe dwarf phenotype and sterility of mutants (Makarevitch

et al. 2012). Interestingly, genome of another monocot crop species, barley, contains two genes encoding enzymes catalyzing the C-6 oxidation reaction. Both these genes (*HvDWARF* and *HvBRD*) are located in close vicinity in the telomeric region of the short arm of the barley chromosome 2H. Various mutations (amino acid substitutions, nonsense mutations, alteration in splicing) identified within both of these genes caused a decrease in accumulation of castasterone and plant growth reduction of various degree, however the barley mutants showed less severe phenotypes when compared with the mutants of the homologous genes in rice and maize (Dockter et al. 2014; Gruszka et al. 2016a). BRs are most probably synthesized in the endoplasmic reticulum (ER) (Vukasinonic and Russinova 2018), however this suggestion has been experimentally confirmed only for the enzyme encoded by the *BR6ox2* gene in Arabidopsis. Farnesylation-mediated post-translational modification of the encoded enzyme (CYP85A2) was shown to be required for its localization in the ER and biochemical function (Northey et al. 2016). Location of the BR biosynthetic enzymatic machinery in the ER would allow formation of a metabolon (complex of enzymes involved in the same biosynthetic pathway) in order to efficiently direct substrates (intermediates) to target enzymes (Vukasinonic and Russinova 2018).

3 Genetic Mechanisms of Regulation of the BR Accumulation

BR exert their biological activity at very low concentrations ($<10^{-9}$ M) and their steady-state level is strictly controlled (Bishop and Yokota 2001; Fujioka and Yokota 2003). It was reported that the accumulation of 6-deoxocathasterone and the C6-oxidation reaction, which leads to castasterone synthesis, constitute the rate-limiting steps during the BR biosynthesis (Nomura et al. 2001). Moreover, the *CPD* and *BR6ox2* genes show cyclic fluctuations of expression level, at the 12-h intervals (Bancos et al. 2006). A separate mechanism of regulation of the BR biosynthesis is the feedback inhibition of the *DWF4* gene expression upon activation of the BR signal transduction, which enables a maintenance of a dynamic homeostasis, also because the DWF4 enzyme catalyzes another rate-limiting step in the BR biosynthesis. Generally, transcript levels of the *BR6ox1, BR6ox2*, *CPD*, *DWF4*, *CYP90D* and *ROT3* genes are down-regulated by bioactive BRs shortly upon BR treatment (Bancos et al. 2002; Goda et al. 2002). Relatively to other BR biosynthetic genes (including *CPD*), *DWF4* is expressed at an extremely low level (Kim et al. 2006). The expression of the *CPD* and *DWF4* genes is strongly repressed by the major BR-regulated transcription factors BZR1 and BZR2 (Wang et al. 2002; He et al. 2005). In contrast, transcription factors CESTA and TCP1 positively regulate expression of the BR biosynthetic genes *CPD* and *DWF4* by binding the conserved motifs in their promoters. In turn, BRs activate the *TCP1* gene expression. Interestingly, TCP1 specifically stimulates the expression of the *DWF4* gene but not the other BR biosynthetic genes. It was also reported that subnuclear localization of

the CESTA transcription factor is regulated by BR, and that CESTA is required for maintaining the balance of BR concentration at an early stage of development. However, transcript level of the *CESTA* gene does not seem to be regulated by BR (Guo et al. 2010; Poppenberger et al. 2011). It was recently reported that also the expression of the *DWF1* gene is down-regulated by application of active forms of BR. The BR-induced inhibition of the *DWF1* expression is mediated in a feedback manner by the major transcription factor involved in the BR response – BES1 (Youn et al. 2018). Activity of the DWF1 enzyme may be also modulated at the protein level – the enzyme is activated by Ca^{2+}/calmodulin (CaM). It suggests that on the long term basis the Ca^{2+} may influence the production or steady-state content of BRs (Du and Poovaiah 2005). This constitutes another level of complexity in the regulation of BR homeostasis, as it was observed that an elevation in the cytosolic Ca^{2+} concentration is induced within seconds after treatment with exogenous BR. The BR-dependent increase in cytosolic Ca^{2+} concentration can mediate the BR effect on gene expression (Zhao et al. 2013). It was shown that the expression of the *CPD* and *DWF4* genes may be stimulated by the Rapid Alkalinization Factor (RALF) peptides, which show inhibitory activity on root and hypocotyl growth through negative effect on cell expansion. This phenomenon may be explained by the fact that the RALF peptides and BRs exert an antagonistic effect on the regulation of genes involved in cell expansion, and these mechanisms form a feedback loop. On the other hand, BRs decrease the mRNA level of genes upregulated by the RALF peptides (Bergonci et al. 2014).

The *cog1* mutant of Arabidopsis was identified through activation tagging and the gene encodes a transcription factor, which acts as a negative regulator of phytochrome (light) signaling pathway. BR levels are significantly increased in this mutant, which is caused by upregulation of the BR biosynthetic genes. Molecular analyses indicated that the COG1 transcription factor binds to promoters of two genes *PIF4* and *PIF5* (*Phytochrome Interacting Factors*), which encode transcription factors redundantly binding to promoters of the BR biosynthetic genes, such as *DWF4* and *BR6ox2* to stimulate their expression. PIF4 and PIF5 are regulators of the BR biosynthesis, what indicates that light signaling is crucial for maintenance of the BR homeostasis (Wei et al. 2017).

The BR accumulation is also regulated based on inter-hormonal crosstalk with auxin. The auxin-stimulated induction of the BR biosynthesis requires the auxin signaling pathway, but not the BR signaling, indicating that the auxin signaling directly regulates the BR biosynthesis. However, auxin relies on BRs for some of its growth-promoting effects and functional BR biosynthesis is partly required for auxin-dependent gene expression (Nakamura et al. 2003; Chung et al. 2011). The *CPD* gene expression is activated by the BREVIS RADIX (BRX) transcription factor, which acts downstream of the auxin signaling (Mouchel et al. 2006). Expression of the *BRX* gene is highly auxin-inducible and the BRX activity is regulated by auxin at both the transcriptional and post-translational level (Scacchi et al. 2009). It indicates that BRX mediates the crosstalk between the BR and auxin metabolic pathways (Sankar et al. 2011). Gain-of-function lines which constitutively and ectopically over-express the *BRX* gene contain significantly higher contents of the

major, biologically active BRs: brassinolide and castasterone (Beuchat et al. 2010). Interestingly, the *BRX* gene expression is induced by auxin, but repressed by BR (Mouchel et al. 2006). Similarly, the *DWF4* gene expression is also up-regulated by auxin signaling through inhibition of binding of the BZR1 transcription factor to promoter of the *DWF4* gene. Interestingly, the other transcription factor, BES1, binds to the *DWF4* promoter regardless of hormonal conditions. On the other hand, BZR1 binds to promoter of the *CPD* gene regardless of BR or auxin treatment. The BES1 transcription factor in combination with the interacting group of factors BES1-Interacting Myc-Like (BIMs) and BR Enhanced Expression (BEEs) bind to the *DWF4* promoter to mediate the up-regulation by auxin and BR-induced down-regulation of this gene (Friedrichsen et al. 2002; Yin et al. 2005). This suggests that the two major transcription factors, BES1 and BRZ1, differently bind to promoters of the BR biosynthetic genes (Chung et al. 2011).

Accumulation of the endogenous BRs may also be regulated based on crosstalk with other phytohormones. Overexpression of the *Isopentenyltransferase* (*IPT*) gene results in an increased cytokinin content, which leads to upregulation of several BR-related genes, including the *DWF5* gene (Peleg et al. 2011). It was previously reported in Arabidopsis and rice that BRs increase the JA content under normal conditions (Müssig et al. 2000; Kitanaga et al. 2006). In barley, it was also found that mutants (both BR-deficient and BR-insensitive) contained significantly lower concentrations of JA under the control conditions, however both the BR-deficient and BR-insensitive barley mutants retained the capacity of significantly increasing the endogenous JA content in response to the drought stress (Gruszka et al. 2016b). It seems that the BR-JA interplay may be quite complicated, as it was reported that exogenous methylJA application significantly repressed expression of BR biosynthesis genes, and consequently decreased the endogenous BR content (Gan et al. 2015).

It has been shown that high temperature can also induce *DWF4* expression, however the detailed mechanisms controlling the BR biosynthesis by environmental factors are still poorly understood (Maharjan and Choe 2011; Wei et al. 2017). Nevertheless, it was recently reported that accumulation of the endogenous bioactive BRs (castasterone and 24-epibrassinolide) is induced by drought stress in barley, both in the wild type cultivar and in the BR-deficient and BR-insensitive mutants (Gruszka et al. 2016b).

Apart from the regulation of the BR biosynthesis pathway, plants have evolved mechanisms of regulation of the BR accumulation, which involve catabolic mechanisms of BR inactivation, however the catabolic pathways are still poorly understood (Du et al. 2017). Accumulation of the biologically active BRs (castasterone and brassinolide) is regulated by activity of the BAS1 hydroxylase, which belongs to the cytochrome-binding protein family. The C-26 hydroxylation of the biologically active BRs is a prerequisite for degradation (Neff et al. 1999; Turk et al. 2003) and is sufficient to abolish their biological activity (Ohnishi et al. 2012). In Arabidopsis the inactivation of BRs is performed by enzymes encoded by two genes, *BAS1* and *SOB7/CHI2* (Neff et al. 1999; Turk et al. 2003), whereas in the rice genome no ortholog of the *SOB7/CHI2* gene was identified. It was reported in rice

that the CYP734A protein family (to which BAS1 belongs) includes multifunctional and multisubstrate enzymes which regulate the endogenous bioactive BR content through inactivation of castasterone and via attenuation of biosynthesis of this compound by decreasing concentration of its precursors. In contrast to BAS1 in Arabidopsis which selectively inactivates castasterone and brassinolide (the most biologically active forms), in rice the CYP734A homolog metabolizes various BR intermediates during the early steps of biosynthesis (Sakamoto et al. 2013). However, BAS1 and its rice homologs share high sequence identity and have similar function, what suggests that the mechanisms regulating the BR inactivation through hydroxylation are conserved in monocots and dicots (Qian et al. 2017). The BAS1-mediated BR inactivation was proven to be induced by light (Nakamura et al. 2005). In contrast to the BR biosynthetic genes *CPD* and *DWF4*, expression of the BR catabolic gene *BAS1* is stimulated by the BZR1 and BZR2 transcription factors via feedback regulatory loop upon BR treatment (He et al. 2005; Sun et al. 2010; Oh et al. 2012a). However, it was recently reported that expression of the *BAS1* gene is negatively regulated by one of the Auxin Response Factors (ARFs) in Arabidopsis. The *BAS1* gene expression is oppositely regulated by BZR1 and ARF7 which both bind to the same motifs in the *BAS1* promoter (Youn et al. 2016). It is also known that BZR1 binds directly to the *ARF7* promoter and suppresses the *ARF7* gene expression (Zhou et al. 2013). ARF7 was also reported to stimulate BR biosynthesis through binding to the *DWF4* promoter, thus ARF7 increases the endogenous BR content via regulation of the BR inactivation (*BAS1*) and BR biosynthesis (*DWF4*) (Youn et al. 2016). Recently, it was reported that *BAS1* expression is also upregulated by Lateral Organ Boundaries (LOB) transcription factor. The *lob* knockout mutants show an organ fusion phenotype, which can be suppressed by the *BAS1* expression (Bell et al. 2012). Therefore, it is suggested that transcriptional regulation of the *BAS1* gene is needed for efficient control of BR response in tissues where cell divisions and elongations are precisely balanced (Youn et al. 2016).

In plants, the KNOX transcription factors are crucial for establishing and maintaining the shoot apical meristem. In rice the *KNOX* gene represses the BR response pathway through transcriptional activation of the BR catabolism genes (Tsuda et al. 2014). It is known that genes of the KNOX family activate the cytokinin but repress gibberellic acid (GA) biosynthesis (Sakamoto et al. 2001; Jasinski et al. 2005). The auxin pathway is also regulated by the KNOX proteins (Bolduc et al. 2012). Hence, the KNOX proteins form a hub in the regulation of various phytohormonal pathways. It was suggested that BR inactivation plays an important role in maintaining the shoot apical meristem functionality (Tsuda et al. 2014).

In was recently reported that overexpression of the Arabidopsis BR-related acyltransferase1 (BAT1), which is known to catalyze a conversion of BR intermediates to inactive acylated conjugates (Schneider et al. 2012; Choi et al. 2013), in creeping bentgrass (*Agrostis stolonifera*) resulted in dwarf phenotype, delayed senescence and improved drought tolerance. This suggests that the BAT1 acyltransferase is functional in dicot and monocot species and that the BR acylation represents a general inactivation mechanism. The overexpression of *BAT1* decreased the endogenous contents of BR intermediates (6-deoxotyphasterol, 6-deoxocastasterone,

typhasterol and to a lesser extent castasterone and brassinolide (Han et al. 2017) (Fig. 8.1). Several other enzymes involved in the regulation of BR accumulation through chemical modifications have recently been identified (Du et al. 2017). BEN1, dihydroflavonol 4-reductase (DFR)-like protein regulates the contents of typhasterol, castasterone and brassinolide (Yuan et al. 2007). Another enzyme – DRL1 is an acyltransferase that regulates the BR homeostasis by mediating the BR conjugation through esterification (Zhu et al. 2013a). The Brassinosteroid Inactovator 1 (BIA1) and Abnormal Shoot 1 (ABS1), which belong to the BAHD family of acyltransferases, are involved in BR acylation, which leads to a decrease in BR content (Roh et al. 2012; Wang et al. 2012a). Interestingly, the BIA2 acyltransferase is involved in the regulation of BR homeostasis and may inactivate bioactive BRs by esterification, particularly in roots and hypocotyls under dark condition (Zhang and Xu 2018).

4 Molecular and Genetic Aspects of BR Perception and Signaling

Intensive studies conducted mainly in Arabidopsis with the genetic, physiological and molecular approaches led to identification and characterization of various components, which take part in the BR signaling, from the ligand perception, via cytoplasmic phosphorylation and dephosphorylation relay, up to the BR-regulated gene expression (Gudesblat and Russinova 2011; Gruszka 2013). BRs are perceived at the plasma membrane by a receptor complex, which includes the BRI1 receptor kinase and one of the small group of protein kinases belonging to the Somatic Embryogenesis Receptor Kinases (SERK) family. The major components of the receptor complex belong to the family of Leucine-Rich Repeat Receptor-like Kinases, which encompasses more than 200 protein kinases in Arabidopsis (Li and Chory 1997; He et al. 2000; Wang et al. 2001).

In Arabidopsis, distribution of the BRI1 receptor is not spatially regulated, the gene is ubiquitously expressed (Friedrichsen et al. 2000), and the gene expression studies indicated that only moderate variation of the *BRI1* transcript levels between organs could be detected (Li and Chory 1997; Goda et al. 2002). However, expression of the *BRI1* gene is under developmental, organ-specific and diurnal regulation (Hategan et al. 2014). Moreover, on the protein level a considerable cell-type specific differences in the BRI1 density on the cell surface could be detected, and it was stated that intensity of the BR signaling is correlated with the abundance of the receptor (Van Esse et al. 2011). The *BRI1* gene expression is also regulated by phytohormones, as BRs downregulate its expression at transcriptional level via feedback mechanism mediated by the BES1 and BZR1 transcription factors (Sun et al. 2010; Yu et al. 2011), while auxin can increase the gene transcription level (Goda et al. 2002; Nemhauser et al. 2004; Sakamoto et al. 2013). It is suggested that the *BRI1* activity is determined in a complex way, similarly to the key BR biosynthetic

genes (Hategan et al. 2011; Zhao and Li 2012). This allows an optimal coordination of the BR accumulation and susceptibility, which underlies regulation of various physiological processes (Hategan et al. 2014). Up to now, over 30 different alleles of the *BRI1* gene have been identified mainly in Arabidopsis, but also in other species. The mutations are localized in various domains of the encoded receptor kinase, which resulted in various degree of phenotype alterations (Gruszka et al. 2011b; Jiang et al. 2013). The BRI1 receptor kinase is composed of three major parts: extracellular LRR domain, single-pass transmembrane domain and cytoplasmic kinase domain (Gruszka 2013). The extracellular domains of the BRI1 protein are mainly responsible for protein interactions during formation of the receptor complex and mediate binding of the BR ligand. BR ligand binds to a hydrophobic surface groove formed by 70-amino acid 'island' domain and the following four Leucine-Rich Repeats (LRRs) (Li 2003; Li and Jin 2006; Witthöft and Harter 2011; Jiang et al. 2013). Two loop domains, which link the island domain with two flanking LRRs of BRI1, undergo a BR-induced local structural rearrangements. BR binding induces transformation of the disordered loops into an ordered domain, which forms a protein-protein interaction platform (Hothorn et al. 2011). Direct binding of the BR molecule by the BRI1 subdomain forms a docking platform for one of the SERK co-receptors, leading to initiation of signaling relay (Hothorn et al. 2011; She et al. 2011). The BR ligand binding by the BRI1 receptor is followed by numerous auto- and transphosphorylation events in the cytoplasmic part of the receptor, which have a regulatory effect on recruiting of the second component of the receptor and function of the receptor complex (Gruszka 2013). Heterodimerization of the BRI receptor kinase with one of four members of the Somatic Embryogenesis Receptor Kinase (SERK) family is required for full activation of the signaling pathway. The activation of the BRI1 receptor kinase by ligand binding results in activation of downstream signaling components only upon transactivation with the SERK co-receptor proteins (Hecht et al. 2001; Gou et al. 2012). In Arabidopsis, the members of the SERK gene family have emerged in a gene duplication event, and the resulting paralogues maintained a functional redundancy (Kim and Wang 2010). The sequence of events initiated by the BR ligand binding by the BRI1 receptor, through the interaction between the BRI1 receptor kinase and one of the SERK co-receptors, auto- and transphosphorylations of various amino acid residues within both components of the receptor complex, up to full activation of the receptor has been described (Gruszka 2013). At the protein level activity of the BRI1 kinase is negatively regulated in a feedback manner by the cytoplasmic protein phosphatase 2A (PP2A). This process is regulated by BRs through stimulation of the Suppressor of bri1 (SBI1) leucine carboxy-methyltransferase, whose function is to methylate the PP2A phosphatase, what facilitates its interaction with BRI1, and ultimately results in dephosphorylation of the receptor kinase (Di Rubbo et al. 2011; Wu et al. 2011). It was reported that BRI1 may physically interact with Ca^{2+}-binding calmodulin (Oh et al. 2012b). It is speculated that the BR-induced increase in cytosolic Ca^{2+} concentration may act through the BRI1-calmodulin interaction to attenuate the BRI1-depenedent phospho-relay cascade (Zhao et al. 2013). Function of the receptor complex is also regulated at the protein level through endocytosis of cell membrane

fragments containing these polypeptides, which leads to the receptor recycling. This process is mediated by the Membrane Steroid-Binding Protein1 (MSBP1), which negatively regulates the BR signaling. MSBP1 specifically interacts with extracellular (Leucine-Rich Repeat) domain of the BAK1 kinase (the major representative of the SERK family, which participates in the receptor complex formation) in a BR-independent manner. MSBP1 attenuates BR signaling through the interaction with BAK1, which results in BAK1 endocytosis and consequently in a suppressed BR signaling by shifting the equilibrium of BAK1 toward endosomes and inhibiting the BRI1-BAK1 association. Thus, MSBP1 acts a negative regulator at an early step of the BR signaling pathway. It is suggested that enhanced response to BR, which was observed in one of the *bak1* mutants (*bak1*^{elg}*, elongated-D*) was due to reduced MSBP1-BAK1 interaction, which resulted in a reduced inhibition of BAK1 activity by MSBP1. Protein encoded by the *elg-D* allele of the *BAK1* gene shows enhances association with the BRI1 kinase (Song et al. 2009; Jaillais et al. 2011). Interestingly, the MSBP1 gene expression is stimulated by light, but inhibited by dark (Yang et al. 2005). The activated BRI1 receptor kinase may also be inhibited by its substrate – the Transthyretin-Like (TTL) protein, which interacts with and is phosphorylated by BRI1. TTL is associated with the plasma membrane and acts as a negative regulator of plant growth through high-affinity interaction with the kinase-active BRI1 (Nam and Li 2004).

BR-triggered activation of the BRI1-BAK1(SERKs) receptor complex leads to initiation of transduction cascade mediated by the cytoplasmic BR-Signaling Kinases (BSKs), which function as positive regulators of the BR signaling. The members of the BSK family transmit the signal between the BR receptor complex and cytoplasmic regulators of the BR signaling (Kim et al. 2009). It was reported that two paralogous proteins, BSK1 and BSK3, interact directly with BRI1 in the absence of BR, whereas upon the ligand binding BRI1 phosphorylates BSK1 inducing its activation and release from the receptor complex (Tang et al. 2008). Another components of the cytoplasmic BR-triggered phosphorylation cascade include two homologous cytoplasmic kinases Constitutive Differential Growth1 (CDG1) and CDG-like1 (CDL1), which also play a role of positive regulators of the BR signaling and are substrates of the BRI1 kinase domain. The activated receptor complex phosphorylates the CDG1 kinase rendering it active. In turn, the phosphorylated CDG1 and CDL1 kinases phosphorylate the BRI1-Supressor1 (BSU1) phosphatase, what stimulates its activity and ultimately leads to the BSU1-mediated dephosphorylation and inactivation of the major negative regulator of the BR signaling pathway – the Brassinosteroid-Insensitive2 (BIN2) kinase (Muto et al. 2004; Kim et al. 2011; Gruszka 2013).

Apart from acting as a major negative regulator of the BR signaling, mainly through phosphorylation of the BR-regulated transcription factors BES1 and BZR1, BIN2 has additional substrates modulating downstream components of the BR biosynthesis and signaling. BIN2 phosphorylates various transcription factors and signaling components thus regulating their activities and providing another point of interactions with other signalosomes (Guo et al. 2013). BIN2 phosphorylates the above-mentioned CESTA transcription factor, which positively regulates the BR

biosynthesis (Poppenberger et al. 2011; Gruszka 2013). CESTA shows nuclear localization which is regulated specifically in reaction to a rapid stimulation of the BR signaling by inhibition of BIN2 activity (Poppenberger et al. 2011). It was reported that one of the BIN2 substrates in also the above-mentioned PIF4 being a basic helix-loop-helix (bHLH) transcription factor regulating cell elongation (Castillon et al. 2007; de Lucas et al. 2008; Oh et al. 2012a). The BIN2 kinase phosphorylates PIF4, what results in targeting this transcription factor for proteasome-mediated degradation, which is responsible for regulating the timing of hypocotyl elongation to late night. It is suggested that a main role of BR in antagonizing light signaling is mediated by inhibition of the BIN2-mediated destabilization of PIF4 (Bernardo-Garcia et al. 2014). However, the BIN2 kinase is a multifaceted protein and apart from being a critical repressor of the BR signaling it also positively regulates the abscisic acid (ABA) responses during germination and plant growth. BIN2 physically interacts with the Abscisic acid Insensitive 5 (ABI5) transcription factor (Hu and Yu 2014). It was shown that ABA stimulates the BIN2 kinase activity (Zhang et al. 2009b). In contrast to the influence of the BIN2 kinase on the BZR1 and BES1 transcription factors in the BR signaling, BIN2 phosphorylates and stabilizes ABI5 in the presence of ABA to mediate response to this hormone, whereas BRs inhibit the regulatory effect of BIN2 on ABI5. It was reported that BRs induce proteasome-mediated degradation of ABI5 (Hu and Yu 2014). Interestingly, cytokinin signaling also promotes the degradation of ABI5 in proteasome (Guan et al. 2014). Hence, BIN2 is a critical node for the BR-ABA antagonism. BIN2 interacts also with the Abscisic acid responsive element Binding Factor 1 (ABF1) and ABF3, which play a crucial regulatory role in ABA signaling. Thus, it is postulated that BIN2 may phosphorylate and activate these factors (Hu and Yu 2014). On the other hand, it was reported that BES1 forms a transcriptional repressor complex with TOPLESS (TPL) and Histone Deacetylase 19 (HDA19) to regulate expression of ABI5 and suppress the ABA signaling (Ryu et al. 2014).

BR-regulated gene expression is mediated mainly by two transcription factors – BZR1 and BES1. It is known that target genes of the BZR1 and BES1 transcription factors encode proteins participating in various processes, including various aspects of morphogenesis, cellular transport, cell wall modifications, cytoskeleton function, chloroplast development, metabolism and response to various (ABA, auxin, cytokinin, ethylene, gibberellin, jasmonic acid) phytohormones, as well as responses to various stress conditions and environmental cues (Zhu et al. 2013b). However, it is also known that they constitute focal points of interactions with various transcription factors and chromatin modifying enzymes, what ultimately results in a complicated network of interactions allowing coordinated regulation of gene expression in response to various cues (Gruszka 2013). BZR1 directly or indirectly regulates expression of about 80% of the BR-controlled genes. BZR1 inhibits expression of at least five BR biosynthetic genes and the BR receptor gene *BRI1,* and this mechanism provides a negative feedback. However, BZR1 positively regulates expression of genes encoding components mediating downstream BR signaling by inhibiting transcription of the *BIN2* gene and activating expression of the *BSU1* gene.

Moreover, BZR1 directly regulates expression of a number of genes involved in biosynthesis of other hormones, such as auxin, GA, ethylene and JA (Sun et al. 2010). Upon the BR perception and signaling initiation, the BIN2 kinase is inactivated and the transcription factors BZR1, BES1 and PIF4 are rapidly dephosphorylated and migrate into the nucleus to form a complex, which synergistically activates a common group of the BR-regulated genes. The function of the BZR1-PIF4 complex on hypocotyl elongation is further enhanced by the abovementioned COG1 transcription factor which stimulates expression of the *PIF4* and *PIF5* genes. It should be kept in mind that the PIF proteins promote the BR biosynthesis, which stimulates BR signaling and in consequence enhances the function of the BZR1-PIF4 complex (Wei et al. 2017). BZR1 and PIF4 directly interact with each other and show synergistic and interdependent relationship in stimulating gene expression and regulating the process of etiolation. BZR1 and PIF4 are crucial for cell elongation in dark but also at high temperature, which both increase the PIF4 accumulation (Wang et al. 2012b). Initially, the PIF proteins were shown to interact directly with phytochrome B to act as downstream components of the phytochrome signaling (Huq and Quail 2002; Shen et al. 2007). Later on, they proved to be key integrators of light and hormonal signalosomes (de Lucas et al. 2008; Bai et al. 2012; Gallego-Bartolome et al. 2012; Oh et al. 2012a, 2014; Bernardo-García et al. 2014). Apart from BZR1, PIF interacts directly with the Auxin Response Factor 6 (ARF6) to regulate a large number of target genes. This indicates that a crosstalk exists among the BR, auxin and phytochrome signalosomes (Oh et al. 2014). The BZR1 and BES1 transcription factors interact at the protein level with the DELLA proteins, which function as negative regulators of plant growth. The DELLA proteins bind the DNA recognition domain of the PIF proteins to form an inactive complex (de Lucas et al. 2008; Feng et al. 2008; Schwechheimer 2008; Alabadi and Blazquez 2009). It is known that the DELLA proteins use the same strategy to suppress the BZR1/BES1 activity as in the case of PIFs (de Lucas and Prat 2014). The DELLA proteins attenuate function of the PIF4 and BZR1/BES1 transcription factors individually, but also inhibit function of the PIF4-BZR1/BES1 complex (Wang et al. 2012b). It is known that light promotes accumulation of the DELLA proteins through reduction of the GA contents (Achard et al. 2007). Moreover, it was reported that function of the PIF4, BZR1 and ARF6 transcription factors is repressed by the DELLA proteins, which function redundantly as negative regulators of the GA signaling. This DELLA-mediated attenuation of the PIF4, BZR1 and ARF6 module's function is released upon the GA perception (Bai et al. 2012; Gallego-Bartolome et al. 2012). This indicates that the PIF4-BZR1-ARF6-DELLA module is a point of convergence of the light, BR, auxin and GA signalosomes, which is crucial for plant growth regulation (Wang et al. 2012b; de Lucas and Prat 2014). It is known that accumulation of the DELLA proteins is regulated by multiple hormonal and environmental signals, including auxin, cytokinin, ABA, ethylene, jasmonate and environmental stresses (Sun 2010; Yang et al. 2012). The PIF proteins are activated by the major regulators of the BR-dependent gene expression – the BZR1 and BES1 transcription factors, what points to a role of the PIF proteins in integration of the signaling pathways. Moreover, PIFs act in a concerted manner with the BZR1 and

BES1 transcription factors to activate auxin biosynthesis and transport at the gene expression level. Auxins play a feedback role in this regulatory module by inducing the GA biosynthesis and the *BZR1/BES1* genes' expression (Fig. 8.1). GA and BRs stimulate plant growth through the BZR-PIF4-mediated activation of cell wall modification, enhancement of the auxin biosynthesis and auxin responsive gene expression. The stimulation of auxin biosynthesis and the auxin-dependent gene expression results in the induction of GA biosynthesis and the *BZR1/BES1* gene expression (Frigerio et al. 2006; Chapman et al. 2012). Therefore, it contributes to release of the DELLA-mediated repression and consequently to enhancement of the BZR1-PIF4 complex formation (de Lucas and Prat 2014).

BRs participate in the hormonal network that includes also the ABA signaling and this inter-hormonal crosstalk plays an essential role during plant development (Rajjou et al. 2012). The ABA and BR signaling pathways are interconnected in an antagonistic manner and the molecular aspects of this interaction are intensively studied (Steber and McCourt 2001; Xue et al. 2009; Zhang et al. 2009b). ABA signaling, which is mediated by the ABA-Insensitive2 (ABI2) protein, stimulates the BES1 phosphorylation, what indicates that ABA inhibits the BR signaling by activating the BIN2 kinase (Zhang et al. 2009b). On the other hand, BR treatment or overexpression of the *DWF4* gene suppress the ABA-mediated inhibition of seedling development (Steber and McCourt 2001; Xue et al. 2009; Zhang et al. 2009b). Recently, it was reported that ABI1 and ABI2, which are negative regulators of the ABA signaling, may significantly promote the BR signaling. ABI1 and ABI2 physically interact and dephosphorylate BIN2, consequently leading to reduced phosphorylation (and increased activity) of BES1. The inhibition of BIN2 by ABI2 is ABA-dependent (Wang et al. 2018).

Genomic studies led to identification of a few thousand target genes (about 5000) of the BZR1 and BES1 transcription factors, which are involved in various signaling pathways, including light, stresses and almost all phytohormones (Guo et al. 2013). The BES1 and BZR1 transcription factors form a point of interactions of various transcription factors and other regulators of gene expression, representing various signalosomes (Gruszka 2013). Besides, BES1 interacts with the transcription co-repressor Myeloblastosis family transcription factor-like 2 (MYBL2) to inhibit expression of BR repressed genes. Interestingly, MYBL2 is a substrate of the BIN2 kinase. However, unlike BIN2-mediated phosphorylation of the BZR1 and BES1 transcription factors, which renders them inactive, BIN2-mediated phosphorylation stabilizes MYBL2 (Ye et al. 2012). Picture of this processes is further complicated by the fact that the *MYBL2* gene is transcriptionally repressed by BES1, whereas MYBL2 protein is co-repressor of BES1 (Guo et al. 2013).

Moreover, BRs regulate gene expression also through histone modifying enzymes and alteration of chromatin structure. The BR-regulated gene expression involves histone modifications including H3K27 demethylation and H3K36 methylations. It is known that BES1 interacts with two proteins: Early Flowering 6 (ELF6) and Relative of Early Flowering 6 (REF6, H3K27 demethylase), which play a positive role in the BR signaling pathway. BES1 accumulates and recruits REF6 to target genes to release the histone repression mark (H3K27 double and triple

methylation) to activate gene transcription. BES1 interacts also with another protein, Interacting-with-Spt6 1 (IWS1), which functions in the transcription elongation process and plays a positive role in the BR signaling (Li et al. 2010). Expression of about 1/3 of the BR-regulated genes is affected in the *iws1* mutant. BES1 recruits IWS1 to promote transcription elongation and stimulate BR-induced gene expression (Guo et al. 2013). On the other hand, H3K36 methylation was found to be a hallmark of positive regulation in the BR response. It was shown that chromatin of the *BRI1* and *DWF11* genes is modified through H3K36 methylation, which positively influences their expression (Sui et al. 2012), however a detailed mechanisms has not been described yet (Guo et al. 2013). Recently, another component of this regulatory system has been identified. The chromatin-remodeling factor PICKLE/Enhanced Photomorphogenic (PKL/EPP1) represses photomorphogenesis in Arabidopsis. The PKL protein level is significantly increased in response to exogenous application of BR or GA (Zhang et al. 2014b). On the contrary, light represses PKL both at the mRNA and protein levels (Jing et al. 2013). PKL physically interacts with PIF3 and BZR1, and therefore constitutes another point of interaction between the light and BR signaling pathways. The PKL-PIF3-BZR1 triad co-regulates skotomorphogenesis by repressing the trimethylation of lysine-27 in the histone H3 in promoters of target genes. Interestingly, DELLA proteins interact with PKL and reduce its DNA chromatin-binding activity (Zhang et al. 2014b). This indicates that DELLAs exert their negative effect on various types of proteins. PKL was also implicated in responses to other phytohormones: auxin, ABA, GA and cytokinin (Fukaki et al. 2006; Perruc et al. 2007; Zhang et al. 2008; Furuta et al. 2011). The PIF3, BZR1 and DELLA proteins regulate the recruitment of PKL to promoters of the target genes, and consequently regulate multiple physiological processes. Thus, PKL plays a prominent role in integrating the light/darkness, BR, GA and other phytohormonal signaling pathways to epigenetically regulate plant growth (Zhang et al. 2014b).

It is known that BRs function synergistically with auxin to promote cell elongation and auxin response mutants have reduced sensitivity to BR (Nemhauser et al. 2004; Vert et al. 2008). BZR1 binds to promoters of many auxin-responsive genes (Sun et al. 2010; Yu et al. 2011). An analysis of promoter sequences of the BR-regulated genes indicated that they are co-regulated by both the BRZ1/BES1 transcription factors and the Auxin Response Factors (ARFs) (Sun et al. 2010). Generally, transcriptional changes occur much more slowly in response to BR than to auxin (Mockaitis and Estelle 2004). Positive interactions between the BR and auxin biosynthetic and signaling processes play significant roles in various developmental processes in plants (Ye et al. 2011; Choudhary et al. 2012; Ryu and Hwang 2013). Auxin response is also dependent on the BR signaling pathway (Zhang et al. 2009c). BR and auxin share a number of early responsive genes, which was manifested by identification of ARF-binding motives within promoters of the BR responsive genes (Nemhauser et al. 2004; Goda et al. 2004). Moreover, the BIN2 kinase interacts directly with Auxin Response Factor 2 (ARF2). The BIN2-mediated phosphorylation of ARF2 leads to loss of its DNA binding capacity and repression of the ARF2 activity. Thus, BIN2 increases expression of auxin-induced genes by the

inactivation of the ARF2 repressor, what results in synergistic stimulation of transcription (Vert et al. 2008; Zhang et al. 2009c). Interestingly, BR and auxin responses are integrated through the actin cytoskeleton, which is regulated by both these hormones and mediates auxin transport and BR signaling (Lanza et al. 2012).

It is known that BRs and GAs enhance plant growth in an additive way, what indicates that these phytohormones function independently at the cellular level. Moreover, expression of numerous genes is coordinately regulated by both hormones (Goda et al. 2008; Zhang et al. 2009c). It was also reported that accumulation of one the biologically active forms of GA (GA_7) in barley is BR-dependent, as it is significantly reduced in BR-deficient and BR-insensitive mutants under optimal watering conditions. However, the GA7 accumulation is significantly induced by drought and this stimulation is even more pronounced in the BR mutants, what indicates that the mutants retained a capacity of increasing the GA_7 content in response to the stress conditions (Gruszka et al. 2016b).

BR may influence the stress responses of plant also by stimulating the jasmonic acid (JA) biosynthesis. The expression of the *OPR3* gene that is required for the JA biosynthesis is induced by BR and JA, depending on environmental and developmental conditions (Müssig et al. 2000). Indeed, it was recently reported in barley that BR-deficient and BR-insensitive mutants contained significantly lower concentrations of this hormone. This indicated that the JA homeostasis is dependent on the normal progress of the BR synthesis and signaling. However, it was reported that the BR-deficient and BR-insensitive mutants retain the capacity of significantly increasing the endogenous JA content in reaction to drought (Gruszka et al. 2016b). BRs stimulate the biosynthesis of ethylene through stabilizing an enzyme, which catalyzes a rate-limiting step in the ethylene biosynthesis pathway. Moreover, these hormones may enhance the biosynthesis of each other (Shi et al. 2006; Hansen et al. 2009).

5 Conclusions

Identification and characterization of new components of the BR signaling pathway is still in progress also in Arabidopsis, however the emerging view indicates that this process is interconnected at many stages with the signal transduction pathways of other phytohormones. Several regulators of the BR signaling form hubs of the interhormonal crosstalk. This interhormonal network of interactions allows the various physiological processes to be regulated in the BR-dependent manner, but also enables a coordinated regulation of the processes in response to various hormones. Ultimately, the interhormonal crosstalk allows an efficient fine-tuning of plant growth and development to constantly changing environmental cues, including stress conditions.

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Chapter 9
Transformation of Matter and Energy in Crops Under the Influence of Brassinosteroids

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Abstract The application of various allelochemicals in agricultural production is carried out primarily to increase the quantity and quality of crop yield. These allelochemicals, which include brasssinosteroids (BRs), can reinforce the resistance of crops to abiotic stresses or increase their competitive ability against other organisms (biotic stresses). In particular, BRs can directly intensify crop physiological processes leading to increased growth and development, which create essential prerequisites for their increased yield. Thus, the use of the BRs in plant protection and agriculture is of particular interest. As yield is the ultimate and most important characteristic related to agricultural production, it represents the end product of transforming matter and energy in plants in the field. In order to obtain better qualitative and quantitative yield results, different crops are often subjected to various concentrations of 24-epibrassinolide (24-EBL). Therefore, this chapter concerns biochemical and biophysical responses of several (maize, soybean, barley etc.) crops treated with a range of concentrations of 24-EBL at various stages of development (seedlings, vegetative stages of plants before flowering and mature field plants). Particular attention is given to the influence of exogenously applied 24-EBL on specified physiological and biochemical parameters (carbohydrates, starch, polyphenols, pigments, proteins, etc.) in selected crops, especially maize, in relation to their likely roles in determining crop biomass accumulation, biomass redistribution, growth, yield and improved resistance to abiotic stresses.

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1 Introduction

Terrestrial plants are thermodynamically open systems which exchange matter and energy with the environment, necessary for survival, growth and reproduction. But unlike moving animals, land-based plants with their sesile habitus and poikilothermal metabolism had to develop a completely different life-style strategy in order to obtain resources for survival and reproduction. It is essential that terrestrial plants differentiate organs from the outside (unlike animals, developed by complex systems of internal organs, Vernadsky 2008). Therefore, the structure of plant organisms is relatively simple in relation to animals, but they have developed a surprisingly complex primary and secondary metabolism, possibly based on the aforementioned specificity of the morphological plant geometry. The mentioned complexity of the metabolism of terrestrial plants is also increased by the basic division of their organs into the above-ground organs, which acquire resources from the atmosphere (light, O_2 and CO_2), the underground root, and the soil (water, minerals). This was due to the fact that the issuing organs, above all the leaves, are autotrophs, which make net exports of newly synthetic organic matter, while the root (as well as some other organs) is heterotrophic, and carries out the net import of newly synthetic organic matter.

All this points to photosynthesis as the basic physiological process in plants, which depends on the homeostasis of the entire plant metabolism. This imposes the process of plant photosynthesis and other catabolic processes in optimum frames, in order to continuously produce organic matter, while on the other hand it "imposes" the "need" for the export of synthetic organic matter from autotrophic to heterotrophic organs of terrestrial plants to ensure their optimum growth and development, including the formation of generative organs necessary for the propagation of plants. These processes are regulated by the negative feedback loop through the so-called "source-sink" relationship (Paul and Foyer 2001), and also through other signal systems (phytohormones and pigment signal systems; Gururani et al. 2015a, b). In addition, photosynthesis is in the leaf cells "bound" with other metabolic processes, which ensures optimal production of assimilates and other products of the leaf metabolism (Noctor and Foyer 1998a), in support of plant growth and development, and within crops to their yield. All these processes of production and redistribution of organic matter in plants must be coordinated, in order to optimize the processes of growth, development and reproduction, which is also reflected as a yield of cultivated (crop) plants. The coordination of the production and the redistribution of organic matter takes place through a complex interplay of internal signal systems

(phytohormones, pigment and other signal cascades) and external stimuli (light, temperature, osmotic, ionic and other factors), including stress, which affect the homeostasis and survival of plants (Lichtenthaler 1996; Gururani et al. 2015a, c).

2 Factors Which Determinate or Limit Bioproduction and Yield of Plants

This homeostatic nature of photosynthesis and other aspects of plant metabolism is subjected to variations of the environmental factors, but also the development dynamics of the plants functions. Although the significance of developmental dynamics (often associated with changes in source-synk relations in plants) is important for overall bioproduction, attention is focused on the effect of the environment (with optimal or stress intensity of ecological factors: Lichtenthaler 1996) on the plant's energetics and the interaction of these processes with their growth and development, determined by phytohormones (Gururani et al. 2015a), phytochrome system (Gururani et al. 2015b), but also with several other signaling systems.

The basic environmental factor that affects plants is light. Light is an energy engine of the photosynthesis process; and also one of the most important environmental inductors of plant developmental processes through their pigment systems (phytochromes, cryptochromes etc.); Further, the photosynthetic apparatus itself, especially the so-called "light phase" of photosynthesis, is subjected to functional inactivation at strong light, either temporary (dynamic) or permanent (chronic), processes associated with the acclimation and/or destruction of the photosynthetic apparatus under of the light stress, (Lichtenthaler 1996). That these processes are not insignificant from the point of view of total organic production, as has been testified both theoretically and experimentally (Werner et al. 2001); it has been found that the usual daytime photosynthesis are lower than of the maximal daily photosynthesis, i.e. the reduction of photosynthesis of healthy plants at the time of the greatest daily insolation also reduced the daily production of organic matter of the plants (native and cultivated) by 8–10%!

When plants are subjected to the simultaneous effects of photoinhibitory stress combined with other types of stress (low and high temperature, osmotic stresses (drought, salinity), xenohemicals (pesticides, toxic metals, radionuclides etc.), biotic stresses), which is a common situation, either in native or cultivated plants (Lichenthaler 1996; Gururani et al. 2015c). These processes lead to the reduction of the photosynthetic electron transport chaine and at the end of the photoxidative degradation of photosynthetic and other cellular plant structures (Noctor and Foyer 1998b), which leads to greater thermodynamic inefficiency of plants as energy systems (Dragicevic 2015).

3 Phytohormones, Particularly Brassinosteroids as the Main Internal Factors in the Co-ordinated Needs for Plant Growth to Continuous Production of Organic Matter Under Usual and Stress Environmental Conditions

Photosynthesis as the main catabolic process of plants, which produces organic matter necessary for the growth and development of plants, is inherently an inefficient process due to photoinhibition and related photo-oxidative processes (Long et al. 2006). This inherently ineffectiveness of photosynthesis and associated organic matter production is even greater in additional stress conditions (Larcher 2003). However, processes of growth and associated plant development require the continuous assimilate flow from leaves to heterotrophic organs, even in stress conditions, as well as during the night when there is no photosynthesis, indicating the importance of the source-sink relationship (Paul and Foyer 2001). At the same time, this problem also points to the discrepancy between the biological productivity of plants and theso called economic productivity of crops, which usually coincides with the so-called harvest index, i.e. with the share of economically exploitable parts of plants (grains, fruits, tubers) in the total weight of crop plants. Quarrie (1997) correctly observed that while the life strategy of native plants is to survive under stress conditions and produces propagules (seed, fruit, tubers) for reproduction, for crop breeders and growers, the main goal is not only that, but also to receive a satisfactory yield of crops over a long period, with a preserved harvest index also in harsh environments and seasons. In short, the demand placed on crops as living systems is in some way "unnatural". What is more, modern high-yielding crop genotypes are most often created by cross-breeding and returning selection, which in turn reduces their ability to survive in abiotic stress conditions.

Since the crops and the environment in which crops grow, in modern agriculture are viewed as one system with these two elements, the environmental stress and plant resistance to it, can be considered in three aspects: (a) Escape from stress (stress escape) (b) Avoiding stress (stress avoidance) (c) Tolerance to stress (stress tolerance). Adaptations to stress, which increase the tolerance of crops to the scarcity (or surplus) of a resource, can consist of different morphological or biochemical-physiological adjustments that act either in the plant (changing the state of their cytoplasme or simplaste), but also from out of it. The synthesis of these protective compounds in the plant may mobilize a significant amount of photosynthates (Kochian et al. 2004; Narula et al. 2009). So, if the synthesis of these compounds would represent the constitutive character of the crop, the crop yield would therefore be reduced in periods when the plants were not exposed to stress. All this points to the significance of manipulation with the status of phytohormones (Gururani et al. 2015a), pigment systems (phytochromes, cryptochromes, etc., Gururani et al. 2015b), in the processes of adapting plants to stress conditions in order to achieve their optimal yield.

How are these necessary prerequisites (changes in genotype (classical selection or GMO mode) or appropriate agro-technical measures) achieved for improved

(quantity and quality) yield of crops? It can best be shown by describing for example the influence of brasinosteroids and other phytohormones on different processes and the development phases of the crops.

3.1 *Influence of Brassinosteroids and Other Phytohormones on Seed Dormancy and Germination*

Regulation of the seed germination rate is very important for a good seedling establishment, resulting in weed control and efficient crop production, especially under suboptimal growth conditions (Finch-Savage and Leubner-Metzger 2006). Researcher previously considered that the GA and ABA interactions regulated germination (Raghavendra et al. 2010), but there was also an insight that other phytohormones (El-Maarouf-Bouteau et al. 2015), particularly of BR positively influenced seed germination in different plant species. Exogenous BR application removes the low germination of gibberellin mutants, and the seed germination of BR-related mutants is more sensitive to inhibition by abscisic acid (ABA) than the wild type (WT) (Xue et al. 2009). Recent results suggests that the antagonistic effect of BRs on seed germination is partially mediated through the *MFT* protein, because application of BRs to *mft* mutants did not antagonize the inhibitory effect of ABA (Xi and Yu 2010). The ABA inhibition of germination was overcome by overexpressing the *DWF4* biosynthetic gene in *Arabidopsis* (Divi and Krishna 2010). All these results show that BRs and some other phytohormones play a roles in seed germination, both under normal and stress conditions.

3.2 *Influence of Brassinosteroids and Other Phytohormones on Plant Architecture and Biomass*

Plant architecture is the three-dimensional organization of the plant, and these includes many different traits, f.e. plant height, branching/tillering pattern, foliar arrangement and morphology, and reproductive organ structure, all depend from action of BSs (Clouse 2011), and other phytohormones. Plant architecture is a complex of many traits of extraordinary agronomic importance with a strong influence on harvest index and grain yield (Reinhardt and Kuhlemeier 2002).

In the field, crops are usually grown at high planting density and high nitrogen input, two factors that influenced stem elongation and lodging. To provide high yield and avoid lodging of crops under described conditions, its manipulated with their pigment signal systems (Gururani et al. 2015b), but in cereal crops semidwarf and/or erect leaf are required as desirable traits (Van Camp 2005). Semidwarf varieties of some cereal crops with enhanced yield and resistance to lodging are in the roots of “green revolution” (Athwal 1971). Green biomass is another important

phenotype, especially in energy crops. BR-deficient and BR-insensitive *Arabidopsis* mutants are generally dwarfed with shorter petioles and hypocotyls. On the contrary, catabolic mutants and transgenic plants with higher BR content generally show increased growth and has elongated organs. BR-deficient and BR-signaling mutants of other dicotyledonous (dicot) plant species, also possessed a dwarf phenotype (Bishop and Koncz 2002). Similar picture are observed in monocotyledonous (monocot) species, f.e. in the rice mutants with a reduced leaf lamina inclination, shortened internodes and more erect leaves (Hong et al. 2004). Conversely, the elongated organ trait of some BR mutant/transgenic plants translates in mutant/transgenic rice plants with increased leaf bending (Park et al. 2006). Leaf angle is an important trait in grass crops because it allows higher density sowing and it have a great influence on biomass and grain yield. Under high planting, the semidwarf rice mutants also show an increased biomass compared with the WT plants (Sakamoto et al. 2006). The mechanisms by which BR regulate lamina joint inclination remain unclear. Reduced leaf angle, as a trait inherited in some BR mutants, is caused by an elongation failure in the abaxial lamina joint cells (Hong et al. 2004), but also other factors may affect lamina joint inclination, and thus influenced the architecture of plants. Differential expression of various component(s) of the BR pathways may explain why some tissues are more sensible than others to changes in BR levels and responses.

With the exception of rice, very little is known about BR pathways and the effect of manipulating them in other monocots. Some of the few BR mutants identified in non-rice monocot species is the semidwarf *uzu* mutant of barley and dwarf maize mutant at *Zm DWF1* gene. Plants of the grass crops with modified BR content have dwarf phenotypes and changed plant biomass yield. In addition, BR can also influence plant branching/tillering, and also in rice affects panicle architecture (Hong et al. 2003). Transgenic dicots and monocots, overexpressing different genes have a higher biomass yield than the WT plants (Wu et al. 2008; Vriet et al. 2013).

Vascular tissues are of great importance for plant growth and development because they provide the flows of water, nutrients, and photoassimilates through the plant and supported it. Mutants lacked BR generally show abnormal mode of vascular differentiation, characterized by proliferation phloem against xylem cells. Consistent with BR importance to xylem development, treatment with the BR biosynthetic inhibitor brassinazole prevents the development of secondary xylem in *Lepidium sativum* (Nagata et al. 2001). Also, BR modulate the number of vascular bundles by influenced early procambial activity, but periodic auxin maxima control their positioning (Fabregas et al. 2010). Many of the BR genes with different functions involved in vascular tissue development (Cano-Delgado et al. 2004). Orthologs of *BRI1* and *BRL* genes also exist in monocots, but their role in vascular development has not been established yet (Cano-Delgado et al. 2010).

3.3 *Influence of Brassinosteroids and Other Phytohormones on Photomorphogenesis*

Shade avoidance is a complex of responses (shade avoidance syndrome: *SAS*) that plants show when their leaves come in the shade of their own leaves or leaves from neighboring plant. *SAS* is an significant determinant of plant architecture and seed and biomass yields. Reducing of *SAS* is a breeding target for seed yield increase, particularly for crops raised at a high planting density. And the opposite, enhancement of *SAS* to increase green biomass production at the expense of grain yield is of interest for the development of energetic crops (Kebrom and Brutnell 2007). Importance of BR in response to shade noticed by the induction of many BR-related genes under the conditions (Kozuka et al. 2010). Many transcription factors connected with *SAS* are influenced by BR or BR-related components and genes (Crocco et al. 2011). BR are required for *SAS* responses to reduce blue light and also by a lowered *R:FR ratio* (Keller et al. 2011). Moreover, the *Arabidopsis* BR inactivation enzyme modulates the change from skoto- to photo-morphogenesis, mainly through FR light-related changes in BR levels (Turk et al. 2003). Many data are pointing toward interactions between light and BR signals. First, a BR-induced genes participated in light responses, and the two important transcription factors of the BR signaling pathway bind to many of them (Yu et al. 2011), and one of them is repress the expression of an positive regulator of photomorphogenesis (Luo et al. 2010). Second, many of the *Arabidopsis* BR-related mutant seedlings show a deetiolation phenotype in the dark (Szekeres et al. 1996). These suggest that BR work as negative regulators of the deetiolation. In support of that, the expression of BR biosynthetic genes in *Arabidopsis* are higher in seedlings raised in dark than light-grown seedlings (Symons et al. 2002).

Also, reduction of BR content reinforce the expression of light-induced genes and photomorphogenesis, but brassinolide treatment suppresses it (Song et al. 2009). However, direct measurements of endogenous contents of BR do not confirm correlation with these gene expression pattern, neither in *Arabidopsis* and in other species. In fact, BR contents were lower in dark-grown *Arabidopsis* seedlings comparing to light-grown control plants (Vriet et al. 2013; Symons et al. 2008).

3.4 *Influence of Brassinosteroids and Other Phytohormones on Photosynthesis*

Photosynthesis is the main producers of carbon assimilates in plants. Photoassimilate production may be enhanced by amplification either efficiency the photosynthesis or the whole plant photosynthetic capacity (by increasing leaf area index (LAI) in different ways) (Van Camp 2005). The prospect of increasing the photosynthetic

efficiency for crop improvement has received much attention in the last near past by finding that usually crop yields are enhanced by a CO_2-induced increase in leaf photosynthesis (Long et al. 2006). Many data indicate the stimulation of photosynthesis by BR. For example, genetically modified rice with overexpressed *OsDWF4/CYP90B1* or its close gene homologs from different plants under the control of a promoter active in stem, roots, and leaves (but not in seeds), showed an increased seed yield and CO_2 uptake, all marks of enhanced photosynthesis (Wu et al. 2008). Surprisingly, an rice mutants with semi-dwarf, erect leaf phenotype also had higher photosynthesis and seed yield under high plant sowing than the WT plants, possibly due to the more erect leaves who do not make shadow on lower leaves (Sakamoto et al. 2006). An inhibitory phosphorylated *Arabidopsis* mutant also show increased photosynthetic rate (Oh et al. 2011). Consistent with BR effect on photosynthesis, it activate the *RUBISCO ACTIVASE* enzym of cucumber (Xia et al. 2009a).

Also, an brassinazole induced gene encoding a protein necessary for proper *Arabidopsis* chloroplast biogenesis (Komatsu et al. 2010), allow further evidence for BR influence on regulation of photosynthesis. It was found that *BES1/BZR2* gene restricted chloroplast development in dark by repressing the expression of two *GLK* transcription factors that function redundantly to promote chloroplast development (Vriet et al. 2013). Delayed leaf senescence, or a *stay-green* trait, is usually considered a good characteristic of crops and constitutes a goal for enhance of crop productivity (Horton 2000). Leaf senescence is a complex process controlled by environment as well as internal factors. BRs may play a role in enhance of leaf senescence because (1) many of the BR-related mutants show a delayed senescence phenotype (Clouse and Sasse 1998), and (2) exogenous BR treatment induces leaf senescence in many plant species (Saglam-Cag 2007).

Although it is many proofs that BR application really improves the photosynthetic efficacy and that BRs regulate the photosynthesis under different conditions (Holá 2011), the precise basic mechanisms of BR-induced effects on photosynthesis remain hypothetical. Rothová et al. (2014) studied the effects of BR application on photosynthesis of maize and spinach. Although the efficacy of the photosynthetic *ETC* responded negatively to BR treatment in both plants, responses of the *PSII* activity were completely different. Similarly, the maize exhibited a positive BR influence on the accumulation of their photosynthetic pigments; but, this was not true for the spinach plants (Rothová et al. 2014). These findings raised an important question which concerns the possible differences in the *PSII* response to BR treatment of various plants, maybe because different phytohormone crosstalks which existing in certain plant species might not occur in other species.

Other phytohormones like as ABA influenced photosynthesis, possible because that the ABA biosynthetic pathways partly overlap with the synthesis of xanthophyll cycle pigments (Zhu et al. 2011). Possibly exist a connection between reduced expression of the gene for an photosynthetic protein in potato transformed plants, with higher content of ABA and resistance to many stresses (Lundin et al. 2007; Gururani et al. 2013). Cytokinins (CK) are phytohormones which primarily influenced plant cell division, but also they play a role in chloroplast biogenesis and in abiotic stress tolerance in higher plants (Rivero et al. 2009). Gibberellic

acids (*GA*) are a group of plant growth substances involved among other processes also in induction of photosynthesis-related processes (Cheikh et al. 1992).

3.5 *Influence of Brassinosteroids and Other Phytohormones on Root Development*

Roots are important for of crop productivity because their role in water and mineral uptake from the soil. BR exert opposite effects on root growth, depending on their applied concentration, it is stimulated by low and inhibited by high concentrations of exogenous BR (Müssig et al. 2003). Many of the *Arabidopsis* and pea BR-deficient mutants have reduced growth and changed development of roots, suggesting a positive influence of BR on that physiological processes at a usual physiological concentrations. It has also been found that BRs interact with auxin to promote lateral root growth and negatively influenced jasmonate inhibition of root growth in *Arabidopsis* (Huang et al. 2010). Several sterol and BR mutants also show changes in root hair formation, suggesting that sterols possibly are needful for correct auxin and ethylene signaling (Souter et al. 2002). In last years, it is assumed that local distribution of structural sterols affected both the initiation and tip growth of root hairs by regulating the vesicular trafficking and plasma membrane performances of root cells (Ovecka et al. 2010). On the contrary, BRs are necessary to maintain position-dependent fate specificity of cells and to control meristem size by improving the cell cycle progress in *Arabidopsis* roots (Gudesblat and Russinova 2011).

3.6 *Influence of Brassinosteroids and Other Phytohormones on Flowering*

Another significant agronomical trait is the flowering time. Floral induction is a complex developmental process that need integration of different endogenous signals and environmental limitations to get that flowering processes are adequately carried out in the appropriate environment (Srikanth and Schmid 2011). Plants that flower late tend to have high total seed production as a result of extended vegetative growth and source strength, but delayed crop flowering is generally undesirable trait. In many BR mutants, the flowering time is delayed, suggesting a role for BR in the control of the trait (Li et al. 2010). For example, *BRI1*-mediated signals promoted flowering in *Arabidopsis* by preventing the expression of the transcription factor *FLC* (Domagalska et al. 2007). Also, histone acetylation dramatically raised at the *FLC* locus of the double mutant, maybe because remodeling of chromatin is part of the BR regulation of flowering. Interactions between *BES1/BZR2* and the chromatin remodeling factors (containing *histone demethylases*), might provide a molecular connection between BR and flowering time. Recently, the role of BR in

regulating the flowering time has been shown to depend on their interaction with gibberellin (Domagalska et al. 2010).

3.7 *Influence of Brassinosteroids and Other Phytohormones on Male and Female Fertility*

Seed production of flowering plants based on the formation of male and female gametophytes of the reproductive organs and is regulated by various external and internal factors. Many of the BR mutants show diminished male fertility. Systematic phenotypic analysis of the male reproductive organs of the mutants uncover defects in their morphology, function, development and growth (Ye et al. 2010). In addition to their role in male fertility, BR also influenced development of female reproductive organ (Perez-Espana et al. 2011). Fertility is also reduced in many BR-deficient rice mutants (Wang et al. 2008), although it remains to be determined do the same function of BRs in *Arabidopsis* both sex gametophyte development is also applies to monocot species. Recently, feminized male flowers found in an maize dwarf mutant (Hartwig et al. 2011), and also in another maize dwarf plants, defective in an BR biosynthetic enzyme (Makarevitch et al. 2012), suggesting an important role of BRs in the control of sex determination in maize.

3.8 *Influence of Brassinosteroids and Other Phytohormones on Source-Sink Relationships, Seed Development and Seed Filling*

Seed yield is the most important agronomical trait in grain crops, and huge efforts are made to enhance it, under both optimal and suboptimal conditions, especially in the major cereal crops in the world (maize, wheat, and rice). In rice, the yield potential consists of a some substantial components: grain weight (controlled by factors of heritability), grain number per panicle, panicle number per plant (connected with tiller number per plant), and proportion of filled grains (influenced by environmental factors) (Sakamoto and Matsuoka 2008). In other important grain crops similar characteristics determined yield. Concerning yield improvement in the major grass crops, increasing in seed number is better option comparing to seed size or weight to limit the possible alterations in different technological characteristics often seen with larger, heavier grains (Fitzgerald et al. 2009).

There is decisive evidence for a BR function in plant seed production. Although are only a few reports regarding seed yield and characteristics of *Arabidopsis* BR-related mutants and transgenic plants, a some examples uncover BR effects on these traits. The *Arabidopsis dwf5* mutant produced irregularly shaped seeds similar to the seeds of corresponding *lk* mutant of pea. Overexpression of *DWF4* in

Arabidopsis much increased seed weight per plant, mainly due to more seeds produced than in WT as a result of an elevated number of branches and siliques. Also, an increased number of siliques and seed yield were observed in genetic transformed *Arabidopsis* plants with overexpress *HSD1* gene (Vriet et al. 2013; Li et al. 2007). Positive or negative regulated genes of BR synthesis and signaling, usually have (except in case of gene redundancy) sterile phenotypes and/or strongly reduced seed yield due to smaller and rounder seeds. Such phenotypes are observed in downregulated mutants (Morinaka et al. 2006). But on opposite, transformed rice, overexpressing an transcription factor, a positive regulator of BR signaling, have larger seeds (Tanaka et al. 2009). These and other examples (Reuzeau et al. 2005) point to the importance of promoters who mediate all these BR-related gene transformations of field crops. Possibly, all these mechanisms of BR influence on seed size can be mediated by their well known effects on cell division, elongation, and differentiation. Alternatively, the effects of BR on seed size might be driven by an enhanced seed filling caused by higher carbon flux (Wu et al. 2008). Also observed dose-dependent, tissue/organ-specific phenotype effects of BR in allelic series of rice *bri1* mutants with phenotype series from sterile dwarfs to fertile *semidwarf* plants, with an erect leaves. If that semidwarf mutant planted at a high density, they show a high grain number, but not observed improvement of seed yield because of the smaller grains. Considering that leaf lamina joints are more sensitive to altered BR-related processes than other tissues/organs (f.e. seeds), rice plants with an erect leaves and no negative effect on seed size were acquired using methods of partial gene suppression, but in these plants raised under high planting density observed 30% increase of grain yield.

Gene duplication may also be exploited for crop improvement (Hong et al. 2003), because different effects of BR in various plant species (Sakamoto et al. 2006). Very high degree of gene duplication in cereals indicates that selective inactivation of some BR-related gene can be widely used as a way to alter the plant architecture in a sophisticated manner. Also, it less well-known consequences of change BR endogenous content on the seed composition. Trials involving exogenous BR application on a crop plants and seeds suggest that BRs might significantly affect it (Janeczko et al. 2009). Additional studies on the BR effects on seed composition are needed if the BR pathway components are to be manipulated for grain crop improvement.

Also, other phytohormones influenced source-sink relationship in plant. For example ABA regulated photosynthesis and related processes. Also should be noted that auxin control of photoassimilate unloading within developing grains of wheat (Darussalam et al. 1998). Besides that it should be noted that reduction transpiration in shaded leaves, caused decrease among other factors, also content of the cytokinin (Pons et al. 2001), but application of BAP removes that symptoms. Importance of cytokinines for source-sink relationship in plants were further considered by Guivarc'h et al. (2002) on tobacco plants transformed by *ipt* gen with tissue specific promoter. In the *ipt* transformed plants, beside many other effects, on the lateral branches of the plants started tuberization, with the high content of extracellular invertases and starch in the "tubers".

3.9 *Influence of Brassinosteroids and Other Phytohormones on Fruit Ripening and Other Economicaly Important Crop Quality Traits*

BR also have a role in stimulating fruit ripening. For example, the ripening period of grape berry was connected with an increase in catasterone levels. Also, exogenous treatment by BR enhanced berry ripening, but application of Brz significantly delayed it (Symons et al. 2006). BRs are also influenced the ripening of tomato fruits, which is accompanied by increased contents of lycopene and carbohydrate and lowered content of chlorophyll and ascorbic acid in tomato pericarp discs externally treated by BR. This BR-induced fruit ripening has been associated with increased ethylene production (Vardhini and Rao 2002).

Data obtained by analysis of the different *Arabidopsis* and tomato BR-related mutants and antisense transgenic plants, indicated BR influence on the plant primary metabolism, f.e. significantly changed starch, sugar and nitrogen compound contents (Schluter et al. 2002; Lisso et al. 2006). Additionally, evidenced a link between BR and carbohydrate metabolism in different *Arabidopsis* mutants with sugar hypersensitivity, that can be rescued by BR application (Laxmi et al. 2004). Evidence also supports a role for BR in nitrogen metabolism (Nam and Li 2004).

Because crucial role for cellulose synthesis and plant cell wall homeostasis has been established for phytosterols and recently, also for BRs (Wolf et al. 2012) it is assumed that brasinosteroids may influenced plant fiber synthesis, such as in cotton. BR-deficient or insensitive *Arabidopsis* mutants contain less cellulose than WT controls, and the expression of the cellulose synthase genes is regulated by *BES1/BZR2* (Xie et al. 2011).

3.10 *Influence of Brassinosteroids and Other Phytohormones on Plant Tolerance to Stress*

Brassinosteroids are plant hormones that are known for a wide range of functions in plant metabolism, growth and development, abiotic and biotic stress tolerance (Bai et al. 2012). Also, some recent data indicated a complex interplay between phytohormones and cellular redox machinery that regulate the response of the photosynthetic apparatus to different abiotic stress conditions (Holá 2011). Furthermore, the expression of plastidial and nuclear photosynthetic genes can be under hormonal regulation (Bartoli et al. 2013). However, the highly complex molecular linkages between the signal connections of various hormones make it difficult to elucidate the clear roles of individual hormones in regulating the expression of different genes and in the regulation of the repair process (Gururani et al. 2015a, c). In addition, the phytohormones interact with each other during episodes of various types of stress, at various age phases of the plants (Kranner et al. 2010; De Bruyne et al. 2014). During the usual conditions of the

environment, interactions of various phytohormones have also been noted, with a particular emphasis on relationship between brassinosteroids and other phytohormones (Hartwig and Wang 2015), which make up the whole "net" of interactions of the phytohormones, ensuring the optimal development of cellular reactions, but also other processes important at the level of the whole plant level during the growth and the development.

Osmotic stresses (drought and salt stress) are one of the most limiting abiotic factors for crop productivity. Whereas many results demonstrate a positive effect of *BR* treatment on plant tolerance to salt and drought stresses (Bajguz and Hayat 2009), only few studies have been performed to evaluate the effects of altered endogenous *BR* content on these traits, but with contradictory results, and the mechanisms involved in these processes remain mainly unknown. BR influenced plant drought tolerance maybe by controlling the morphology and physiology of stomata, but the results are controversial (Schluter et al. 2002). Another possible molecular mechanism that links BRs with abiotic stress tolerance involves endoplasmic reticulum signals. Also, BR-treated cucumber showed improved utilization of absorbed light energy in chloroplasts and reduced drought-induced photoinhibition (Xia et al. 2009a). BR analogues modulated salt stress by affects synthesis of ethylene and polyamine in lettuce (Serna et al. 2015). And, in addition, BRs influenced different aspects of plant cell alternative respiration (Derevyanchuk et al. 2017) in salt stress conditions. Among other phytohormones, ABA is a well-known as in their significant role in plant reaction to different stresses and senescence, particularly by induction of stomatal closure. Divi et al. (2010) emphasize the importance of the common effects of ABA with brasinosteroids, ethylene and SA in plant resistance to salinity stress, but Ha et al. (2014) indicate a positive regulatory function of strigolactone (SL) in ABA mediated response to salinity stress. Also, other phytohormones increase the resistance of metabolism to osmotic stress factors (Holá 2011; Vriet et al. 2013; Gururani et al. 2015a).

Thermal (heat and cold) stresses have a high impact on seed yield. Crops are particularly sensitive to thermal stresses during their reproductive stages (Zinn et al. 2010). Exogenous BR treatment significantly enhance plant tolerance to both heat and cold stresses (Ogweno et al. 2008). By contrast, a few studies exploring the effect of altering plant endogenous BR contents and their influence on thermotolerance, showed somehow contradictory results (Divi et al. 2010). Also, an *Arabidopsis* mutant has an increased tolerance to cold compared with WT controls, whereas the transgenic plants overexpressing *At BRI1* have the opposite phenotype, which correlated with an increased expression of stress-inducible genes and transcription factors regulating them in the *bri1-9* mutant compared with the *BRI1*-overexpressing plants (Kim et al. 2010). Divi et al. (2010) emphasizes the importance of common action of ABA with BR, ethylene and SA in plant resistance to high-temperature stress. Also, other phytohormones increase the resistance of metabolism to extreme temperature stress (Holá 2011; Vriet et al. 2013; Gururani et al. 2015a).

Reactive oxygen species (*ROS*) play a role in both plant growth and development and stress responses (Apel and Hirt 2004). Trials with BR external treatments and with the *Arabidopsis det2* mutant point out on BR role in the plant responses to

oxidative stresses. Enhanced oxidative stress response in the *Arabidopsis* BR-related mutants associated with a constitutive increase in *SOD* enzyme activity and *catalase* transcript levels, suggest that longterm BR deficiency results in a constant in vivo physiological stress in the plants (Cao et al. 2005). These results indicate that endogenous BR levels are negatively correlated with the plant tolerance to stress, but BR levels positively correlated with an increased tolerance of cucumber treated with 24-EBL and *Brz* to photooxidative stress (Xia et al. 2009b), maybe via the production of antioxidants that protect cells from damage. These data highlight some differences in the effect between BR application and manipulation of the endogenous BR level and/or between plant species. Also, other phytohormones increase the resistance of metabolism to oxidative stress (Holá 2011; Vriet et al. 2013; Bajguz and Hayat 2009).

High concentrations of metals, including those essential for growth, have a toxic effect on plant metabolism. Many trials conducted in different crops show that BR interfere with the uptake of heavy metals and promote their detoxification, particularly by enhance production of antioxidant enzymes and the accumulation of proline under Cd and Al-induced (Janeczko et al. 2005; Ali et al. 2008; Hasan et al. 2008, 2011) metal toxicity. Pesticides (include herbicides, fungicides, and insecticides) play a major role in agriculture by reducing crop yield losses, but these molecules can also have a negative effect on the crop and can be detrimental to human health and the environment. BRs have been shown to reduce the damages caused by pesticides by accelerating their catabolism, consequently reducing their residual levels in the plants (Xia et al. 2009c). Other phytohormones also act as protective agents against xenobiotic stress (Holá 2011; Gururani et al. 2015a).

Pathogen attacks are one of major limiting factors of crop productivity. In the evolutionary arms race between plants and their pathogens, plants have evolved a highly sophisticated defense system in which plant hormones play a pivotal role. The hormones salicylic acid, jasmonate, and ethylene are well known regulatory signals of the plant's immune response, and pathogens can antagonize it by affecting its hormone homeostasis. More recently, other plant hormones, including BRs, have been implicated in plant defense mechanisms (Pieterse et al. 2009).

4 Plant Growth, Bioproduction and Crop Yield Influenced by Brassinosteroids in Different Environmental Conditions and Development Stages: An View

From previous findings, it is clear that the production of organic matter and associated plant growth, development and yield has complex polygenic properties which is influenced by different environmental factors, especially stressful ones, often exceed the physiological reaction of the plants, which, as an open system, moved to new balance, i.e. their homeostasis is a dynamic category, as Lichtenthaler (1996) observes. Quarrie (1997) goes a step further, considering in the context not just the ecological resistance of plants to stress in terms of survival and producing

generative propagules (seed, fruit, tubers), but also achieving economically satisfactory yields over a longer period, with preserved harvest index.

Such an approach imposes the creation of not some individual crop traits, determined by one or several genes (such as the production of osmolites or other small protective molecules), but rather of a complex crop ideotype, as the "ideal" morphological and physiological form of plant crops, adapted to the prevailing agroecological conditions in specific production region. Although the BR related pathways were connected with different important morphological traits of crops, close to their ideotype (Hong et al. 2004; Wu et al. 2008; Schulz et al. 2012; Feng et al. 2012) or their improved development changes (Hartwig et al. 2011; Ye et al. 2010), through genetic transformation, raised hopes for increasing the crop yield (Oh et al. 2011, 2012), problem still remains. There is a necessity for developmentally and environmentally induced promoters, as activators of the introduced gene "at the right time," because the transformation of crops with constituent promoters does not solve the problem of the necessary phenotypic plasticity of crops, as a prerequisite for good and stable yields.

An attempt to improve yields through an external application of brasinosteroids (Khripach et al. 2000), as well as other growth regulators, despite some very imaginative ways of application, also does not provide sufficiently reliable results. Thus, although the central role of brasinosteroid phytohormones in the regulation of plant metabolism is practically proven, there is still a need to improve the methodology of better defining the crop ideotyping to improve yields.

Progress in the study of brasinosteroids is taking place, by monitoring the a molecular paradigm, which "suggests" (beginning with the seminal work of Watson and Crick), that the phenotype of living organisms is determined by their genetic inheritance. This is essentially true, but as we noted at the outset, the phenotypic plasticity of plants is a highly variable category, moreover due to the old observation that the sesile plants are differentiated from the outside (Vernadsky 2008), for the exploitation of external resources from the atmosphere and the soil needed for their growth and development. Therefore, plants are significantly exposed to variations of the environmental factors, so the variability of their phenotype and the associated bioproduction of plants is surprisingly large, which can only compare phenotypic plasticity and bioproduction of prokaryotic microorganisms. Phenotypic plasticity of plants, due to the need for economic predictability in plant production (Quarrie 1997), imposes some other methodologies, in addition to the methods of molecular genetics. The specificity of the plants, in addition to their phenotypic plasticity, is also reflected in the fact that they possess some molecular markers, which can be easily followed by biophysical methods, such as, for example, chlorophyll fluorescence (Lichtenthaler 1996; Baker 2008), thermal imaging etc., which can under certain conditions be used as a non-destructive method for assessing plant bioproducts.

Also, the poikilotermic energy of the plants indicates their great dependence on the external temperature variations and other energy factors, which opens the way and the application of thermodynamics in the estimation of their yield, as the economically most important phenotypic characteristic of the crop.

4.1 Plant Growth and Bioproduction Influenced by Brassinosteroids at Seed and Seedling Stages

According to the results shown in Waisi (2016) and Waisi et al. (2017a, b), control samples of maize hybrid ZP434 are characterized by higher values of the plumule and radicle mass, compared to hybrid ZP704. Also, for hybrid ZP434, it can be concluded that lower concentrations of 24-EBL- a (5.2×10^{-15}, 5.2×10^{-13} and 5.2×10^{-12}) had a stimulatory effect on the plumule biomass, while the stimulatory effect in the radicle was present both at low and at higher concentrations of the 24-EBL (5.2×10^{-15}). Hybrids ZP434 and ZP704 differed in response to the concentrations of the 24-EBL, It is known that brasinosteroids and auxins act synergistic when it comes to cell proliferation (Zhang et al. 2009), and the elongation, and increase in the mass of the seedlings can be associated with the BRs induced genes which are known to be early auxin gene. It has been proven that brasinosteroids, if exogenously added to the plant, can inhibit the growth of roots and lateral root formations (Clouse and Sasse 1998), which is in line with the results obtained at higher concentrations of the 24-EBL. Greatest influence on the initial stages of the development of the seedlings has crossed signal pathways of brasinosteroids and other essential phytotohormones and probably, lower concentrations of 24-EBL are influencing the expression of the gene together with auxinins and gyberellins, influencing elongation of the seedlings, while its high concentrations probably favor jasmonate and activation of DELLA proteins, negative regulators of giberelin (Gallego-Bartolomé et al. 2012).

The obtained results of mass accumulation during germination of the seed of two maize hybrids were used for the evaluation of the so-called Vigor Index II, the common parameter in agronomy for estimating seed germination (Fig. 9.1).

Hybrid ZP434 had higher SVI-II values at lower concentrations of 24-EBL compared to control samples, while hybrid ZP704 had a lower SVI-II value at all concentrations compared to control samples. These results confirm the assumption that lower concentrations of the 24-EBL could improve the vigor of the seedlings and the initial phase of growth and the development of seedling with lower vigour II. It is known from the literature that seedlings with increased biomass in early stages of development, longer plumule and radicle and high percentage of germination can be identified as seedlings that will develop in the future in more resistant plants with higher growth (Mondo et al. 2013).

The content of several sugars in the samples of seeds, plumule and radicle of both hybrids (ZP434 and ZP704) for the entire concentration range of 24-EBL, as well as samples not treated with 24-EBL were determined. Results are shown in Table 9.1.

Hybrids react differently to the highest concentration of 24-EBL when it comes to the content of glucose and fructose in plumule and radicle. Observing the content of sucrose (Table 9.1), it can be concluded that the content is the same in both the plumule and the radicle relative to RoS (rest of seedling), which indicates the use of sucrose in the elongation of the parts of the seedlings of both hybrids. Analyzing the

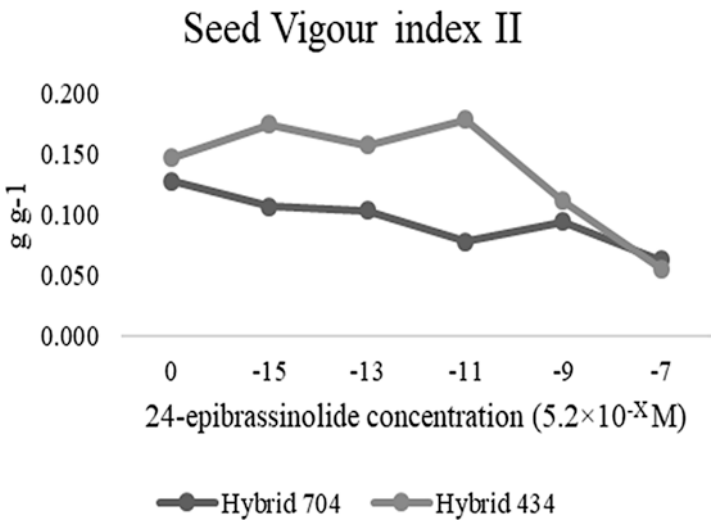

Fig. 9.1 The effect of different 24-EBL concentrations on Vigor Index II (g g^{-1}) of hybrids ZP434 and ZP704

content of disaccharides in the samples of both investigated hybrids, sucrose is present at the highest concentration, which explains the high content of glucose and fructose molecules (monosaccharides that build sucrose molecules) in these samples. Furthermore, the arabinose content in plumule of hybrid ZP704, treated with a 24-EBL concentration of 5.2×10^{-9}–5.2×10^{-15} M is higher than the control sample. Arabinose is part of the biopolymer of hemicellulose and pectin, which are involved in the construction of the cell wall of plants. The increased content of this sugar in the mentioned samples has a positive effect on plant growth (Waisi 2016).

Observing the ZP434 hybrid, the highest trehalose content was in RoS in the control sample, in RoS at the lowest concentration of 24-EBL, and also in the radicle and plumule at the higher concentration of 24-EBL.

All values of the trehalose content are higher in all parts of the seedlings of the ZP704 hybrid, comparing to hybrid ZP434, except for control RoS and radicle treated with 5.20×10^{-7} concentration (Table 9.1). The effect of trehalose can be attributed to the formation of membrane bonds or the ability to modify the solvation layer of the protein. Trehalose occupies a minimum of 2.5 times the volume of fructose and glucose. Therefore, because of its high hydration volume, trehalose can replace more water molecules than fructose and glucose (Sola-Penna and Meyer-Fernandes 1998). For the above reasons, trehalose can have a major influence on thermal activation in the dehydration process and it can also be concluded that it may have an effect on the possible change in the reaction mechanism during dehydration and thermal stress (Waisi et al. 2017), Higher content of different sugars, starches (Janković 2013) and specialy sucrose is essential in the process of drought tolerance. The increase in the content of sucrose in the radicle and plumule is

Table 9.1 Contents of various sugars determined in seedlings parts (with three different monitored 24-EBL concentrations (5.20×10^{-9}, 5.20×10^{-12} and 5.20×10^{-15} M)) including control samples for both hybrids [±(..) – SD: standard deviation value (σ)]

Concentration	Raffinose	Arabinose	Trehalose	Glucose	Fructose	Sucrose
ZP434 hybrid (mg kg^{-1} of dry matter)						
Control radicle	7.998 ± 0.027	55.197 ± 0.153	62.397 ± 0.017	68.540 ± 0.187	254.377 ± 0.160	842.125 ± 0.342
Control plumule	12.737 ± 0.117	81.446 ± 0.107	43.87697 ± 0.016	222.65 ± 0.016	271.266 ± 0.015	649.208 ± 0.076
Control RoS	51.122 ± 0.810	283.082 ± 0.848	73.894 ± 0.052	1046.265 ± 0.015	261.206 ± 0.164	130.057
5.20×10^{-7} radicle	18.535 ± 0.067	21.648 ± 0.135	83.094 ± 0.002	141.704 ± 0.142	432.331 ± 22.108	4385.532 ± 0.538
5.20×10^{-11} radicle	9.547 ± 0.065	15.922 ± 0.029	31.532 ± 0.031	39.697 ± 0.136	243.859 ± 0.022	2993.569 ± 0.416
5.20×10^{-7} plumule	56.149 ± 0.109	56.609 ± 0.457	127.867 ± 0.061	2386.577 ± 0.274	2698.74 ± 0.175	2103.836 ± 0.491
5.20×10^{-11} plumule	18.137 ± 0.064	40.867 ± 0.130781	14.731 ± 0.057	113.786 ± 0.16	286.91 ± 0.088	3174.901 ± 0.420
5.20×10^{-7} RoS	48.853 ± 0.525	40.177 ± 0.044	39.66 ± 0.052	894.366 ± 0.225	149.678 ± 0.17	45.8835 ± 0.344
5.20×10^{-11} RoS	37.333 ± 0.662146	563.5635 ± 0.24	7.029 ± 0.013	79.45 ± 0.049	38.772±0.0517	87.8414 ± 0.527
ZP704 hybrid (mg kg^{-1} of dry matter)						
Control radicle	28.440 ± 0.401	32.567 ± 0.027	84.908 ± 0.075	51.907 ± 0.053	61.414 ± 0.084	2527.665 ± 0.09
Control plumule	65.758 ± 0.26	8.81 ± 0.056	53.221 ± 0.113	76.563 ± 0.103	110.202 ± 0.039	187.272 ± 0.077
Control RoS	21.86013 ± 0.449	20.737 ± 0.028	92.747 ± 0.004	1042.716 ± 1.273	749.213 ± 0.074	46.924 ± 0.593
5.20×10^{-7} radicle	48.803 ± 0.303	37.251 ± 0.04	57.353 ± 0.301	2202.367 ± 1.519	2724.65 ± 0.135	2479.99 ± 14.675
5.20×10^{-11} radicle	29.735 ± 0.351758	47.734 ± 0.585	181.724 ± 0.198	1001.867 ± 0.441	1009.431 ± 0.127	891.498 ± 0.073
5.20×10^{-7} plumule	57.412 ± 1.125	4.629 ± 0.012	374.509 ± 0.136	151.133 ± 0.033	166.087 ± 0.074	353.441 ± 0.382
5.20×10^{-11} plumule	64.02 ± 0.204	147.426 ± 0.211	31.469 ± 0.029	1034.767 ± 0.163	604.33±0.363	5919.5 ± 1.038
5.20×10^{-7} RoS	39.798 ± 0.083	23.443 ± 0.001	95.56 ± 0.304	972.086 ± 1.234	329.43 ± 0.091	160.498 ± 0.687
5.20×10^{-11} RoS	27.269 ± 0.222	13.075 ± 0.008	178.597 ± 0.032	749.213 ± 0.074	292.532 ± 0.345	75.529 ± 0.150

probably due to the effect of sucrose on the development of the embryo, which in the results coincides with the initial stages of tolerance of the seed to desiccation.

Table 9.2 shows the percentage of the redistribution of the important minerals in all parts of the seedlings, at the tested concentrations of 24-EBL (5.2×10^{-9}, 5.2×10^{-12}, 5.2×10^{-15}) and the control samples of the seedlings. In Waisi et al. (2017), we can see that the content (regardless of the effect of the 24-EBL) Fe, K and P is higher for hybrid ZP704 than the content in hybrid ZP434. It can be seen that for all control samples, regardless of the concentration, the content Fe higher for hybrid ZP704 than hybrid ZP434 for all parts of seedlings. However, the highest Fe content was identified at a concentration of 24-EBL of 5.2×10^{-12} for ZP704. Changes in the level of iron, especially for hybrid ZP434, can be attributed to the inhibition of the growth of the parts of the seedlings, which occurs due to the limited content of phosphorus and can be attributed to the phosphorylation regulatory mechanism.

Table 9.2 Effect of different concentrations of 24-EBL on content of micronutrient and heavy metals (mg/kg) in different seedling parts of ZP704 and ZP434 hybrids

Concentration	Mn	Na	Zn	Cu	Cr	Ni
ZP434 hybrid (mg kg^{-1} of dry matter)						
Control radicle	19	46	34	33	32	35
Control plumule	38	38	50	56	50	34
Control RoS	43	17	16	12	17	31
5.20×10^{-9} radicle	18	30	24	27	26	30
5.20×10^{-12} radicle	19	61	31	32	22	31
5.20×10^{-15} radicle	16	43	32	34	43	30
5.20×10^{-9} plumule	34	45	44	41	38	34
5.20×10^{-12} plumule	34	25	39	38	45	37
5.20×10^{-15} plumule	33	33	38	37	32	37
5.20×10^{-9} RoS	48	25	32	32	35	36
5.20×10^{-12} RoS	48	14	30	30	34	32
5.20×10^{-15} RoS	51	24	30	29	25	33
ZP704 hybrid (mg kg^{-1} of dry matter)						
Control radicle	23	31	28	27	26	26
Control plumule	39	25	67	68	62	34
Control RoS	37	44	5	5	12	40
5.20×10^{-9} radicle	24	44	33	30	34	23
5.20×10^{-12} radicle	31	43	38	33	13	15
5.20×10^{-15} radicle	25	50	30	37	14	12
5.20×10^{-9} plumule	26	27	35	40	40	46
5.20×10^{-12} plumule	23	25	33	24	9	7
5.20×10^{-15} plumule	18	28	33	28	6	2
5.20×10^{-9} RoS	50	29	32	31	26	31
5.20×10^{-12} RoS	46	32	29	43	79	78
5.20×10^{-15} RoS	57	22	37	35	80	86

Results are expressed in percentage (%) Sum of the shoot, root and RoS is 100% for every trial combination

Namely, the inhibition can be attributed to the toxic effects of iron that are probably no longer in the complex phosphate system, which increases the individual influence and bioavailability of phosphorus (Celik et al. 2010). Based on the results, it can be concluded that the 24-EBL has a greater effect on ZP434 hybrid in reducing the toxic effects of the above elements. This decrease is probably associated with a lower Ionic adoption and an increase in ATP activity. Regulatory activity of H + -ATPase, not only facilitates the absorption of nutrients but also controls water fluxes, which indirectly influences dehydration processes (Sze et al. 1999).

For ZP704, it can be stated that every concentrations of 24-EBL are influencing redistribution of Zn and Mn between shoots and roots. Generally, vegetation are accumulating higher amounts of Zn within the shoots than within the roots, and that is the case with the control samples. Within the case of lowest implemented 24-EBL concentration, apparent blocking of distribution of Cu was found within each hybrids, which would possibly suggest that maize plants could gain best of growth in polluted soils. It is widely recognized that 24-EBL can reduce the toxic effect of Cd (Hayat et al. 2007). In case of seedlings treated with the lowest concentrations of 24-EBL it is apparent that the accumulation of Cr and Ni might be blocked inside the seeds. Similar inhibitory role of BRs at the uptake of Ni was also mentioned through Sharma and Bhardwaj (2007).

These important insides to redistribution of highly toxic elements are leading to conclusion that maize treated with 5.2×10^{-15} M of 24-EBL could survive much polluted soils due to its capability to block toxic factors before they reach plumule and radicle, what could guard plants in stressed situations. Outcomes confirmed that redistribution of essential factors stayed in a regular variety, while the accumulation of potentially toxic factors was blocked in seeds, which could allow seedlings regular growth and development, and protection against toxic metals. As a confirmation of such speculation, lower concentrations of 24-EBL (5.2×10^{-13} and 5.2×10^{-12} M) had stimulatory impact on ZP434 maize seedlings length (Table 9.2), whilst the weight of the shoot remained unchanged. Treatment of seedlings of both hybrids with various concentrations of 24-EBL is affecting stability of Cu, so we are able to anticipate that 24-EBL have defensive effect in terms of avoidance of possible toxic effect of Cu. Transport of Na under saline conditions is still poorly understood, however it's far suggested that vegetation could have compartments for reserving Na. This likely is helping plants to overcome environmental stress which includes salinity. Considering the fact that 24-EBL is influencing relocation of Na into root, in particular within the case of ZP704 and within the case of ZP434, treated with 5.2×10^{-12} M concentration of 24-EBL, we are able to expect that maize seedlings handled with lower concentrations of 24-EBL could have higher possibilities to emerge in saline habitats (Gomes 2011).

Also, the polyphenol profiles of above mentioned hybrids was examined. The general reasons for the resistance of ZP434 hybrid to stress conditions, in relation to ZP704 (control samples), could be identified through differences in polyphenol profiles of both plumule and radicle (Table 9.3). It has been found that hybrid ZP434 contains more highly polar phenolic compounds than hybrid ZP704. High concentrations of 24-EBL (5.20×10^{-7}, 5.20×10^{-8}) inhibitively affect the content of poly-

Table 9.3 Qualitative polyphenolic profile of maize seedling extracts attached to control samples

ZP434 plumule	ZP434 radicle	ZP704 plumule	ZP704 radicle
Ferulic acid	Ferulic acid	Ferulic acid	Ferulic acid
Protokatechic acid	p-Coumaric acid	Chlorogenic acid	Protokatechic acid
Vanillic acid	Vanillic acid	Sinapic acid acyl-β-D-glucoside	Tangeritin
2-O-feruloyl hydroxycinnamic acid	Galvanic acid		
2-o-caffeoyl hydroxycinnamic	Gentisic acid		
3-o-feruloylquinic-acid			
4-methoxycinnamic acid	4-hydroxybenzoic acid		
Cinarin	4-methoxycinnamic acid		
Kaempferide	3 4 5-trimethoxycinnamic acid		
Rutin	Cinarin		
Kaempferol 3-O-rutinoside			

phenols in hybrid ZP434, and the lowest 24-EBL concentration (5.20×10^{-7}) has an inhibitory effect on polyphenol content in hybrids of ZP704 (Waisi et al. 2015a).

Free radicals are species (atoms, molecules or ions) containing at least one unwanted electron in an external electronic envelope, which makes them very reactive, unstable and have high energy potential. One or more unconnected electrons mean a free and very unstable valence, which makes free radicals bind to the molecules they are in contact with, especially for proteins, lipids and rich biomolecular structures. In addition, there is a tumultuous chain reaction and numerous damage to the cells that in this way become faster and enter into degenerative processes. In living organisms, the level of free radicals and other reactive species are controlled by a complex antioxidant defense system that reduces damage to biomolecules. It has been found that different concentrations of the 24-EBL have a different effect on the content of ROS and RNS. Also, hybrid ZP704 reacts differently in relation to hybrid ZP434 when it comes to the amount of ROS and RNS when is exposed to the same concentrations of 24-EBL (Waisi 2016).

4.2 Plant Growth and Bioproduction Influenced by Brassinosteroids at Whole Plant Stages

Consideration of bioproductivity at the level of whole individual plants includes several specific categories: (a) photosynthesis and energetics at leaf level; (b) redistribution of assimilates and dry masses synthesized in leaves and other heterotrophic organs through the source-sink relationship; (c) the growth and development of autotrophic leaves and heterotrophic organs, as well as, methods to follow these

processes: (1) measurement of the intensity of photosynthesis and energy of leaves and whole plants; (2) analysis of the growth and chemical composition of leaves and other plant organisms, etc.

The factors influencing these processes are the same as those affecting plants in phytocoenoses (e.g. light intensity, temperature, nitrogen and other nutrients contents, also contents toxic elements, osmotic status (drought, salinity) and physical properties (soil compaction) of the substrate were plants grown etc.), but with isolated single or small number plants, which grow in pots of defined volumes, on defined soils it is easier to follow these processes. Such an approach was used extensively earlier (Poorter and der Verf 1998; Qereix et al. 2001), wherein the plants were exposed by various manipulative approaches or treatments (Sun et al. 1999; Nakano et al. 2000; De Groot et al. 2003), such as destructive and non-destructive mechanical manipulations with a leaf or root status (removal or shading of leaves, i.e., growth of plants in vessels of varying volumes), exposure of plants to different temperatures, nutritive or light intensities during growth, use of genetically modified plants with altered activity of genes of important for photosynthesis or carbohydrate metabolism and other manipulative approaches. These approaches were tested in maize plants (*Z. mays* L.) exhibited by various manipulations of the status of the leaf and roots, treated with 24-EBL, as well as brassinosteroid biosynthesis inhibitor propiconazole (PZR; Hartwig et al. 2012).

4.2.1 Plant Growth and Photosynthesis Influenced by Brassinosteroids, Type of Lighting of Leaves and at Ample Nitrogen Nutrition at Whole Plant Stages

During trials, in the full sunlight grown plants (Table 9.4), the parameter of Chla fluorescence and photosynthesis in maize plants slowed down but at the end of the experiment the highest values of this parameter were found in plants treated with ample nitrogen («+N»). In the shade grown plants, the same parameters rose up, also to the highest values in plants treated with ample nitrogen («+N») (Nikolić et al. 2013). The plants were grown in $V = 20$ L pots, with treatments of 24-EBL (+ BRs treatment: $\approx 4 \times 10^{-9}$ M), additional nitrogen nutrition, (+N treatment, equivalent to dose of 100 kg N/ha) and growth conditions equivalent to field plants (Tables 9.4, 9.5, and 9.6).

Considering Table 9.5, we can observe that the RWC parameter for all treatments of high light grown plants remained unchanged during the trial and was high and similar for the different treatments. The RWC parameter for all treatments increased in shade plants and reached high values by the end of the trial. In maize plants grown under full daylight different treatments had different influence on dry matter partitioning (Table 9.5), but it depended on light growth environments of plants. In both light environments ample nitrogen nutrition has positive influence on dry matter accumulation and growth, but in different manner in depending on the light environment and presence or absence of additional «BRs» treatment (Tables 9.5 and 9.6).

Table 9.4 Average values ($\underline{X}\pm\sigma$) of parameters of fluorescence of Chla and photosynthesis (Maxwell and Johnson 2000) in the maize (ZP434) plants growed in the whole sun light (PAR max≥1500 μmol m^{-2} s^{-1})

	Whole sun light plants					Shade plants				
P/T	A	B	C	D	E	F	G	H	I	J
Fv/Fm (r.u.)	0.776 ± 0.033	0.810	**0.831 ± 0.032**	*0.737*	0.740	0.792	*0.753*	**0.806 ± 0.024**	0.767	0.756 ± 0.012
		0.021		*0.015*	0.039	0.014	*0.019*		0.018	
Fv/F_0 (r.u.)	3.542 ± 0.712	4.317	**5.100 ± 1.322**	2.979	*2.908*	3.804	*3.062*	**4.230 ± 0.540**	3.312	3.100 ± 0.200
		0.597		0.239	*0.593*	0.322	*0.315*		0.342	
Fv'/Fm' (r.u.)	**0.525 ± 0.077**	0.402	0.500 ± 0.082	0.360	*0.350*	*0.440*	0.458	**0.570 ± 0.055**	0.472	0.535 ± 0.079
		0.071		0.067	*0.136*	*0.043*	0.095		0.104	
Φ PS_2 (r.u.)	**0.580 ± 0.180**	0.258	0.392 ± 0.104	0.265	*0.157*	*0.260*	0.316	**0.447 ± 0.081**	0.308	0.406 ± 0.093
		0.111		0.190	*0.063*	*0.040*	0.120		0.078	
qP (r.u.)	0.690 ± 0.140	0.625	**0.792 ± 0.210**	0.729	*0.458*	*0.590*	0.669	**0.783 ± 0.058**	0.650	0.752 ± 0.091
		0.159		0.208	*0.084*	*0.100*	0.134		0.034	
NPQ (r.u.)	*0.520 ± 0.080*	1.971	1.704 ± 0.505	1.952	**2.526**	*0.440*	1.132	0.869 ± 0.169	*1.540*	0.914 ± 0.408
		0.590		0.202	**0.471**	*0.040*	0.404		**0.309**	
RFD_{730} (r.u.)	2.800 ± 0.141	3.025	*2.462 ± 0.571*	**3.416**	3.042	**3.390**	*2.081*	2.366 ± 0.265	2.596	2.223 ± 0.258
		0.685		**0.419**	0.479	**0.310**	*0.483*		0.184	
ETR (μmol m^{-2} s^{-1})	**192.31 ± 60.76**	*70.79 ± 29.01*	107.66 ± 28.75	72.84 ± 25.37	93.47 ± 18.66	*85.75 ± 14.23*	92.95 ± 36.46	**138.81 ± 31.50**	89.28 ± 18.31	120.75 ± 38.53

A (Control: K_1; 5th week after germination), B (K_2; 3rd week after "A"), C (+N treatment; 3rd week after "A"), D (+BRs treatment; 3rd week after "A"), and E (+N, +BRs treatments; 3rd week after "A") and in the shade (PAR max<400 μmol m^{-2} s^{-1}): F (control: K_1; 5th week after germination), G (K_2; 3rd week after "F"), H (+N treatment; 3rd week after "F"), I (+BRs treatment; 3rd week after "F") and J (+N, +BRs treatments; 3rd week after "F") from 2nd week after germination to the end of trial and exposed to the different additional treatments

Bold: maximal values in a series. Italic: minimal values in a series

P parameters, *T* treatments

Table 9.5 Average values ($\underline{X}\pm\sigma$) of parameters of plant dry matter partition, total dry matter, and RWC parameter of leaf water regime in the maize (*Z. mays* L.; cv. ZP434) plants grown in the full sun light (PAR max≥1500 μmol m^{-2} s^{-1})

	Whole sun light plants						Shade plants					
P/T	A	B	C	D	E	F	G	H	I	J	K	L
LMR (gg^{-1})	*0.332 ± 0.160*	**0.470 ± 0.105**	0.405 ± 0.067	0.397 ± 0.065	0.422 ± 0.04	0.387 ± 0.032	*0.332 ± 0.160*	0.557 ± 0.033	**0.598 ± 0.035**	0.529 ± 0.059	0.539 ± 0.068	**0.598 ± 0.050**
SMR (gg^{-1})	*0.137 ± 0.083*	**0.471 ± 0.094**	0.437 ± 0.079	0.456 ± 0.109	0.420 ± 0.065	0.437 ± 0.104	*0.137 ± 0.083*	0.358 ± 0.020	0.319 ± 0.072	0.362 ± 0.105	**0.365 ± 0.086**	0.315 ± 0.038
RMR (gg^{-1})	**0.531 ± 0.240**	*0.059 ± 0.036*	0.158 ± 0.091	0.147 ± 0.073	0.158 ± 0.034	0.176 ± 0.093	**0.531 ± 0.240**	0.084 ± 0.038	*0.083 ± 0.052*	0.109 ± 0.053	0.090 ± 0.020	0.087 ± 0.024
TDW (g)	*0.61 ± 0.32*	12.23 ± 4.72	36.16 ± 10.67	39.99 ± 15.77	36.79 ± 16.46	**43.00 ± 16.45**	*0.61 ± 0.32*	5.93 ± 1.07	11.49 ± 4.79	**15.51 ± 3.67**	13.79 ± 6.22	9.64 ± 5.13
RWC (%)	–	*95.39 ± 4.80*	96.43 ± 2.80	95.63 ± 1.31	95.96 ± 1.67	**97.65 ± 0.16**	–	*86.31 ± 2.76*	94.93 ± 1.33	96.98 ± 0.32	**98.12 ± 0.19**	96.88 ± 0.82

A (control: K; 2nd week after germination), B (K_1; 3rd week after "A"), C (K_2; 3rd week after "B"), D (+N treatment; 3rd week after "B"), E (+BRs treatment; 3rd week after "B"), and F (+N, +BRs treatments; 3rd week after "B") and in the shade (PAR max<400 μmol m^{-2} s^{-1}): G (control: K; 2nd week after germination), H (K_1; 3rd week after "G"), I (K_2; 3rd week after "H"), J (+N treatment; 3rd week after "H"), K (+BRs treatment; 3rd week after "H"), and L (+N, +BRs treatments; 3rd week after "H"), during 2011 summer and exposed to the different additional treatments

Bold: Maximal values in a series. Italic: Minimal values in a series

P parameters, *T* treatments

Table 9.6 Growth (RGR parameters; mg g^{-1} day^{-1}) of plants, raised on different light environments (whole sun light: PAR max≥1500 μmol m^{-2} s^{-1}; shade: PAR max<400 μmol m^{-2} s^{-1}), treated by ample BRs (~4 × 10^{-9} M) and N (equivalent to dose of 100 kg N/ha) treatments

Growth of whole sun light plants	Growth of shade plants
$RGR_{SUN\ (B\text{-}A)}$ = 146.67 mg g^{-1} day^{-1}	**$RGR_{SHADE\ (H\text{-}G)}$ = 114.76 mg g^{-1} day^{-1}**
$RGR_{SUN\ (C\text{-}B)}$ = 32.00 mg g^{-1} day^{-1}	$RGR_{SHADE\ (I\text{-}H)}$ = 17.43 mg g^{-1} day^{-1}
$RGR_{SUN\ (D\text{-}B)}$ = 33.71 mg g^{-1} day^{-1}	$RGR_{SHADE\ (J\text{-}H)}$ = 27.43 mg g^{-1} day^{-1}
$RGR_{SUN\ (E\text{-}B)}$ = 34.00 mg g^{-1} day^{-1}	$RGR_{SHADE\ (K\text{-}H)}$ = 22.57 mg g^{-1} day^{-1}
$RGR_{SUN\ (F\text{-}B)}$ = 36.00 mg g^{-1} day^{-1}	*$RGR_{SHADE\ (L\text{-}H)}$ = 8.00 mg g^{-1} day^{-1}*

Treatments: A (control: K; 2nd week after germination), B (K_1; 3rd weeks after "A"), C (K_2; 3rd weeks after "B"), D (+N treatment; 3rd weeks after "B"), E (+BRs treatment; 3rd weeks after "B"), and F (+N, +BRs treatments; 3rd weeks after "B") and in the shade (PAR max<400 μmol m^{-2} s^{-1}): G (control: K; 2nd week after germination), H (K_1; 3rd weeks after "G"), I (K_2; 3rd weeks after "H"), J (+N treatment; 3rd weeks after "H"), K (+BRs treatment; 3rd weeks after "H"), and L (+N, +BRs treatments; 3rd weeks after "H")
Bold: Maximal values in a series. Italic: Minimal values in a series
GP RGR growth parameters, *LT* Light treatments

In any case, it should be noted that the growth is more pronounced in full sunlight plants, but that the difference between treatments with nitrogen, 24-EBL or together is more pronounced in plants grown in the shade. The question remains, whether the treatment of 24-EBL plants and nitrogen in earlier maize phases (before the age of 2 weeks), when the growth was pronounced ($RGR_{SUN\ (B\text{-}A)}$ = 146,67 mg g^{-1} day^{-1} and $RGR_{SHADE\ (H\text{-}G)}$ = 114,76 mg g^{-1} day^{-1}) would give even higher results. It is known that BRs interact with other signaling molecules on their growth and accumulation of plant mass (Zhang et al. 2009), so the question arises as to whether the efficiency of the nitrogen nutrition and its use for the growth of maize plants would be greater in the earlier treatment of the 24-EBL.

4.2.2 Plant Growth and Photosynthesis Influenced by Brassinosteroids Under Restriction of Root Growth and at Whole Plant Stages

Nikolić et al. (2014) notes that the accumulation of absolute fresh and dry weight (g) of the plant organs (leaves, stems, roots) and the whole plant is at the very least at the start of experiments with plants grown in the pots of least volume (V = 5 L) (Table 9.7), which is a common situation. However, this is not the case with the relative mass (gg^{-1}) of the plant organisms, and also with the differential Gibbs energy (J mol^{-1} K^{-1}) of the leaves and stems, which has the lowest values in plants grown in larger vessels (V = 11 L) at the beginning of the experiment. As far as the maximum values of different parameters are concerned, we notice (Table 9.7) that they are most represented in plants exposed to manipulations with the status of BRs (treatments of 24-EBL or PZR) at the end of trial (End 24-EBL, 5L; End 24-EBL, 11L; End PZR, 11L), which is very interesting, as the plants were exposed to low-temperature episodes for maize (t = 10–15 °C) during the sampling period for the estimation of growth parameters, indicating that although the total energy balance

Table 9.7 Average values of parameters of maize hybrid ZP505 plant growth and matter partitioning and thermodinamic changes during manipulation of root status and plant content of BRs

T/P	Start K, 5L	Start K 11L	End K 5L→11L	End K 5L	End K 11L	End 24-EBL 5L→11L	End 24-EBL 5L	End 24-EBL 11L	End PZR 5L→11L	End PZR 5L	End PZR 11L
1	*4.94*	9.13	29.28	14.06	36.18	28.74	13.38	**38.58**	29.41	12.80	30.65
2	*0.46*	0.81	2.83	1.96	3.19	3.11	2.04	3.49	3.12	1.91	**3.55**
3	0.353	*0.508*	0.387	0.360	0.520	**0.298**	0.320	0.472	0.290	0.307	0.292
4	0.561	0.587	0.567	0.492	0.584	0.571	0.481	0.563	0.590	*0.457*	**0.608**
5	*3.24*	6.45	34.42	15.01	38.95	31.94	15.85	**46.01**	31.2	16.45	32.41
6	*0.23*	0.35	1.52	1.16	1.61	1.56	1.22	**1.98**	1.53	1.36	1.69
7	0.158	*0.271*	0.168	0.157	0.252	0.138	0.142	0.253	**0.133**	0.232	0.175
8	0.280	*0.254*	0.305	0.291	0.295	0.286	0.288	0.319	0.289	**0.325**	0.289
9	*0.68*	2.28	4.50	8.09	5.09	5.15	**9.11**	4.79	3.61	8.66	3.99
10	*0.13*	0.22	0.64	0.86	0.66	0.78	**0.98**	0.73	0.64	0.91	0.60
11	*0.614*	0.349	0.512	0.516	0.358	0.520	0.482	0.353	0.437	0.667	**0.319**
12	0.159	0.159	0.128	0.216	0.121	0.143	**0.231**	*0.118*	0.121	0.218	0.103
13	-	-	3.9	5.5	*3.8*	4.8	3.9	4.4	4.3	**6.8**	*3.8*
14	*8.86*	17.86	68.2	37.16	80.22	65.83	38.34	**89.38**	64.22	37.91	67.05
15	*0.82*	1.38	4.99	3.98	5.46	5.45	4.24	**6.20**	5.29	4.18	5.84
16	0.306	0.253	0.239	0.356	0.222	**0.204**	*0.368*	0.226	0.270	0.368	0.218
17	0.907	0.923	0.927	0.893	0.932	0.937	*0.889*	0.931	0.918	0.890	**0.933**

Legends: *T* Treatments, *P* Parameters: 1: FW (g) leaves; 2: DW (g) leaves; 3: ΔG_{105} leaves (J mol^{-1} K^{-1}); 4: LMR (g g^{-1}); 5: FW (g) stem; 6: DW (g) stem; 7: ΔG_{105} stem (J mol^{-1} K^{-1}); 8: SMR (g g^{-1}); 9: FW (g) root; 10: DW (g) root; 11: ΔG_{105} root (J mol^{-1} K^{-1}); 12: RMR (g g^{-1}); 13: V root (ml); 14: TFW (g); 15: TDW (g); 16: ΔG_{105} tot (J mol^{-1} K^{-1}); 17: $a_{W\,tot}$ (r.u.). FW, DW: Fresh and dry weight of plant parts. 5L, 11L, 5L→11L: plants grown in pots volume of 5L, 11L and first in pots of 5L, and after repotting in pots of volume of 11L. Start, End: Start and end of trial. 24-EBL, PZR: Treatments of plants by 24-EBL (≈10^{-7} mol) and propiconazole (≈10^{-6} mol). LMR, SMR, RMR: Relative weight (gg^{-1}) of plant parts, leaf, stem and root. ΔG_{105}: Differential Gibss energy (J mol^{-1} K^{-1}) of plant parts or whole plant. $_{W\,tot}$ (r.u.): Relative content of plant water. Bold: Maximal values in a series. Italic: Minimal values in a series

of the plants was negative (the positive values of the ΔG_{105} thermodynamic parameters: Sun 2002), they grew (Table 9.7). How is it possible?

Fluorescence parameters (Frachebaud et al. 2002) are usually considered representative for the evaluation of photosynthesis of C_4 plants, such as maize). We find that (Table 9.8) most of the maximum values are related to plants treated with 24-EBL, while the lowest values are observed in plants treated with PZR (Hartwig et al. 2012), indicating that the externally added 24-EBL has a protective effect on photosynthesis under maize unfavorable conditions (t = 10–15 °C), which is in accordance with the literature data (Vriet et al. 2012).

It can be seen in Table 9.9 that the nitrogen content is the lowest in the control samples at the end of the trial, while the highest value of N content was registered in the leaves of transplanted maize plants, treated by 24-EBL, as well as in the stem and root of plants treated with PZR, all at the end of the experiment. As for the P

Table 9.8 Average values of parameters of fluorescence of Chla measured at youngest full developed leaves of same maize plants as in Table 9.7

Treatments during trial	Fv/Fm (r.u.)	Fv/F_0 (r.u.)	ΦPS_2 (r.u.)	qP (r.u.)	NPQ (r.u.)	ETR (μmol electrons m^{-2} s^{-1})	RFD_{730} (r.u.)
Start K 5→11	0.813	4.361	0.091	*0.278*	3.077	28.90	3.690
Start K 5	0.812	4.361	**0.206**	0.389	3.217	**49.06**	4.739
Start K 11	0.794	4.078	0.156	0.383	2.989	42.43	4.335
End K 5→11	0.786	3.756	0.100	0.305	3.144	21.55	3.925
End K 5	**0.839**	5.250	0.104	0.389	3.376	28.75	4.300
End K 11	0.793	3.836	0.107	0.389	2.944	33.56	3.711
End 24-EBL 5→11	0.836	5.117	0.180	**0.500**	3.876	45.53	5.228
End 24-EBL 5	0.837	**5.283**	0.151	0.333	**5.111**	39.35	**6.444**
End 24-EBL 11	0.792	3.822	0.088	0.389	3.126	22.77	3.788
End PZR 5→11	0.805	4.137	0.091	0.444	3.182	27.17	4.067
End PZR 5	*0.753*	*3.066*	0.153	0.472	3.194	38.55	4.183
End PZR 11	0.785	3.667	*0.081*	0.389	*2.799*	*18.47*	*3.485*

Bold: Maximal values in a series. Italic: Minimal values in a series

Table 9.9 Averaged values of content of nitrogen (N), phosphorus (P) and potassium (K) (%, w/w)

Treatments during trial/elements and plant parts	N (%, w/w)			P (%, w/w)			K (%, w/w)		
	L	R	St	L	R	St	L	R	St
Start K 5	2.90	1.88	0.95	1.22	0.76	1.84	4.72	**4.81**	4.84
Start K 11	4.03	2.44	2.76	1.44	**0.99**	2.08	4.87	4.53	4.36
End K 5→11	4.68	1.15	4.11	**1.82**	0.30	**2.34**	4.94	4.17	4.56
End K 5	*1.76*	2.20	1.20	0.74	0.21	1.18	4.72	4.36	4.73
End K 11	4.82	*0.75*	*0.64*	1.79	0.49	2.43	4.46	4.74	**5.18**
End 24-EBL 5→11	**6.23**	2.69	2.25	1.76	0.62	2.18	*4.42*	4.25	4.98
End 24-EBL 5	3.15	2.24	2.35	*0.60*	*0.18*	*1.01*	4.52	4.47	4.93
End 24-EBL 11	5.61	2.68	5.59	1.70	0.46	2.16	4.72	*4.16*	4.33
End PZR 5→11	1.83	1.37	4.21	1.65	0.81	2.12	**4.98**	4.59	5.03
End PZR 5	1.81	0.76	1.28	0.64	0.25	1.23	4.83	4.80	*4.27*
End PZR 11	4.50	**2.82**	**4.64**	1.44	0.36	2.07	4.91	4.26	5.13

Bold: Maximal values in a series. Italic: Minimal values in a series
L, R, St leaves, roots, stems of the maize (ZP505) plants

content in the organs of the maize plants, we notice a certain opposite trend, that the content of phosphorus is highest in the stem of maize control plants at the beginning of the experiments, grown in pots of V = 11 L, as well as at the end of the experiment, also in control transplanted maize plants. In contrast, the lowest P content is in the plants treated with 24-EBL at the end of the experiment. The influence of various manipulations of the root status and the content of BRs in maize plants on the potassium content is somewhat similar to the phosphorus redistribution situation.

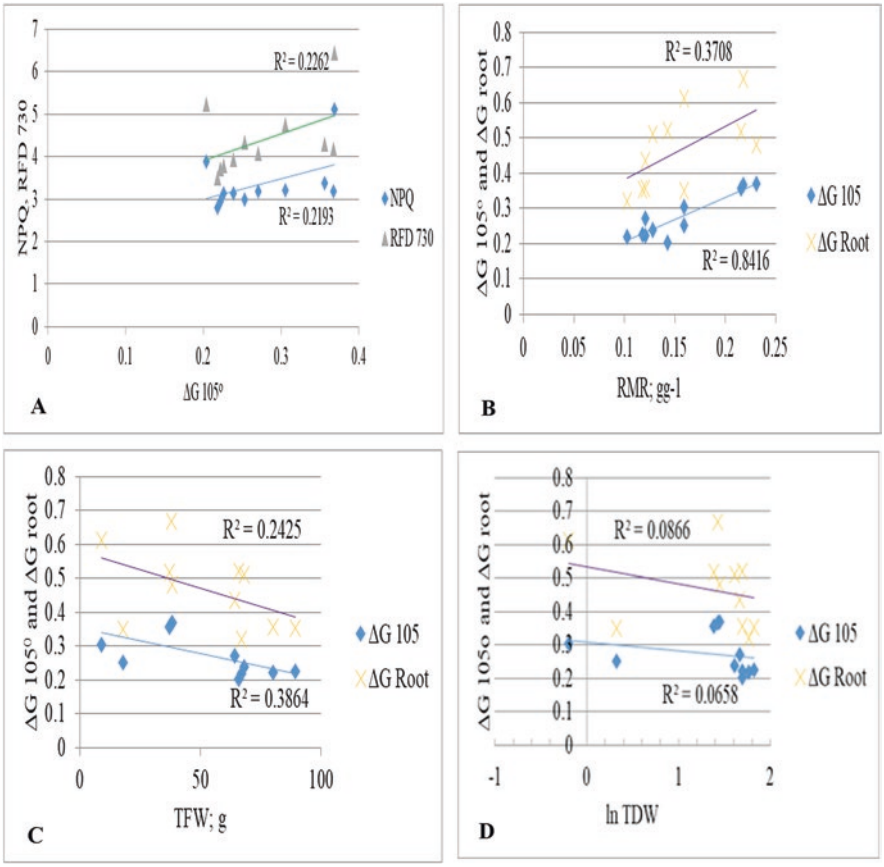

Fig. 9.2 (**a**) Regresion between termodynamic parameter ΔG 105^0 and photosynthetic parameters NPQ and RFD 730; (**b**) Regresion between RMR parameter of plant mass alocation and termodynamic parameters ΔG 105^0 and ΔG root; (**c**) Regresion between TFW parameter of plant mass acumulation and termodynamic parameters ΔG 105^0 and ΔG root; (**d**) Regresion between ln TDW parameter of plant mass acumulation and termodynamic parameters ΔG 105° and ΔG root

Correlative dependence of energy parameters (fluorescence parameters Chla and ΔG 105° parameters of thermodynamics) and the parameters of accumulation and mass distribution in maize in conditions of unfavorable temperatures (t = 10–15 °C) as well as the root status manipulation can be seen from Fig. 9.2.

First, we note (Fig. 9.2c, d) that the regression between the parameters of accumulation of fresh (TFW; g) and dry mass (ln TDW; g) and thermodynamic parameters ΔG 105° (the change of Gibbs free energy parameter calculated as difference beetwen values of G assesed at 105 °C and room (25 °C) temperatures, corresponds to the sum of the free energy of evaporation of the free apoplastic and simplistic, i.e. intracellular water of whole plants; Sun 2002) and ΔG root (equivalent values of ΔG 105° calculated for plant roots) of whole plants and roots are negative and weak,

which means that the energy (photosynthesis and respiration) of whole plants and roots in these conditions are not related to mass accumulation and growth of corn. However, if we look at the regression dependence of the root mass fraction (RMR; gg^{-1}) and ΔG 105° and ΔG root thermodynamic parameters (Fig. 9.2b), we note that this relationship is positive and statistically significant, especially in relation to the proportion of the root mass fraction (RMR; gg^{-1}) and ΔG 105° (J mol^{-1} K^{-1}) thermodynamic parameter, which refers to the biosynthetic capacity of the whole corn plant (Sun 2002). Last, but not least, we note (Fig. 9.2a) and not such a large, but statistically significant regression link ΔG 105° parameter of thermodynamics and photosynthetic parameters NPQ and RFD_{730}. These parameters indicate the protective processes in photosynthesis (Lichtenthaler and Miehe 1997; Frachebaud et al. 2002; Baker 2008), which is understandable in for maize unfavorable temperatures (t = 10–15 °C) conditions. Because we are even before (Table 9.8) concluded that the photosynthesis and protective processes associated with photosynthesis were positively acting externally by the 24-EBL, which shows once again that this class of phytohormones has a protective role in stress conditions (Bajguz and Hayat 2009; Vriet et al. 2012; Gururani et al. 2015a). So the preserved photosynthetic functions of corn plants exposed to suboptimal temperatures would be an explanation for their still sustainable growth, even in the negative maize plant energy status.

4.3 Plant Growth, Bioproduction and Crop Yield Influenced by Brassinosteroids at Field Agrophytocenosis Stage

Before we turn to the consideration of the comprehensive aspects of the effects of brasinosteroids on plant growth, bioproduction and crop yield in field agrophytocoenosis, we give some partial examples. Nikolić and Waisi (2012), examined the results from micro-trials, in two apple orchards. Plots were treated with combinations of half of the usual dose of mancozeb and tebuconazole fungicides with 24-EBL based preparation, also with other non-standard fertilizers (based on plant extracts). Control treatments were treated with half doses and a full usual dose of these fungicides. First, was assessed the yield of fruits per hectare, and the apples were sampled for determination of average fruit weight, pH and Brix's index of refraction in extracts of fruit pulp. Also efficacy of these procedures to plant protection of apple leaves and fruits from notorious phytopatogenic fungus *Venturia inaequalis* (Stevanović et al. 2012) was assessed. In first orchard, evaluated yield/ha of 24-EBL treated apples is same as in control plots, with comparable pomological and fruit quality parameters of apple. In second orchard, evaluated yield/ha of 24-EBL treated apples was higher by almost a quarter than then apple yield from control plots (treated by half and full doses of fungicides) and other treatments, also with comparable pomological and fruit quality parameters of apple fruits. From the point of view of plant protection, these procedures are also satisfactory with a 78,71% and 77,69% plant

protection efficacy using 24-EBL+half fungicide doses for treatment in leaves and fruits (compared to an 84,17% and 87,90% efficacy when using full fungicide doses for treatment) in the first orchard, which is a satisfactory result. These results are very similar to findings by other researchers (Clouse and Sasse 1998; Khripach et al. 2000).

Also, we examined influence of BRs based preparation on yield and yield components in soybean and barley. Three soybean genotypes were treated (ZP-015, "Nena", and "Laura") with 24-EBL based, and with other non-standard fertilizers (based on plant extracts), as an type of biofortification. With this approach we found that it is to a lesser extent affected by alterations in P_{phy} (content of phytic phosphorus), an important factor which restrains the availability of mineral nutrients. Only at the Zn level, this dependence is significant, where lowering the P_{phy} at the same time increases Zn concentration in grains. Moreover, the influence of β-carotene is significant for availability of mineral nutrients, but more important is that its increase is linked with parallel Fe increase, mainly in grains with higher weight, as part of better yielding potential It is significant to underline that the ratio between P_{phy}, β-carotene and the mineral nutrients could be altered to some degree by applying foliar fertilizers to potentially increase the availability of mineral nutrients, but it also depends on the soybean variety. 24-EBL based preparation and the plant extract ("Zircon") were efficient for decrease of mentioned ratio for ZP-015 and "Nena" grains, as well as some plant extracts ("Zlatno inje" and "Zircon") were efficient for "Laura". Also, correlation between 1000 grain weight (as significant yield component) and grain content of β-carotene and Zn in soybean is very significant (Dragičević et al. 2016b).

In the late winter of two different years, we sown hull-less barley (*Hordeum vulgare* L. *var. nudum*; cv. "Apolon"), and after that in the spring of the years, we treated the crop with 24-EBL based preparation, and also with other non-standard fertilizers (based mainly on plant extracts and other phytohormones). After harvesting in the summer we assessed yield (at 14% grain moisture content; kg ha^{-1}) and determined different chemical ingredients in the barley grains. Obtained results (Dragičević et al. 2016a) indicate that year affects barley grain yield and its chemical composition, with the highest impact obtained for Si under unfavourable conditions. The applied treatments were the most effective regarding the grain yield and increase in the grain quality mainly when reducing the P_{phy}/β-carotene ratio and increasing the GSH content, thus increasing the potential bioavailability of the examined mineral elements. What is more, the stress resulting from high amounts of precipitation could be mitigated by application of an fertilizers by increasing potential bioavailability of P, Mg, Ca and Fe. Generally, 24-EBL preparation influenced content of P_i, Zn and Fe, and other fertilizers mainly affected potential availability of some other mineral elements BAP (Ca, Mn, Si and GSH).

From previous field trials carried out on one fruit (apple) and two field crops (soybean and barley) we indicated that when compare with other non-standard fertilizers, the preparation based on 24-EBL affects not so much the yield, as it does the quality and chemical composition of crops (Nikolić and Waisi 2012; Dragičević et al. 2016a, b), and acts to protect the crops in stressful conditions (Stevanović et al. 2012).

Also, in research conducted on seedling stages of maize (Waisi et al. 2015a, 2017a), we found changes in the chemical composition of the maize seedling, influenced different concentrations of 24-EBL. Based on preliminary results, we set up in 2014 and 2015 comprehensive field trials with two maize hybrids (ZP434, ZP341) treated with different concentrations of the 24-EBL-based preparation as well as with propiconazole (Waisi et al. 2015b).

Tables 9.10 and 9.11 show that the highest concentration of 24-EBL (5.2×10^{-7} mol) has an inhibitory effect on the yield and yield components, which is in agreement with previous findings that the physiological response of the plants (inhibitory or stimulating) to BRs depends on the concentration of the applied phytohormone. Thus (Müssig et al. 2003) observed that high concentrations of BRs act inhibitory to root growth, although this response also depends on the genotype and age of the plants. It is possible that the variability of the response to the action of one and the same concentration of BRs applied depends on the number of receptors for BRs (*BRI*$_1$ protein) in an plant tissue (van Esse et al. 2012), and it is possible that it depends on the activity of the genes for the synthesis of brassinosteroides (Bancos et al. 2002), which are categories that depend on the genotype and age of the plants. We think that this problem has not been considered in detail in terms of molecular eco-physiology (Stitt and Sonnewald 1995; Kutschera and Wang 2012), which is a relevant approach for transmitting the findings of molecular and biochemical analyzes of the action of BRs (or any important regulatory molecule) to the level of agrophytocoenosis, which is essentially determinant for the yield and quality of maize grains to human nutrition and other uses. What is worth mentioning here is that the most applied concentrations (5.2×10^{-7} M) of 24-EBL (Tables 9.10 and 9.11) they reduce the number of kernels per row of maize cob, which is a high-heritability traits, significant for the final yield of maize. We see (Tables 9.10 and 9.11), that although there are variations in the maximum values of the yield param-

Table 9.10 Averaged values of different yield characteristics of ZP434 hybrid in 2014 field trial

Treatments during trial	Yield at 14% of grain moisture (t/ha)	Weight of cob (g)	Ratio of weights of grain/cob (%)	Rows in cobs	Kernels per row
Control	19.44 ± 0.88	63.73 ± 3.40	87.94 ± 0.93	15.33 ± 1.63	37.62 ± 4.34
10^{-7} of 24-EBL	12.01 ± 1.85	40.27 ± 6.38	85.92 ± 0.34	14.17 ± 1.95	32.67 ± 6.04
10^{-9} of 24-EBL	19.58 ± 2.04	63.87 ± 4.55	87.69 ± 1.88	15.58 ± 1.56	40.33 ± 4.61
10^{-11} of 24-EBL	19.97 ± 1.22	66.27±4.09	87.74 ± 0.75	15.83 ± 1.55	40.17 ± 4.62
10^{-13} of 24-EBL	17.23 ± 0.40	56.13 ± 2.34	87.1 ± 1.18	15.75 ± 1.48	39.12 ± 4.80
10^{-15} of 24-EBL	20.04 ± 0.10	65.6 ± 2.43	88.17 ± 1.39	15.17 ± 1.01	40.21 ± 4.02
10^{-6} of PZR	18.22 ± 0.13	66.4 ± 3.12	88.28 ± 1.47	15.92 ± 1.50	39.79 ± 3.40
10^{-7} of PZR	18.67 ± 1.04	62.67 ± 2.27	87.1 ± 0.32	15.58 ± 1.56	39.67 ± 4.22

Bold: Maximal values in a series. Italic: Minimal values in a series

Table 9.11 Averaged values of different yield characteristics of ZP341 hybrid in 2014 field trial

Treatments during trial	Yield (t/ha) of calculated at 14% of moisture	Weight of ear (g)	Ratio of weights of grain/whole ear (%)	Number of rows in cobs	Number of kernels per row
Control	17.28 ± 1.59	60.8 ± 4.85	87.06 ± 0.93	14.38 ± 0.53	38.25 ± 1.06
10^{-7} of 24-EBL	*11.46 ± 1.46*	*41.67 ± 6.00*	*85.58 ± 1.59*	*12.75 ± 1.66*	*36.38 ± 1.59*
10^{-9} of 24-EBL	16.84 ± 2.04	59.47 ± 7.42	86.73 ± 1.42	15.08 ± 1.56	39.17 ± 3.80
10^{-11} of 24-EBL	18.03 ± 1.41	61.67 ± 4.47	87.38 ± 0.48	14.75 ± 1.29	**41.42 ± 3.89**
10^{-13} of 24-EBL	17.77 ± 0.83	62 ± 0.80	**88.01 ± 1.72**	14.83 ± 1.17	42.17 ± 3.67
10^{-15} of 24-EBL	17.44 ± 1.91	59.93 ± 4.92	87.3 ± 0.35	14.75 ± 1.65	39.54 ± 3.93
10^{-6} of PZR	**19.2 ± 1.62**	**65.2 ± 3.20**	86.54 ± 1.07	**15.17 ± 1.66**	40.71 ± 3.63
10^{-7} of PZR	18.03 ± 1.37	63.33 ± 2.95	86.23 ± 0.99	14.67 ± 1.63	38.17 ± 4.52

Bold: Maximal values in a series. Italic: Minimal values in a series

eters and the yield components in the response of these two hybrids to the effect of the 24-EBL concentrations, most of these highest values in both genotypes are observed at lower concentrations of 24-EBL (5.2×10^{-13} and 5.2×10^{-15} M) or even in the presence of PZR, which is a BRs biosynthesis inhibitor (Hartwig et al. 2012).

When considering the chemical composition of maize seeds, we notice that there is a difference in the chemical composition of the grains of different maize hybrids (Tables 9.12 and 9.13) exhibited by various treatments of BRs. We note the hybrid ZP434 (Table 9.12) that contains the elements of the elements (K, Ca, Mg, Fe, Zn, Si) is elevated in treatments that are associated with a lower content of BRs in the plant (5.2×10^{-15} M of 24-EBL or 10^{-7} M of PZR), while treatment with also low concentrations of 24-EBL (5.2×10^{-11} and 5.2×10^{-13} M) increases the content of total polyphenols, total protein, total oil and GSH. This means that the treatment of maize hybrid ZP434 (drought-resistant) with low concentrations of BRs represents a biofortification (Welch and Graham 2004; Dragicevic and Stojkovic 2016) of the chemical composition of the grain of this hybrid.

This means that the treatment of maize hybrid ZP434 (drought-resistant) with low concentrations of BRs represents a biofortification (Welch and Graham 2004; Dragicevic and Stojkovic 2016) of the chemical composition of the grain of this hybrid. In contrast to the accumulation of nutrients in grains of hybrid ZP434 treated with various concentrations of 24-EBL, in the grain of hybrid ZP341 (Table 9.13), we note the accumulation of some (total phenols, GSH, Mg) nutrients in untreated control plants as well as other nutrients (total proteins, total oils, Pphy, Pi, Fe, Zn, Si) in maize plants treated with higher concentrations of 24-EBL (5.2×10^{-7} and 5.2×10^{-11} M), which means that such nutrient accumulation seems to be the result of the toxic effects of high concentrations of 24-EBL. In contrast, the treatment of hybrid plants ZP341, a biosynthesis inhibitor BRs (Hartwig et al. 2012) PZR (10^{-6} or 10^{-7} M of PZR) reduces the content of most organic nutrients, while the inhibi-

Table 9.12 Average values of relative content (% against control) of different chemical and biochemical parameters in crude extract of ZP434 maize grain from 2014 field trial

Relative content of different compounds (% of control)	Treatments during trial							
	Control	10^{-7} of 24-EBL	10^{-9} of 24-EBL	10^{-11} of 24-EBL	10^{-13} of 24-EBL	10^{-15} of 24-EBL	10^{-6} of PZR	10^{-7} of PZR
Starch	**100**	98.19	99.60	98.86	95.51	98.39	*95.17*	98.86
Total phenols	100	99.73	94.51	**148.63**	95.88	114.01	*92.03*	96.98
Moisture	100	**111.06**	*96.48*	104.52	108.04	108.04	110.05	105.02
Total proteins	*100*	108.72	101.19	105.58	**118.42**	102.51	115.42	107.47
Total oil	100	101.45	95.65	97.10	**105.80**	102.90	98.55	*94.20*
Pphy	100	100.73	95.62	*95.25*	99.03	102.31	103.16	**108.03**
Pi	100	**111.59**	100.29	96.01	107.98	98.10	97.44	*77.01*
GSH	100	122.21	*87.11*	110.69	**130.92**	107.73	104.02	117.43
K	100	99.33	95.76	98.25	96.19	**100.67**	97.99	*93.82*
Ca	100	79.90	122.53	145.37	478.45	89.92	*68.50*	**275.82**
Mg	100	95.62	*78.81*	100.80	93.66	96.95	108.98	**112.02**
Fe	*100*	103.57	111.33	156.34	208.87	322.84	319.21	**384.17**
Zn	100	73.04	*49.26*	55.97	49.31	91.75	62.74	**118.40**
Si	100	118.65	88.89	80.20	88.01	99.16	*77.66*	**127.72**

Absolute values of control of different parameters: 1. Starch: 74.60%; 2. Total phenols: 260.05 μg/g; 3. moisture: 9.95%; 4. Total proteins: 7.16%; 5. Total oil: 3.45%; 6. Pphy: 3.22 mg/g; 7. Pi: 0.36 mg/g; 8. GSH: 1053.63 nmol/g; 9. K: 3185.12 mg/g; 10. Ca: 36.38 mg/g; 11. Mg: 384.64 mg/g; 12. Fe: 5.08 μg/g; 13. Zn: 6.10 μg/g; 14: Si: 23.88 μg/g
Bold: Maximal values in a series. Italic: Minimal values in a series

tion of mineral nutrient absorption comes at the highest concentration of 24-EBL (5.2×10^{-7} M) (Table 9.13).

4.4 Plant Growth, Bioproduction and Crop Yield Influenced by Brassinosteroids: Conclusions

In contrast to the molecular paradigm, to present the usual method of testing brassinosteroids, as probably the key signal molecules in the development of plants (Zhang et al. 2009; Vriet et al. 2013), with the intention of optimizing the performance of the plants for a better yield (Vriet et al. 2012) and crop resistance to stressful episodes (Bajguz and Hayat 2009), we approached the issue from the other side. Namely, terrestrial plants (which include practically all crops) are thermodynamically open systems (like other living organisms) which, for reason of their survival, growth and reproduction, exchange matter and energy with the environment. But unlike moving animals, land-based plants with their sesile life formes and poikilothermal metabolism had to develop a completely different life-style strategy in order to obtain survival and reproduction resources. This allows approaching to the problem from the cybernetic point of view, watching plants as well as black and/or

Table 9.13 Average values of relative content (% against control) of different chemical and biochemical parameters in crude extract of ZP341 maize grain from 2014 field trial

Relative content of different compounds (% of control)	Treatments during trial							
	Control	10^{-7} of 24-EBL	10^{-9} of 24-EBL	10^{-11} of 24-EBL	10^{-13} of 24-EBL	10^{-15} of 24-EBL	10^{-6} of PZR	10^{-7} of PZR
Starch	100	*99.37*	101.55	101.69	99.58	102.04	**102.61**	101.55
Total phenols	**100**	**100**	94.13	90.62	91.50	93.55	94.72	*82.40*
Moisture	100	102.78	101.39	**104.17**	101.39	98.15	*98.61*	*98.61*
Total proteins	100	**105.61**	102.07	97.32	108.11	98.90	*91.34*	101.95
Total oil	100	93.42	89.47	**101.32**	90.79	89.47	*86.84*	93.42
Pphy	100	**101.25**	96.48	100.34	98.86	94.09	96.70	*95.23*
Pi	100	**122.82**	*84.46*	84.95	87.42	99.26	117.02	110.49
GSH	**100**	87.44	82.88	79.66	73.26	84.89	*53.79*	82.38
K	100	105.17	98.89	86.36	*76.64*	89.60	**105.47**	88.30
Ca	100	*30.88*	43.33	86.28	**118.35**	43.63	32.08	32.43
Mg	**100**	*79.83*	90.87	84.34	96.90	88.53	82.63	89.75
Fe	100	*53.71*	67.29	**155.44**	142.75	71.87	60.35	101.22
Zn	100	*81.15*	97.39	**159.54**	–	–	–	92.54
Si	100	**109.27**	97.91	79.49	*64.95*	69.44	76.68	85.90

Absolute values of control of different parameters: 1. Starch: 70.95%; 2. Total phenols: 243.62 μg/g; 3. moisture: 10.80%; 4. Total proteins: 8.20%; 5. Total oil: 3.80%; 6. Pphy: 3.45 mg/g; 7. Pi: 0.28 mg/g; 8. GSH: 1908.14 nmol/g; 9. K: 2895.06 mg/g; 10. Ca: 138.36 mg/g; 11. Mg: 436.60 mg/g; 12. Fe: 8.47 μg/g; 13. Zn: 3.98 μg/g; 14: Si: 23.63 μg/g
Bold: Maximal values in a series. Italic: Minimal values in a series

gray boxes (Ashby 1957), examining their entrances and exits without extensive examination of the structure, imposed by the molecular paradigm. Such an approach is "outdated" nowadays, but until recently (Lang and Thorpe 1985) it was legitimate in the physiology of plants. Such an approach requires looking at the plant as a whole, rather than as a mechanism, which entails a different choice of observation methods, such as, for example, thermodynamics, fluorescence chlorophyll, thermovision, growth analysis, and the similar "non-molecular" techniques, which are followed by the reaction of plants as whole systems, at the level of seed and seedlings, whole plants and agrophytocenosis. Such an approach is also used in research on the effects of brasinosteroids, especially in the so-called crosstalks of brasinosteroides with other phytohormones (Sankar et al. 2011), similar to earlier studies of metabolite fluxes in the cells, although more formal approaches to this problem are possible (Grover 2014).

But, the insights from the Sect. 4.1. that the processes in the seed and seedling system that develop under the influence of the different 24-EBL constellations are defined as almost "perfect" (R^2 = 1000) a correlation enthalpy-entropy effect (Janković and Waisi 2017; Janković et al. 2014; Waisi 2016), point to the possibility that the problems of the development of the plant under the influence of brassino-

steroids can be achieved through purely energy-cybernetic considerations. That's the case before, which is independent of the approach described by Flock et al. (2014) considering that the stabilization of complex metastable biological structures can be achieved only in two ways: (a) by increasing the enthalpy of binding (sub) units of complex biological structures, or (b) by reducing the entropy loss in binding of (sub) units of these metastable biological structures. After all, it follows from one form of the Second Law of Thermodynamics. In this context, it is more clearly seen not a new approach, but an different angle of view, which on brasinosteroids is seen as signaling network modulators, which coordinate the plant in the system, but not so much the system of gene and protein (as in the molecular paradigm), but the system of fluxes of energy and matter. If we analyze the plant at a higher level, as a system of whole, individual plants (see Sect. 4.2.), we note that despite the various manipulations of the status of leaves and roots, and whether or not the plant is in a state of stress, the system of the whole plant is very dependent on the interplay of energy production and the transformation of that energy into the redistribution of masses between plant organs and invested in plant growth (see Figs. 9.1 and 9.2).

Finally, at the level of plants associated in agrophytocenoses, besides the case of the effects of brasinosteroids on other cultures (Nikolić and Waisi 2012; Stevanović et al. 2012; Dragičević et al. 2016a, b), we notice that at apparently small differences in the bioproduction (see Tables 9.10 and 9.11) of maize crops treated with different concentrations of 24-EBL and PZR, we see a great diversity of maize plant response and their metabolic processes (synthesis of various groups of compounds such as total phenols, proteins and oils and the absorption of various elements) on different BRs treatments (Tables 9.12 and 9.13). All this points to the "network" of the signal (made by brasinosteroids, other hormones, and also non-hormonal signal paths) that are "hiding" behind this phenomenon, but which point to no determinism (which implies a molecular paradigm), but on the stochasticity of these processes, directed to the flows of energy and matter. After all, just as on one level the interrelations of brasinosteroids and e.g. auxines (Li et al. 2005; Sankar et al. 2011; Sakamoto et al. 2013), and also other phytohormones (Hartwig and Wang 2015) directs the development and growth of plants, at the second level, the "switching" of plants from one energy state to another occurs. Developmental and structural organizations changes (Waisi et al. 2017b) are determining the changes in the bioproduction of plants, from a quantitative, but also qualitative point of view.

This approach is very reminiscent of attitude of Amzallag (2001) about dual function of BRs as molecules influence plant growth and development, depending on the situation (usual or stress) in which the plants were found, maintaining both the homeostasis of the plants or allowing them to move to a new balance, as Lichtenthaler (1996) concluded for different reasons. Therefore, the doubts about the possibility of modulating the yield of plants with brasinosteroids, which in the conclusion of their work are expressed by Hola et al. (2010) and they are not unreasonable.

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Chapter 10
Brassinosteroid Regulated Physiological Process: An Omics Perspective

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Abstract Transcriptomes is referred to an entire set of transcripts and their number present in a cell at a particular developmental phase or physiological state. Study of the transcriptome is necessary to identify different genes and their functions, and elucidating various signalling pathways. The key intend of transcriptomics is to index all sort of transcripts (coding and non-coding RNAs) to establish the transcriptional organization of genes. Genes act as blueprint whereas proteins act as a functional unit of cell that is regulated by gene expression/repression. Proteomics is a broad scale analysis of a complete set of proteins (proteome) in a cell, tissue or organ at a particular time. As proteins are final product of a gene they are closer to the function as compared to genes. Hence, this "*omics"* study will facilitate more rapid advancement in understanding of different biochemical pathways of plants. Brassinosteroids (BRs), a class of plant hormone regulates various developmental and physiological processes. This chapter deal with the application of transcriptomics and proteomics to elucidate the hormonal targets for growth and development of plants.

Keywords Brassinosteroids · Transcriptome · Proteome · Plant physiology · Phytohormones

1 Introduction

Transcriptomics is the study of transcriptome of an organism by using different techniques. Transcriptome is the sum of all RNA transcripts including coding mRNAs and non-coding RNAs such as microRNAs (Lin et al. 2013). The information of an organism is stored in DNA which is expressed through transcript mRNA which act as a transitory intermediate molecule during information transfer whereas

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non-coding RNA perform other functions. Measurement of genes of an organism from different tissues, condition and time provides a valuable information regarding regulation of genes. Genes act as blueprint of cell which gets transcribed into mRNA. The change in the expression level of a transcript during any phase of development and under different conditions is studied to understand various mechanisms.

Different techniques have been developed for transcriptome analysis like hybridization or sequence based approaches. In hybridization based approach a set of transcript is hybridized to a complementary probe with the advancement in sensitivity of fluorescence detection and accuracy of transcript measurement. In seq-based approach the sequence of cDNA is directly determined. Due to low throughput, high cost and non-quantitative nature, the tag-based approach was introduced to overcome these limitations. At present, RNA seq is the most advanced high throughput DNA sequence method which allows the survey of the transcriptome with a high throughput and in quantitative manner.

Proteomics is the study to measure global protein expression in a cell, tissue or organ, even in an organism. Many advanced proteomics techniques such as mass spectrometry (MS) based two-dimensional gel electrophoresis (2-DE) and shotgun proteomics as well as protein microarray. Proteomics not only can be used for global proteome profiling but also post translational modification (PTM) discovery (Lin et al. 2015). The present chapter deals with application transcriptomic and proteomic approach to analyze the BR-regulated physiological processes in plants.

2 Transcriptome Analysis of BR-Regulated Physiological Processes

Brassinosteroid is a class of phytohormone that regulates various processes in plants including cell division, elongation, photomorphogenesis, senescence and vascular development (Yin et al. 2005). Transcription factors accumulates upon BR binding, like BES1 (*BRI-EMS-Suppreseor 1*) and BZR1 (brassinazole resistant 1) that control the expression of various genes responsible for cell elongation, BR synthesize other cellular process (He et al. 2005; Yin et al. 2005). BES1 and BZR1 share similarity in their DNA binding property and transcriptional activities (Sun et al. 2010; Yu et al. 2011). Both transcription factors co-regulate numerous light and BR responsive genes by interacting with PIF (phytochrome interacting factors) family of bHLH factors (Oh et al. 2012; Bernardo-Garcia et al. 2014).

2.1 Growth and Development

BRs are known to induce the expression of different genes which codes for different enzymes for cell wall loosening, thus promoting cell elongation (Coll-Garcia et al. 2004; Goda et al. 2004). Analysis of promoter of *TCH4* gene coding for enzyme

xyloglucan endotrans glycosylase (XET) is induced by BRs. It reveals that a 102 bp promoter fragment generates response to BRs (Iliev et al. 2002). There lies an inconsistency during BR-dependent gene expression upon BR treatment in different genotypes, environmental condition, developmental stages and tissue (Mussig and Altmannn 2003).

Lisso et al. (2005) used gene signaling components BRI1, BAK1 (BRI1 associated receptor kinase), BIN2 (Brassinosteroid insensitive 2), BZR1 and BES1 to screen BR related genes. The *BRI1* gene was found to be allied with 1179 genes. Out of 23 known BR-inducible genes 11 were identified by these transcript co-responses. BAK1 was found to show a co-response with 720 genes. The 301 co-responding genes were identified as result of intersection gene query with BRI1 and BAK1. The presence of 8 of 23 identified BR-regulated genes after intersection analysis confirms a strong relation of BRI1 and BAK1 expression.

BR-mutants are expected to possess reduced transcript level of BR-induced genes whereas BR-treated plants have increased transcript levels. Reduced transcript levels of 23 genes were identified in at least two BR-mutants. BR-responsive genes were also included amongst these such as *KCS1* (*3-ketoacyl CoA synthase 1*) and *TIP2.1* (d-TIP) (Coll-Garcia et al. 2004), four aquaporins (*TIP1.1*, *PIP1.2*, *TIP2.1* and *TIP1.2*), genes apparently involved in cell wall modifications (*AGP21, AGP9*, *FLA2*) (Fig. 10.1). 14- and 19- day-old plants presented more pronounced fold changes in growth-associated genes when compared to 28-day-old plants, perhaps due to the reduction in growth rates of older plants. Out of 23, 6 genes displayed

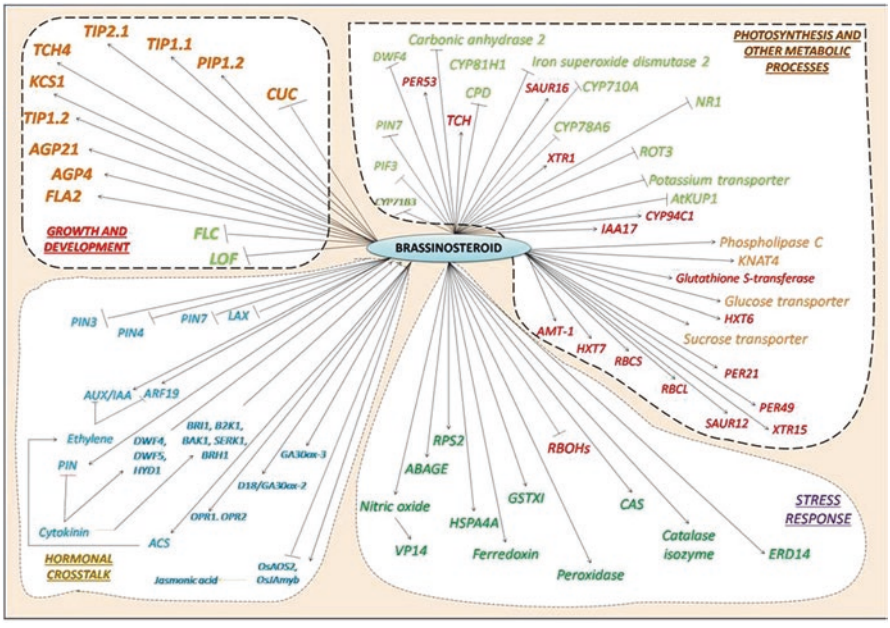

Fig. 10.1 An array of genes regulated by brassinosteroid

higher mRNA levels in BR-treated plants. Three genes were highly expressed in BR-mutants and weakly expressed upon BR-treatment. Eighteen genes did not show signs of BR-responsiveness or displayed a variable transcript levels.

Seed priming is an important technique used to increase the performance is terms of its germinatibility, vigorness, uniformity and stress tolerance. The seed is treated with water to initiate the metabolic activities and allowed to dry prior to complete seed germination, or emergence of radical, as a result the seed again enters a phase with no metabolic activities (quiescent stage) (Varier et al. 2010; Paparella et al. 2015). Where priming has a positive effect in enhancing seed germination, on the other hand it also reduces seed longevity (viability). In non-primed seeds, there are three BR genes which are highly expressed in *Arabidopsis* plant with reduced seed longevity. These genes are *BEN1* (AT2G45400) (Yuan et al. 2007), *DWF1* (AT3G19820) (Fujioka and Yokota 2003), *EXO* (AT4G08950) (Coll-Garcia et al. 2004) which are responsible of BR synthesis or signalling. Along with it four cell wall modification related genes *TRG1/XYL1* [(AT1G68560) (Sechet et al. 2016; Shigeyama et al. 2016)], *EXPA1* [(AT1G69530) (Li et al. 2002)], *EXPA2* [(AT5G05290) (Yan et al. 2014)] and *DUF642* [(AT5G11420) (Zúñiga-Sánchez et al. 2014)] (Fig. 10.1) are also expressed. Thus, it could be inferred that BR and cell wall genes might determine longevity by participating in regulatory networks. The application of BRs also influences the seed longevity in primed seeds. BR reduces the seed longevity after priming in a concentration dependent manner. And the reduction of endogenous concentration of BR enhances seed longevity (Sano et al. 2017).

BR does have a potent role in process of seed germination. Germination rate decreases in BR deficient mutants (*CYP85a1/a2* and *det2*) (Sano et al. 2017). The BR deficient *Arabidopsis* mutant (*det2* mutants) and BR-insensitive mutant *bri1* are sturdily repressed by ABA as compared to wild type. Along with it treating with BR in a GA deficient mutant ga1-3 induces the germination (Steber and McCourt 2001).

Organ boundary formation is another developmental aspect affected by BRs. CUC (cup-shaped cotyledon) and LOFs (Lateral organ fusion) are essential for arresting growth and formation of organ boundary. BZR1 represses CUC directly and LOF gene indirectly as CUC is a requisite for LOF expression. Low accumulation of BZR1 in organ boundary cells allows the expression of CUC genes. Activation of BR signalling represses CUC gene expression leading to organ fusion (Gendron et al. 2012).

BRI1 induces a large number of genes responsible for cell elongation and cell wall organization like *TCH4* which codes for xyloglucan endotransglycosylase, putative expansin which share homology with *ZmExp2*, *KCS1* encoding fatty acid elongase 3-ketoacyl CoA synthase that function in wax biosynthesis (Xu et al. 1995; Todd et al. 1999; Im et al. 2000) (Fig. 10.1). BR also influenced the flowering in plants. Transgenic lines of *Triticum aestivum* examined by real time PCR analysis reveal that over-expression of *TaBRI1* (*Triticum aestivum* BRI1) induced flowering at early stage (Singh et al. 2016). Moreover, the size and number of the silique per plant also increased as compared to the wild type consequently, resulting in enhanced total seed yield in *TaBRI1* over-expressing transgenic plants. The increase

in seed mass was attributed to an increase in number of seeds. *TaBRI1* transgenics are hypersensitive to exogenous epibrassinolide (EBL) and the root length decreases with the application of EBL (Lin et al. 2013). Using small RNA microarray approach, many microRNAs, including miR-395a, miR824, miR169a, miR160, miR-156, and miR-159, regulated by EBL for root growth were screening out (Lin et al. 2013). Especially, miR-395a which suppresses GUN5 expression and its downstream signal transduction is to regulate seedling development (Lin et al. 2013). Enhancement in *TaBRI1* expression leads to better stress tolerance by preserving the membrane integrity. Hence, *TaBRI1* gene can be exploited for conferring abiotic stress tolerance. BRI1 also participate in flowering process by enhancing the flowering time through repressing the expression of FLOWERING LOCUS C (FLC) (Singh et al. 2016; Fig. 10.1).

2.2 Cell Differentiation

BRs regulate the genes associated with xylem formation (Fukuda 1997). BR deficient mutants exhibit unequal cambium division (Szekeres et al. 1996). Procambial cell differentiation into xylem elements is BR regulated via regulation of HD-Zip TF (Ohashi-Ito et al. 2005; Ohashi-Ito and Fukuda 2010). BRs also encourage autophagy during programmed cell death (PCD) in tracheary elements undergoing differentiation process via transcriptional regulation of RabG3b, a small GTP-binding protein, (Kwon et al. 2010).

2.3 Different Gene Families Regulated by BR

Goda et al. (2004) in his study revealed a large number of genes regulated by BR. Some of the important genes of this study are:

(a) Genes down-regulated at different stages of growth: *CYP81H1* (P450 monoxygenase), *SAUR-36* (auxin up RNA gene promote leaf senescence), *CYP71B3* (P450 monoxygenase), *CYP78A6* (P450 monoxygenase), *PIN7* (auxin efflux carrier protein), *AtKUP1* (High affinity potassium transporter), *NR1* (Nitrate reductase 1), *CYP710A* (P450 monoxygenase), *DWF4* (BR biosynthesis), *ROT3* (Leaf polar elongation), *CPD* (BR biosynthesis), *PIF3* (Phytochrome interacting factor 3), actin depolymerizing factor like protein, UDP-glucose glucosyl transferase, carbonic anhydrase 2, iron superoxide dismutase 2, potassium transporter (Fig. 10.1).
(b) Genes up-regulated at different stages of growth: Putative pectin acetylase, putative calcium binding protein, *TCH2* (calmodulin related protein), *XTR1* (xylogucan endotrensacetylase 1), putative disease resistance protein, *PER53* (class 3 peroxidase), *SAUR-16* (auxin inducible SAUR gene family), *CYP94C1*

(P450 monoxygenase), Putative pectin esterase, *IAA17* (auxin response gene), putative β-amylase, GA regulated protein homolog, monosaccharide transporter, biogenesis of cell wall, β-glucosidase like protein, quinine oxidoreductase-like protein, blue copper binding protein, calmodulin, phosphoinositide-specific phospholipase C, DNA binding protein, HOMEOBOX PROTEIN KNOTTED-1 LIKE4 (*KNAT4*), glucose transporter, sucrose transporter, *HXT6* (high affinity hexose transporter), *HXT7* (high affinity hexose transporter), ammonium transport protein (*AMT-1*), glutathione-S-transferase, glycerol 3 phosphate dehydrogenase, *PER21* (class3 peroxidase), *PER49* (class3 peroxidase), putative ABC transporter, *IAA15*, putative ethylene response element binding protein, *XTR15* (xylogucan endotrensacetylase 15), *SAUR12* (auxin inducible SAUR gene family) (Fig. 10.1).

3 Photosynthesis

Role of BRs in regulating photosynthesis has been widely studied. However, its transcriptome analysis remains unexplored. BRs regulate photosynthetic capacity, to a no end in sight extent, regulating the rubisco carboxylation rate and ribulose 1,5-bisphosphate (RuBP) regeneration. The increase in maximum rubisco carboxylation rates ($V_{c,max}$) and initial rubisco activity is attributed to an increase in mRNA profusion and protein level of rubisco small and large sub-unit. EBL promotes photosynthetic electron transport rate as well as the expression of genes coding for calvin cycle enzymes involved in RuBP regeneration (Xia et al. 2009).

Increase in BR signalling mediated by *BRI1* gene, *SLBRI1* over-expression in tomato (*Solanum lycopersicum*) plants reveals that transgenic plants exhibit increased growth and development plus photosynthetic capacity. The leaf area, size of flower, petal and style of transgenic plants also increase as compared to wild type plants. CO_2 assimilation rate and the net photosynthetic rate in *SLBRI1*-overexpressing plants also increases, indicative of a fact that regulation of photosynthetic capacity in tomato is associated with enhanced BR signaling mediated by *SlBRI1* over-expression (Nie et al. 2017). Data related to the transcriptome study of BR-mediated regulation of photosynthesis remains insufficient and thus, it becomes an important area for further research.

4 Stress

BRs regulate various cellular and physiological processes in response to stress conditions (Xia et al. 2009). BRs regulate a numerous genes associated with stress response. In a study, Li et al. (2016) established that EBL up-regulates 29 genes related to photosynthesis during stress conditions. Genes related to chloroplast organization and photosynthetic apparatus were up-regulated in presence of chilling

stress upon BR application. Five genes were linked with the up-regulation of PSII, transfer activity and PSII reaction centre (Li et al. 2016). Furthermore, BR-mediated up-regulation of genes related to cellular redox homeostasis such as peroxidase (POX), catalase (CAT) isozyme, ferredoxin and glutathione S-transferase (GST), was also observed. RBOHs (respiratory burst oxidase homologs) involved in reactive oxygen species (ROS) production in plants are down-regulated suggesting a reduction in ROS accumulation by BRs (Marino et al. 2012; Li et al. 2016). *CAS* (Capana00g001365; calcium-dependent protein kinase) responsible for maintaining cytoplasmic calcium concentration for inducing reaction that provides stress tolerance is also up-regulated by BRs (Li et al. 2016; Fig. 10.1).

BR not only up-regulates various genes in response of abiotic stress but also in presence of biotic stress. Biotic stress response genes like *HSFA4A* (Heat stress transcription factor A-4a), *ERD14* (early response to dehydration14), and *RPS2* (Ribosomal protein S2) are up-regulated by BR (Fig. 10.1). Apart from it, defense response to fungi, sexual reproduction and cell wall loosening are also regulated. Expression of ABA related gene, *ABAGE* (ABA glucosyl ester) which is a hydrolysable ABA conjugate that accumulates and bust into free ABA upon encountering stress conditions is also increased by BR. Along with it, DPA which is an end product of ABA catabolism, indicating ABA level before breakdown also increases (Burla et al. 2013; Divi et al. 2016).

4.1 Br Signalling in Relation with Stress Hormone ABA

Due to a well-established role of BRs in ameliorating stress it becomes important to understand BR cross talk with ABA– the stress hormone at transcript level. Abiotic stresses as well as phytohormones like BR and ABA could induce brassinosteroid-signaling kinase 5 (BSK5, one of the key components of BR signaling pathway) transcripts in *Arabidopsis* (Li et al. 2012). Germination rate is remarkably inhibited in presence of salt stress in bsk5 mutant (*Arabidopsis* loss-of-function mutant) probably due to enhanced ABA accumulation through up-regulation of *ABA3* and *NCED3* expression. Despite of antagonistic relation between ABA and BR in the regulation of some physiological responses there are several ABA-responsive genes and ABA biosynthetic gene that are up-regulated by BR (Divi et al. 2010). Stress significantly increases endogenous ABA level and its content is further enhanced by BR treatment (Kurepin et al. 2008). BR increase stress tolerance by enhancing endogenous ABA content. Likewise, exogenous application of 10 nM BL to high temperature exposed *Chlorella vulgaris* enhances ABA content and heat stress tolerance (Bajguz 2009). However, effects of BR on stress tolerance are reduced by ABA (Ahammed et al. 2015). Zhou et al. (2014) demonstrated that production of H_2O_2 was essential for BR and ABA-induced oxidative stress tolerance, and BR-induced H_2O_2 might act as a trigger for ABA biosynthesis which further elevates the H_2O_2 concentration and extend stress tolerance. Nitric oxide plays an imperative role in BR-mediated stress tolerance as well as in interaction between

ABA and BR (Zhang et al. 2010a; Cui et al. 2011). BR mediated increase in NO production, up-regulates the expression of vp14 (ABA biosynthetic gene), triggering endogenous ABA level, and thereby, inducing water stress tolerance in maize (Fig. 10.1). BR is incapable of alleviating water stress in absence of ABA which was confirmed by inhibiting ABA biosynthesis using fluridone (Zhang et al. 2010a). The genes co-regulated by BR and ABA are mainly regulated in an opposite manner (Huang et al. 2008). Thus, additional investigations are required to expand our understanding on BR-induced stress tolerance mechanisms.

5 Crosstalk with Other Phytohormones

Phytohormones regulate various metabolic processes in plants. Due to the potential role of BR in regulating wide array of metabolic processes, the interaction of BR with other phytohormone becomes an area of special interest as BR regulates the synthesis and inactivation of many phytohormones. The crosstalk of BR with various phytohormone has been discussed in the following section:

5.1 Auxin

Auxins are known for their role in regulating various physiological processes like root formation, apical dominance, tropic response and senescence. BRs and auxin act synergistically in promoting hypocotyl elongation (Sasse 1999). Crosstalk between BR and auxin reveals a numerous facets of plant growth and developmental processes regulated by these hormones (Chaiwanon and Wang 2015). The maintenance of threshold level of BR is a requisite for optimal action of auxin for root growth and *BRAVIS RADIX (BRX)* gene is responsible for it. Auxin induces *BRX* gene whereas BR meekly represses it (Mouchel et al. 2006). *BRX* positively regulates genes responsible for BR biosynthesis i.e. *CPD* and *DWF4* (Tanaka et al. 2005) which suggest a relationship between auxin signaling and BR biosynthesis (Mouchel et al. 2006). Additionally, expression of BR biosynthetic genes is regulated by auxin thus, establishing a direct relationship of auxin with BR biosynthesis (Chung et al. 2011; Fig. 10.1). Treatment with auxin considerably increases the *DWF4* transcript levels in *Arabidopsis* plants resulting in augmentation in BR biosynthesis probably through induction of BRX protein (Chung et al. 2011). Nevertheless, upon synthesis of optimal amount of BR, feedback inhibition of *DWF4* gene by BR occurs (Saini et al. 2015). Hence, it gets confirmed that BR and auxin act antagonistically in regulating *DWF4* (Maharjan and Choe 2011; Maharjan et al. 2011). The auxin transporters are also regulated by BR where expression of numerous genes responsible for auxin transport such as *PIN3, PIN4, PIN7* and *LAX* gene is repressed by BRs (Nemhauser et al. 2004; Fig. 10.1). The differential regulation of *PIN* genes in response to stress and phytohormone has been established.

Hence, the crosstalk between BR and auxin in conferring abiotic stress tolerance through regulation of auxin transport can be further investigated.

There is wide array of genes commonly regulated by auxin and BL together (Goda et al. 2004), like *CYP814F* and genes for nicotianamine synthase, trehalose-6-phosphate phosphatase, uclacyanin, putative respiratory burst oxidase protein B, sharing similarity to proteins induced by jasmonate, cys proteinase-like protein, are all down-regulated by both BR and auxin. Furthermore, certain genes like *IAA3*, *IAA5*, *SAUR-10*, *SAUR-25*, *SAUR-9*, *SAUR-AC1*, *SAUR7*, *XTR6* and the genes coding for putative β-ketoacyl-CoA synthase, putative protein, *BAS1*, putative protein kinase, Cys proteinase, Gibberellin-2-oxidase, stress response calcineurin β like protein, Class III peroxidase *PER62*, Berberin bridge enzyme like protein are constitutively up-regulated by both BR and auxin (Goda et al. 2004; Fig. 10.1).

The coordinated regulation of genes by auxin and BR acts through ARF binding sites. Auxin-mediated degradation of AUX/IAA co-repressors modulates the activator ARFs (auxin response factors) (Guilfoyle and Hagen 2007). There is a competition between repressor ARFs like ARF2 and activator ARFs for binding with at AuxREs at the promoter site of various genes. BIN2 phosphorylates ARF2 and result in elimination of repressor ARF2 from DNA. Hence, BR releases the brake and promotes the expression of auxin (Vert et al. 2008).

5.2 Gibberellins

GAs and BRs are known to regulate common physiological responses. Both GA- and BR-deficient mutants produce dwarf phenotypes (Sun 2011; Clouse 2011). Additionally, there is a synergistic approach between both the hormones in promoting hypocotyl elongation of light-grown *Arabidopsis* seedlings (Tanaka et al. 2003). Likewise, BRs intercede the action of GA in promoting skotomorphogenic developments in etiolated seedlings (Alabadi et al. 2004), indicative of a fact that the there is not a constant parallel relationship between the two pathways.

BR induces the expression of GA biosynthetic gene, *D18/GA3ox-2* and promotes the accumulation of GA (Fig. 10.1). However, a GA inactivation gene, *GA2ox-3* gets activated at high concentration of BRs and results in the inhibition of cell elongation. Though, GA blocks BR signalling along with its biosynthesis in a feedback inhibiting loop but supplying high GA concentration assist in promoting cell elongation through activation of primary BR signaling pathway, suggesting a crosstalk between the two in regulating cell elongation (Tong et al. 2014). BR, IAA, and GA interface during cotton fiber development has been studied in *Gossypium hirsutum* (Hu et al. 2011). Treatment of BR and auxin resulted in the down-regulation of *GhGAII* (a class of DELLA proteins) throughout cotton fiber instigation and elongation, indicating an important role of the two hormones in the improvement of cotton fiber through genetic intonation of phytohormone scheme. However, GA up-regulated the expression of *GhGAI1* and *GhGAI3* during cotton fiber initiation which is a redundant attribute for fiber initiation (Hu et al. 2011) which suggest a

role of GA and BR in regulating the development of cotton fiber. BR also induce GA20-oxidase gene (*At GA20ox1*) (Bouquin et al. 2001), ACS (ACC synthase) gene in *Vigna radiata* (Yi et al. 1999) and GA20ox8 (GA inactivating enzyme) (Schomburg et al. 2003).

BR and GA act synergistically in causing cell expansion during photomorphogenesis through an agreement of BR-mediated activation of BZR1 and GA-mediated inactivation of transcription regulators of DELLA (Gallego-Bartolomé et al. 2012). The requirement of BR signalling has been suggested in GA mediated cell elongation, while BR or active BZR1 are capable of suppressing the dwarf phenotype deficient in GA. DELLA inhibits BZR1-DNA binding by directly interacting with BZR1. Thus, impairing the cascade of signals required for elongation of cell and seedling etiolation (Bai et al. 2012; Gallego-Bartolomé et al. 2012; Li and He 2013).

5.3 Cytokinin

BR crosstalk with cytokinin in regulating development of lateral root operates through inflection of auxin transport at transcript level. BR enhances the expression of *PIN* genes which codes for auxin efflux carriers (Saini et al. 2015) and aids in maintaining optimum concentration of auxin for the development of root primordium (Bao et al. 2004). Conversely, cytokinin down-regulates the expression of *PIN* genes and disturbs the accumulation of auxin thereby, inhibiting the induction of lateral root primordial which suggests an opposite interaction between BR and cytokinin (Benjamins and Scheres 2008). Overexpression of *isopentyl transferase*, *IPT* gene in rice plants enhanced the level of cytokinin and increased its tolerance to drought stress. The rise in cytokinin level corresponds with the up-regulation of various genes responsible for BR biosynthesis (*DWF4, DWF5, HYD1)* and genes involved in BR signalling (*BRI1, BZR1*, *BAK1, SERK1*, *BRH1)*. Hence, BR-CK crosstalk founds to be responsible for a considerable elevated grain yield via alteration of source–sink relations, therefore, boosting the drought tolerance (Peleg et al. 2011; Fig. 10.1).

5.4 Ethylene and Jasmonic Acid

Brassinosteroid interacts with ethylene and regulate different developmental processes. BR is considered to influence shoot gravitropism negatively, while ethylene promotes it (Vandenbussche et al. 2013). Suggesting, an antagonistic relation between BR and ethylene in shoot gravitropic responses through the involvement of auxin signalling genes (Guo et al. 2009). *AUX/IAA* and *ARF7/ARF19* negative and positive regulators of auxin signaling, respectively are up-regulated by BR whereas, down-regulated by ethylene hence, affecting the shoot gravitropic responses (Vandenbussche et al. 2013; Fig. 10.1). The positive relation between BR

and ethylene has also been established. BR up-regulates ethylene biosynthesis through enhanced expression of gene which is essential to be expressed for ethylene production i.e., *1-aminocyclopropane-1-carboxylate synthase (ACS)* (Muday et al. 2012). Ethylene and BR are found to regulate the hyponastic growth synergistically. Ethylene promotes hyponastic growth attained by plants to muddle through the biotic and abiotic stresses, (Polko et al. 2013). *ROT3/CYP90C1* codes for an enzyme that intercedes the C-23 hydroxylation of BR. Any alteration in *ROT3* causes inhibition of BR biosynthesis and reduction in hyponastic growth, suggesting a regulatory function of BR during ethylene-induced hyponastic growth (Polko et al. 2013; Fig. 10.1).

The crosstalk between BR and JA also suggests the regulation of innate immunity during *Meloidogyne graminicola* infection in rice (Nahar et al. 2013). Lower concentration of BR reduced the transcript level of *allene oxidase synthase2 (OsAOS2)* and *OsJAmyb* responsible for JA biosynthesis and signalling, respectively (Lee et al. 2001; Mei et al. 2006). However, the transcript level increased along with the increase in BR concentration. BL induces *OPR1* and *OPR3* genes coding for 12-oxophytodiensic acid reductase involved in jasmonic acid biosynthesis (Biesgen and Weiler 1999; Mussig et al. 2000; Goda et al. 2002; Fig. 10.1).

6 Proteome Analysis of BR Regulated Physiological Processes

Proteomics is the outsized scale analysis of a complete set of proteins (proteome) in a cell, tissue or organ at a particular time. As proteins are final product of a gene and they are closer to the function as compared to genes. Hence, this "*omics"* study will facilitate more rapid advancement in understanding of different biochemical pathways of plants. Inconsistencies between transcript and protein levels have been reported earlier in different organisms when microarray data of RNA expression is compared with proteomic profiling data (Ideker et al. 2001; Griffin et al. 2002; Tian et al. 2004; Huber et al. 2004). Transcriptome and proteome data of *dl2* mutant in rice revealed that 98 mRNAs and 141 proteins were expressed differently between the mutant and its wild-type control (Peng et al. 2015). Nevertheless, there was an overlap of only two genes amongst the proteome and transcriptome profile, suggestive of a weak relationship between transcript and protein levels. Li et al. (2016) also reported a similar result while working on rice pistil response during early post-pollination. In this study, 962 transcripts and 167 proteins had discrepancy in their expression and no more than 12 genes showed changes in expression at both transcript and protein levels. This incongruity is suggestive of giving more relevance to proteomic data towards biological responses as compared to microarray data because proteins are the ultimate functional product of genes and not RNAs. Thus, the data obtained from this study sheds light on the molecular system of BR responses.

Studies combining the protein mapping along with transcript expression profiling are still inadequate in plants (Rossignol et al. 2006). Thus, an approach employing the proteomics technique to study BR-responsive proteins would enhance our knowledge of molecular basis of BR responses in addition to post-transcriptional regulation in plants. Proteomic changes can be analyzed quantitatively using different methods, one such method is 2-DE. Two-dimensional gel electrophoresis (2-DE) has emerged as a powerful technique in proteomics to display the differential expression and post-translational modifications of proteins. By employing 2-DE technique, thousands of proteins could be separated on the basis of their charge and size (O'Farrell 1975; Unlu et al. 1997). Two-dimensional (2-D) DIGE is the recent improvement of this technique (Tonge et al. 2001). In this updated technique, different fluorescence dyes (Cy2, Cy3, and Cy5) are used for covalently labelling the proteins samples, they are mixed together, and separated in a single gel of 2-DE. Scanning of gel is done at a particular wavelength to take the gel image having identical protein spots and the intensities of these spots in different samples and the quantified could be directly compared using image analysis software (Tonge et al. 2001). Immunoblotting is used to analyze the same samples and confirms the precision of 2-D DIGE for quantitative proteomic analysis (Alfonso et al. 2007; Deng et al. 2007) or metabolic stable isotope labelling (Kolkman et al. 2005). 2-D DIGE not only detects the change in quantity of protein but also the post-translational adjustments altering the size or charge of the protein (Kolkman et al. 2005; Casati et al. 2005).

In *Arabidopsis* and rice, 42 and 36 BR-responsive proteins, respectively were identified (Deng et al. 2007; Wang et al. 2010). iTRAQ approach is another technique for quantitative proteome analysis and using this technique Li et al. (2016) identified a total of 840 proteins, and out of these 88 proteins displayed co-regulation by both BR deficiency and BR insensitivity. A mild *OsBRI1* (*Oryza sativa Brassinosteroid Insensitive 1*) mutant, naming *d61-1* possess less sensitivity towards BRs as compared to the wild-type plants (Yamamuro et al. 2000). Though approximately 600 proteins were identified having changed expression in *d61-1* but the change of expression was less than twofold in 92.3% proteins. As we know, BR signalling pathway modulates BR biosynthesis in a type of feedback regulation (Wang et al. 2002). An increase of about 4 and 30-fold in the bioactive cathasterones was observed amongst *d61-2* (*intermediate*) and *d61-4* (*severe*) mutants, respectively (Nakamura et al. 2006; Yamamuro et al. 2000). These studies suggest a rise in endogenous BR content in the BR-insensitive mutants moreover; BR content is closely correlated with severity of mutant phenotypes. Thus, it could be inferred that loss of BRI1 activity in BR signalling mutants could be compensated up-to a certain level by escalating the endogenous BRs.

Dimethyl labeling coupled with phosphopeptide enrichment is a powerful quantitative proteomics method for BR-regulated phosphosingaling study (Lin et al. 2015). Dimethyl labeling is a low-cost and fast quantitative proteomics method. Using the method, Lin et al. (2015) identified a total of 1104 unique phosphorylated peptides from 739 unique phosphoproteins in BR-regulated *Arabidopsis*. Using bioinformatics approach, BR-induced phosphorylated proteins and expressed genes were compared and a new BR signaling pathway was constructed (Lin et al. 2015).

BRs are known to regulate various physiological processes in plants. BRs increase the proteins for tubulin, glyceroaldehyde 3-phosphate dehydrogenase, homeodomain leucine zipper protein, di-hydroflavonol 4-reductase, Pyruvate decarboxylase 1, glutathione S-transferase, and RuBisCO (large subunit). Thus, affecting the various cellular processes, cell structure, photosynthesis and stress response (Yang and Komatsu 2004; Fig. 10.2).

6.1 Growth and Development

The increase in growth by BRs has been extensively studied (Fariduddin et al. 2000; Siddiqui et al. 2018b) but the molecular approach to understand the mechanism of this increment is still lacking. Proteome study of plants has been conducted to elucidate the role of different proteins during growth and development. DREPP protein is recognized as a plasma membrane polypeptide which is developmentally regulated (Logan et al. 1997) and it has been reported during various proteomic studies (Carter et al. 2004; Marmagne et al. 2004; Nelson et al. 2006), where its RNA was found to be induced by BR (Goda et al. 2004). DREPP and DREPP2 over-expression suppresses the *det2* and *bri1-5* mutant phenotypes, respectively and confirms that DREPP have a part to play in BR-mediated promotion of plant growth (Tang et al. 2008; Wang et al. 2010). OsGRP1 possess promotive effect on

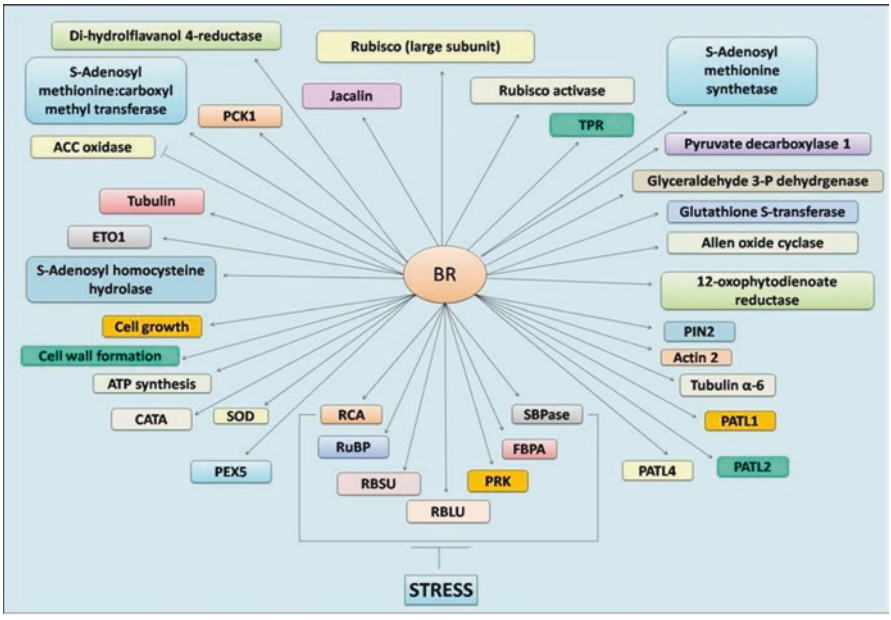

Fig. 10.2 An array of proteins regulated by brassinosteroid

cell expansion and elongation. Over-expression of OsGRP1 in *bri1-5,* considerably increases the cell size and suppresses the dwarf phenotype. It is quite interesting to note that the *OsGRP1* RNA level remained unaffected upon treating with BR treatment or in the BR-mutant. However, BR treatment as well as BR-mutants did affect the protein level thus, suggesting a post-translational level regulation of *OsGRP1* by BR (Wang et al. 2010; Fig. 10.2). Tang et al. (2008) detected both BAK1 and BZR1 on 2-D DIGE images with unswerving BR-regulated changes at phosphorylation level. Apart from BAK1 and BZR1, various other BR responsive proteins were also identified though their exact role in BR-regulated cell response remains unclear. These proteins are PCK1 (phosphoenol pyruvate carboxykinase1; Fig. 10.2) which directs the conversion of oxaloacetate to pyruvate and via gluconeogenesis it takes part in promoting early seedling growth in *Arabidopsis* (Rylott et al. 2003; Penfield et al. 2004). A RanBP1 domain-containing protein which modulates the activity of RAN (Ras-like GTPase) (Sazer and Dasso 2000) playing a role in varied processes, like nucleo-cytoplasmic transport, assemblage of spindle and post-mitotic nuclear envelope (Sazer and Dasso 2000), functioning of ATPase (AAA-type) family protein and two putative tetratricopeptide repeat (TPR) proteins occupying a role in biosynthesis and signal transduction of various phytohormones, for example ETO1 (ethylene over producer1) in ethylene production (Wang et al. 2004), and jacalin known to bind glycoproteins (Fig. 10.2), however their exact role in plants still remains unclear.

Deng et al. (2007) identified four proteins i.e. tubulin, dihydroflavonol reductase, calmodulin and GST to be regulated by BRs. Regulation of these proteins share a similarity with the ones earlier reported by Konishi and Komatsu (2003), α-tubulin homolog and *S*-adenosylmethionine synthetase ortholog were also found. Proteins identified by Deng et al. (2007) intercedes BR-mediated regulation of intracellular signalling, cytoskeleton, secretion and vesicle trafficking and phytohormone biosynthesis. Two proteins each for calmodulin-like and 14-3-3 was considered to be involved in signal transduction or cellular regulation. Regulatory role of calmodulin in BR biosynthesis has been proved via interaction with gene responsible for BR biosynthetic enzyme, DWF1 (Du and Poovaiah 2005). Intracellular calcium fluxes are also affected by BR (Allen et al. 2000). The BR mediated regulation of calmodulin might be responsible for feedback regulation of BR biosynthesis along with other cellular and metabolic responses regulated by BRs. 14-3-3 proteins are involved in signalling processes that get attach to phosphorylated proteins to regulate various cellular processes such as cell division, signal transduction, transcription, and metabolism (Tzivion and Avruch 2002).

Three cytoskeletal proteins which are induced by BR, i.e. actin 2, tubulin α-6 chain, and tubulin β-4 chain were identified (Deng et al. 2007). Sec14 proteins are involved in regulation of signal amid lipid metabolism and membrane trafficking (Cockcroft 1998; Li et al. 2000; Routt and Bankaitis 2004; Phillips et al. 2006). Three BR-inducible Sec14-like proteins (PATL1, PATL-2, and PATL-4) were also found (Fig. 10.2), which help in BR-mediated growth response (Deng et al. 2007).

Binding of PATL1 to phosphoinositides was indicated by vesicle co-sedimentation assays (Peterman et al. 2004). Localization of PATL1 during cell plate formation at late telophase was confirmed by immunolocalization studies, it is the phase in which active vesicle trafficking and vesicle fusion occur. Role of BR-mediated PATL proteins in vesicle trafficking and cell elongation remain as an important area to be explored further for better understanding.

6.2 Photosynthesis

The role of BRs in regulating photosynthesis under normal and stress conditions are well established (Siddiqui et al. 2018a, b). Epibrassinolide significantly increased phosphorylation of polypeptides of the precursor of Rubisco SU (small subunit) and the precursor of chloroplast fructose-1,6-bisphosphate aldolase. BR also caused inconsequential increase in phosphorylation of the chloroplast fructose-1,6-bisphosphate aldolase, and of three isoforms of Rubisco LU (large subunits). Conversely, decrease in phosphorylation was evident only in precursor of α-subunit of Rubisco-binding protein (Fedina et al. 2008). Protein content of rubisco LU, rubisco SU and rubisco activase had a different pattern when compared with its transcript levels. Endogenous BR levels did not alter the protein level of Rubisco LU and Rubisco SU. However, BR deficiency increased and over-expression of *Dwarf* decreased the protein content of rubisco activase in tomato plants, respectively (Li et al. 2016).

6.2.1 Photosynthesis in Presence of Stress

Photosynthesis is highly affected during stress conditions. Proteomic investigation has proved that some isoforms of the Rubisco LU and Rubisco SU decrease in different plants in presence of stress (Ahsan et al. 2010; Wang et al. 2015). Rubisco activase (RCA) regulates the activity of rubisco. RCA gets easily dissociated by stress, and results in reduction of photosynthetic capacity (Raines 2011). Proteomic analysis of *V. vinifera* and *Agrostis sp*. revealed a significant reduction in RCAs abundance (Liu et al. 2014; Xu and Huang 2010), which is in agreement with the retarding activities of RCAs (Crafts-Brandner and Salvucci 2002; Salvucci and Crafts-Brandner 2004). It is quite interesting to note that numerous isoforms of RCA increased in presence of high temperature in *O. sativa*, *C. spinarum*, and *T. aestivum* (Han et al. 2009; Zhang et al. 2010b; Majoul-Haddad et al. 2013; Wang et al. 2015). Moreover, stress exposure marks the reduction of several enzymes involved in ribulose bisphosphate (RuBP) regeneration, such as phosphoribulokinase (PRK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) A/B subunits, fructose-bisphosphate aldolase (FBPA) sedoheptulose-1,7-bisphosphatase

(SBPase) (Lee et al. 2007; Ahsan et al. 2010; Sharmin et al. 2013; Wang et al. 2015; Fig. 10.2). These enzymes determine carbon assimilation and photosynthesis rate by regulating carbon flux in Calvin cycle (Rokka et al. 2001). BR-treatment re-upregulated the ten proteins that were earlier down-regulated due to exposure of plant to chilling conditions. Mostly, these proteins were involved in cell growth, cell wall formation, ATP synthesis, generation of stress response, and methionine assimilation (Huang et al. 2006).

In transgenic *O. sativa* plants the decrease in SBPase results in the decrease of photosynthetic capacity (Harrison et al. 1997), whereas SBPase over-expression enhances the photosynthesis in presence of high temperature stress (Feng et al. 2007) and it was suggested that SBPase has a protective role on photosynthetic machinery in the presence of heat stress. Two photosynthesis-related enzymes (phosphoglycerate kinase and PRK) displayed a similar kind of observation in *O. meridionalis* (Scafaro et al. 2009), indicating that response towards heat is regulated at both RNA and protein levels.

6.3 Stress

Protective role of BRs in conferring stress tolerance has been widely studied (Hayat et al. 2007, 2010). However, the research is mostly confined to the studies analyzing the activities of antioxidant enzymes. The application of proteomics to BR mediated stress tolerance remains at the budding stage. BRs are known to induce various heat shock protein/chaperones to confer thermal tolerance (Dhaubhadel et al. 1999). BR induced various proteins such as SHEPHERD (SHD), luminal binding protein 2 (BiP2), HSP70 and heat shock like protein 2 (Fig. 10.2). SHD is a GRP94 (HSP90-like protein) ortholog. Essentiality of SHD for CLAVATA signalling pathway was revealed during genetic studies of shd mutants in *Arabidopsis*. This pathway involves a peptide hormone and a receptor kinase having structural similarity to BRI1 (Ishiguro et al. 2002). SHD plays a role in folding or complex formation of CLAVATA proteins (Ishiguro et al. 2002).

Superoxide dismutase (SOD), one of the key antioxidant enzymes catalyzes the conversion of the harmful O_2 into O_2 or less harmful H_2O_2, thus protecting the cell from oxidative stress. Catalase (CAT) scavenges H_2O_2 and decomposes it into oxygen and water. BR application promotes the activities of SOD, CAT and APX in maize and rice plants in presence of abiotic stress (Li et al. 1998). However, the exact mechanism explaining the BR mediated increase in the activities of these enzymes is still unknown. Hou et al. (2017) reported several differentially phosphorylated antioxidant proteins for example CATA, SOD, and PEX5 (Fig. 10.2), indicating that the activities of these antioxidant enzymes may perhaps be due to BR-induced protein phosphorylation. CATB (catalaseB) is ABA-dependent in its function and prevents the disproportionate accumulation of H_2O_2 in presence of

stress (Yu et al. 2011). CATs become targets of heavy metal toxicity, leading to retardation in seed germination rate. The physical organisation of glycolate oxidase (H_2O_2 producer) and CAT presents explicit mechanism accountable for H_2O_2 level adjustments (Zhang et al. 2016). Furthermore, a possible PEX5-CATA-SOD interface has been proposed by Hou et al. (2017). Hence, application of BR regulates the antioxidant enzyme activities *through* altering the phosphorylation status and/or antioxidant enzyme intensity, thereby conferring tolerance against various stresses.

6.4 Crosstalk with Other Phytohormones

BRs are involved in regulation of various phytohormones. However, there is a high scarcity of proteomic studies related to BR crosstalk with other phytohormones. However, few studies have been put forward in this context and have been discussed in the following section.

The differential regulation of PIN genes, responsible for auxin transport in plants has been observed in BR-treated, or BR-biosynthetic or signalling mutants. Additionally, the BR-induced accumulation of PIN2 protein (root tip to the elongation zone) elevates the plant tropistic responses (Fig. 10.2) and invigorating the expression and diffused localization of ROP2 proteins (Li et al. 2005). ROP2 overexpression promotes the PIN2 protein polar accumulation in the root elongation zone and also enhances the gravitropic response (Li et al. 2005). Thus, indicating an existence of relationship between BR and auxin during tropistic responses.

BR down-regulates ACC oxidase (At1g62380) proteins, this enzyme is known to convert ACC to ethylene. This observation suggests a BR-ethylene cross-talk for better understanding. There is a possibility that reduced ACC oxidase accumulation promotes the channelling of methyl groups from ethylene biosynthesis to other methylation processes such as phospholipids, pectin, and lignin (Deng et al. 2007). Thus promotes growth, as methylation occupies a seat during synthesis of various compounds. This hypothesis was further confirmed with the increased accumulation of enzymes such as *S*-adenosyl-L-methionine: carboxyl methyltransferase, *S*-adenosylmethionine synthetase, and *S*-adenosyl-L-homocysteine hydrolase belonging to *S*-adenosyl-L-methionine cycle (Deng et al. 2007; Fig. 10.2). BR also up-regulates two proteins involved in jasmonic acid (JA) biosynthesis, allene-oxide cyclase and 12-oxophytodienoate reductase 1 (OPR1; Fig. 10.2). Thus, there is a possibility of BR-mediated induction of JA biosynthesis. BRI1 receptor kinase recognizes both BR and systemin (wounding response peptide signal) in tomato plants (Howe and Ryan 1999; Szekeres 2003; Wang and He 2004), and systemin trigger the wounding responses through induction of JA biosynthesis. Moreover, systemin overexpression enhances stem elongation in a JA-dependent manner (Howe and Ryan 1999; Li et al. 2003; Wang and He 2004). Hence, there is a possibility that BR might promote cell elongation and defense responses via induction of JA biosynthesis.

7 Conclusions

Brassinosteroids are known to regulate wide range of physiological processes in plants. Application of "omics" technique acts as an important tool to determine the changes in expression of various gene and protein during a particular process. The study which combines the transcriptomics and proteomics tool will help in elucidating the molecular aspect of BR regulated processes. These techniques could be further exploited in different plant breeding programmes for improving the quality and quantity of horticultural crops.

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Chapter 11
Interplay Between Antioxidant Enzymes and Brassinosteroids in Control of Plant Development and Stress Tolerance

Mohammad Yusuf, Qazi Fariduddin, Tanveer Alam Khan, Mohammad Faizan, and Ahmad Faraz

Abstract Brassinosteroids (BRs) is a naturally occurring phytohormone of steroidal nature, which take part in the regulation of growth and development of plants through their life cycle. In the present era, availability of a larger number of biotic and abiotic factors restrict the gross production of principal crops. Handful of literature revealed that BRs play vital role in modulating the plant response to various abiotic stresses through alteration in the activities of antioxidant enzymes and proline metabolism by inducing expression of genes involved in defense and antioxidant responses in plants. This plant steroid also found to be very successful in mitigating the damage caused by the oxidative stress under varied unfavorable environmental conditions. These days most debatable part in the BRs research field is the molecular mechanisms associated with the enhanced activities of antioxidant enzymes and proline accumulation in plants under various developmental and environmental cues. Here, we will shed lights on the action mechanisms by which BRs enhanced the activities of antioxidant enzymes and proline accumulation under both stress and stress-free conditions and cross talk with other plant hormones. Therefore, understanding the physiological, biochemical and molecular aspects of BRs would help in developing abiotic stress tolerance in plants in a more significant manner.

Keywords Abiotic stress · Antioxidant system · Brassinosteroids · Plant · Tolerance

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1 Introduction

It was an effort of Mitchell and co-workers over the period of almost 30 years to extract specific organic compounds with active growth-promoting abilities from Brassica pollen after screening nearly 60 species of plants, and were named brassin. Mitchell et al. (1970) endorsed hormonal status to the brassins because this organic compounds was isolated from a plant and induced measurable growth responses when applied in minute quantities to the plants. Assuming the potential applications of brassins in agriculture, efforts were made by the USDA to purify 4 mg of brassins from 500 lbs of bee-collected brassin pollen. Purified brassins were converted into purified brassin crystal and then named brassinolide, active component of brassinosteroids (Grove et al. 1979). This discovery of brassinosteroids leads to the first polyhydroxysteroidal plant hormone. Till now, it is believed that more than 70 analogues of brassinosteroids have been isolated from the tissues of different plants (Kutschera and Wang 2012). In 1980s, BRs research mainly focused on the effective physiological roles and its possible applications in the improvement of crop productivity. In 1990s, many researchers from Japan focused on the studies that revealed the BR biosynthetic pathway and its physiological functions of plant through identifications of various BR biosynthetic and signal transduction mutants (Li et al. 1996; Szekeres et al. 1996; Clouse et al. 1996). With the identifications of key mutants for the enzyme involved in biosynthesis of BR (Li et al. 1996; Szekeres et al. 1996), it has been widely accepted by plant biologist as phytohormone in line with the other well-known hormones such as auxins, gibberellins, cytokinins, ethylene and abscisic acid. There are various precursors for BR biosynthesis namely campesterol, sitosterol and cholesterol (Diener et al. 2000; Shimada et al. 2003; Kim et al. 2004; Taiz and Zeiger 2006). However, the most common precursor of biosynthetic pathway of BR originates from campesterol. This campesterol is converted to campestanol with the help of enzyme the DET2 (De-etiolated-2) enzyme, 5-α-reductase. In the next step, an intermediate product, castasterone is formed from the campestanol and this castasterone is the immediate precursor of brassinolide. This castasterone converted into brassinolide, active component of brassinosteroids via two different pathway namely late C-6 oxidation pathway and early C-6 oxidation pathway. However, later contributed more significantly in brassinosteroids biosynthesis.

It well documented that physiological roles of BRs depend on the exogenous application of BRs on various plants. Moreover, efficacy of exogenous application depends on the concentration of BRs, plant species and time of application. Bajguz (2007) reported that physiological processes including growth and development are controlled by BRs and it also influences the germination of seed, cell division and elongation, flowering and reproductive development, senescence, banding, vascular development, membrane polarization, proton pumping, source and sink relationship (Arteca 1995; Marquardt and Adam 1991; Meudt 1987). BRs significantly stimulates the growth of young vegetative tissues (Sasse 1991). Additionally, endogenous biosynthesis of BRs showed significant relationship in the regulation of cell

expansion and cell division and also interact with other plant hormones to showed physiological responses. Zurek and Clouse (1994) believed that presence of BR increased extensibility of soybean epicotyl due to increased levels of Mrna by BRU1. Moreover, He et al. (1996) revealed that cytokinins inhibits the process of senescence whereas, 24-epibrassinolide reverses the same process. Exogenous sourced BR application also stimulated the differentiation of the tracheary element in *Helianthus tuberosus* and *Zinnia elegans*, two major model systems for xylogenesis. BRs also can accelerate senescence and regulate abiotic and biotic stress responses, including responses to temperature extremes, salt and drought stresses, and pathogen attacks (Clouse and Sasse 1998). Consistent with the effects of the exogenous BR application, BR deficient and insensitive mutants show inhibited cell elongation phenotypes such as dwarfed stature, reduced male fertility, and unexpanded leaves. Overexpression of genes regulating the rate-limiting steps of BR biosynthesis or signal transduction showed physiological effects on plant growth and development similar to exogenous BR application.

2 Physiological Role of Brassinosteroids Under Abiotic Stress

In the recent past, brassinosteroids are considered as "master regulators" due to their pivotal role in conferring tolerance against various abiotic stresses, such as salt (Nunez et al. 2003; Ozdemir et al. 2004; Hayat et al. 2010; Gomes 2011), drought (Li and Van Staden 1998), chilling (Dhaubhadel et al. 1999; Yu et al. 2002), temperature extremes (Fariduddin et al. 2014), and heavy metals (Bajguz and Hayat 2009). Although brassinosteroids are more known as endogenous regulators that induce dramatic growth and development in plants whereas, exogenously sourced BR application through varied mode such as seed soaking, root treatment, and foliar spray showed significant modulation of physiological traits under various abiotic stress conditions in different plant species. Each mode of application has its own advantages and disadvantages however, out of various mode of application, foliar spray is most commonly in practice by agronomist. Moreover, modulation of physiological traits in different plant species under abiotic stress conditions by exogenous application of brassinosteroids are summarized in Table 11.1 and Fig. 11.1.

3 Effect of Brassinosteroids on Antioxidant System and Metabolites Under Abiotic Stress

Research development over the year showed significant impact of BRs and its analogues on the plants exposed to various environmental cues (Fariduddin et al. 2014). BRs showed involvement in the regulation of reactive oxygen species (ROS)

Table 11.1 Modulation of physiological traits in different plant species under abiotic stress conditions by exogenous application of BRs

BR concentration and analogues	Mode of BR application	Abiotic stress imposed	Plant species tested	Effect on physiological traits	References
24-EpiBL, 28-HomoBL (3 μM)	Pre-sowing seed soaking	**Salt stress**	Rice	Restored pigment levels Increased NR activity	Anuradha and Rao (2003)
24-EpiBL and 28-HomoBL (0.5, 1 and 3 μM)			Rice	Enhanced levels of nucleic acids and soluble proteins	Anuradha and Rao (2001)
BR (5 μM)			Beans and Barley	Enhanced betaine level and chlorophyll content	Akram and Ragab (2006)
28-HomoBL 10^{-10} and 10^{-8} M)			Chickpea	Increased activities of CA and NR	Ali et al. (2007)
24-EpiBL (3 μM)			Rice	Increased protein content and activities of APX	Ozdemir et al. (2004)
24-EpiBL (10^{-8} M)			*Triticum aestivum*	Increased maximum quantum yield of PSII and leaf water potential	Yusuf et al. (2017a, b)
24-EpiBL (1 and 2 μM)	Through root		*Brassica napus*	Increased germination rate and seedling growth	Kagale et al. (2007)
24-EpiBL (0.5, 1, 3 μM)			Cyanophyta	Improved growth	Saygideger and Deniz (2008)
24-EpiBL (0.0125, 0.025, and 0.0375 mg L^{-1})	Foliar spray		Wheat	Improved leaf area, photosynthetic rate and Fv/Fm efficiency	Shahbaz et al. (2008)
24-EpiBL			Wheat	Enhanced chlorophyll a and b contents, while decreased transpiration rate and stomatal conductance	Qayyum et al. (2007)
24-EpiBL (0.5 mg L^{-1})			Pepper plants	Significant rise in chlorophyll a and b concentrations	Houimli et al. (2010)

(continued)

Table 11.1 (continued)

BR concentration and analogues	Mode of BR application	Abiotic stress imposed	Plant species tested	Effect on physiological traits	References
24-EpiBL and 28-HomoBL (10^{-8} M)			*Brassica juncea*	Increased leaf water potential, chlorophyll content, net photosynthetic rate, and stomatal conductance	Wani et al. (2017)
				Decreased electrolyte leakage	
28-HomoBL (10^{-8} M)			*Triticum aestivum*	Increased CA activity and maximum quantum yield of PSII	Hayat et al. (2014)
				Decreased lipid peroxidation	
24-EpiBL (10^{-8} M)			*Triticum aestivum*	Increased maximum quantum yield of PSII and leaf water potential	Yusuf et al. (2017)
24-EpiBL (10^{-8} M)			*Cucumis sativus*	Increased activities of CA, NR, and efficiency of PS II	Fariduddin et al. (2014)
BL (0.05 ppm)			Cowpea	Increased total soluble protein content	El-Mashad and Mohamed (2012)
				Decreased lipid peroxidation	
24-EpiBL (10^{-7} and 10^{-9} M)			Rice	Increased protein and total chlorophyll content	Sharma et al. (2013)
				Decreased lipid peroxodation	
24-EpiBL and 28-HomoBL (10^{-8} M)	Seed soaking	**Drought stress**	*Oryza sativa*	Improved net photosynthetic rate, internal CO_2 concentration and stomatal conductance	Farooq et al. (2009)
28-HomoBL (2 and 3 μM)			*Sorghum vulgare*	Increased soluble proteins and proline content	Vardhini and Rao (2003)
24-EpiBL (2 and 3 μM)					
24-EpiBL (1 μM)			*Arabidopsis thaliana*	Modulation of growth, and morphological changes	Kagale et al. (2007)
			Brassica napus		

(continued)

Table 11.1 (continued)

BR concentration and analogues	Mode of BR application	Abiotic stress imposed	Plant species tested	Effect on physiological traits	References
24-EpiBL and 28-HomoBL (0.5, 1, 2 μM)			*Raphanus sativus*	Increased chlorophyll content and protein content Decreased lipid peroxidation content	Mahesh et al. (2013)
BL (1 ppm)			Groundnut	Increased relative water content	Savaliya et al. (2013)
BL (0–0.4 mg/L)	Through root		*Robinia pseudoacacia*	Increased soluble sugar and proline content, and gas exchange traits,	Li et al. (2008)
24-EpiBL (1 μM)	Foliar spray		Tomato seedlings	Increased net photosynthetic rate and relative water content	Yuan et al. (2010)
28-HomoBL (0.01 μM)			*Brassica juncea*	Increased NR and CA activity Increased SPAD chlorophyll, stomatal conductance, and net photosynthetic rate	Fariduddin et al. (2009)
BL (0.1 mg/L)			*Glycine max*	Increased quantum yield of PSII, enzymes activities, soluble sugar and proline content	Zhang et al. (2008)
28-HomoBL (1 and 5 μM) 24-EpiBL (1 and 5 μM)			*Phaseolus vulgaris*	Modification of root nodulation, endogenous ABA and cytokinin, nitrogenase activity	Upreti and Murti (2004)
BL			Papaya	Alteration in chlorophyll metabolism and leaf ontogeny	Gomes et al. (2013)
Heavy metal stress					
24-EpiBL 28-HomoBL (1, 2, 3 μM)	Seed soaking	Cd	Raphanus sativus	Improved growth biomarkers and proline accumulation	Anuradha and Rao (2007)
24-EpiBL (10 nM)	Through root		*Cucumis sativus*	Improved the protein content	Jakubowska and Janicka (2017)

(continued)

Table 11.1 (continued)

BR concentration and analogues	Mode of BR application	Abiotic stress imposed	Plant species tested	Effect on physiological traits	References
24-EpiBL and 28-HomoBL (10^{-8} M)	Foliar spray		Tomato cultivars	Improved photosynthetic machinery and leaf water potential	Hasan et al. (2011)
24-EpiBL and 28-HomoBL (10^{-8} M)			*Solanum lycopersicum*	Improved stomatal conductance and water use efficiency	Hayat et al. (2012)
24-EpiBL (10^{-6} M)	Seed soaking	Ni	*Vigna radiate* cultivars	Improved nitrogen metabolism and proline content	Yusuf et al. (2012)
28-HomoBL (10^{-8} M)	Foliar spray		*Triticum aestivum*	Increased SPAD chlorophyll content and photosynthetic efficiency	Yusuf et al. (2011)
24-EpiBL (10^{-11} M)			*Brassica juncea*	Improved protein content and growth biomarkers	Kanwar et al. (2012)
24-EpiBL (1 μM)			*Brassica juncea*	Increase membrane stability	Ali et al. (2008)
				Decrease electrolyte leakage	
				Improved photosynthetic efficiency	
28-HomoBL (10^{-6} M)	Seed soaking	Cu	*Brassica juncea*	Improved activities of NR and CA	Fariduddin et al. (2009)
				Higher net photosynthetic rate, stomatal conductance, and water use efficiency	
24-EpiBL (0.01 μM)	Foliar spray		*Cucumis sativus*	Decrease electrolyte leakage	Fariduddin et al. (2013)
				Improved protein content and gas exchange traits	
24-EpiBL (10^{-9} M)	Through nutrient medium		*Raphanus sativus*	Reduces Cu uptake and its distribution	Choudhary et al. (2012a, b)
				Modulates IAA and ABA profiles	
28-HomoBL (10^{-8} M)	Foliar spray	Mn	*Brassica juncea*	Increased leaf water potential and stomatal conductance	Fariduddin et al. (2015)
				Decrease electrolyte leakage	

(continued)

Table 11.1 (continued)

BR concentration and analogues	Mode of BR application	Abiotic stress imposed	Plant species tested	Effect on physiological traits	References
28-HomoBL (10^{-8} M)	Foliar spray	Low temperature	*Cucumis sativus*	Increased chlorophyll content and photosynthetic efficiency	Fariduddin et al. (2011)
24-EpiBL (0.1 μM)			*Cucumis sativus*	Change in electron transport rate	Xia et al. (2009)
24-EpiBL (0.1 μM)			*Cucumis sativus*	Recovery of photosynthetic apparatus by balancing the electron partitioning and carboxylation	Jiang et al. (2013)
24-EpiBL (0.05, 0.10, and 0.15 mg/L)			Grapevine plant	Increased soluble sugar and proline content	Xi et al. (2013)
24-EpiBL (0.01, 0.1 and 1.0 mg/L)	Foliar spray	High temperature	*Lycopersicon esculentum*	Change in CO_2 gas exchange	Ogweno et al. (2008)
BL (10^{-6} M)			*Brassica napus*	Change in endogenous ABA content	Kurepin et al. (2008)
28-HomoBL (0.01 μM)			*Brassica juncea*	Protect photosynthetic machinery	Fariduddin et al. (2014)
24-EpiBL (0.05, 0.1, 0.5, 1.0, and 1.5 mg L^{-1})			*Cucumis melo*	Restore the inhibition of photosynthesis	Zhang et al. (2013)

metabolism through the expression of many antioxidant genes which increases the activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) (Cao et al. 2005; Ogweno et al. 2008). Moreover, both BRs and ROS act as a secondary messenger for the induction and regulation of antioxidant systems to confer tolerance against various abiotic stress conditions (Mazorra et al. 2002). Recently the role of BRs and its analogues in the modulation of both enzymatic and non-enzymatic components of antioxidant defense system in abiotic stressed plants are well documented. El-Khallal et al. (2009) reported that brassinolide restored deleterious impact of salt stress in *Zea mays* by modulation the activities of antioxidant enzymes. In another study, treatment with 28-HomoBL elevated antioxidative enzyme activities (SOD, CAT, GR, APX, and GPX) in the seedlings of Zea mays exposed to salt stress (Arora et al. 2008). In a field experiment, brassinolide increased the activities of CAT, SOD, and GR whereas, at the same time downgraded the POD and PPO activities in the two varieties (CSH-5 and CSH-6) of sorghum plants grown under saline conditions (Vardhini 2011).

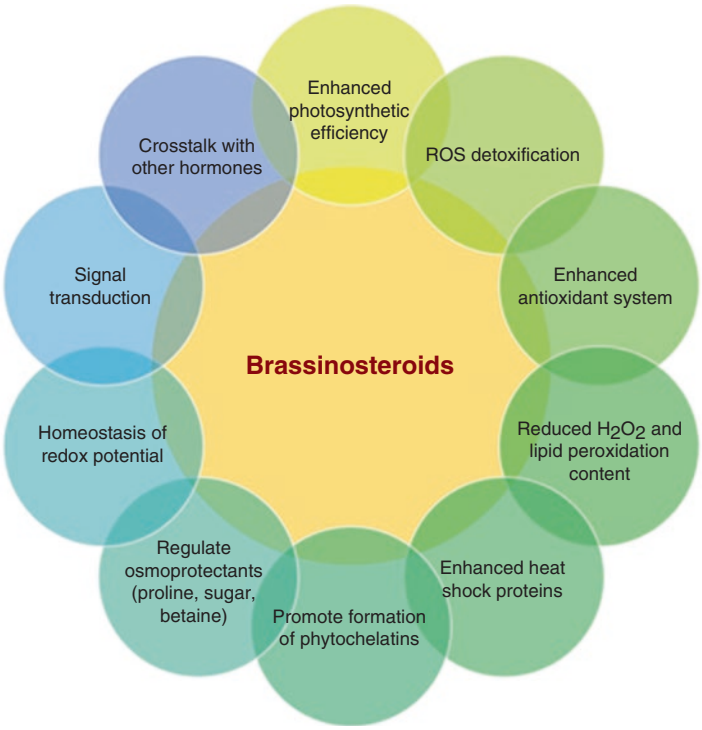

Fig. 11.1 Abiotic stress protection mechanism by brassinosteroids

Exogenous BRs (0.005, 0.01, 0.05, 0.1, and 0.2 mg/L^{-1}) confer tolerance to *Cucumis sativus* seedlings against salt stress by elevating the activities of CAT, POD, SOD and therefore, significantly lowered the salt injury index and increased the metabolites accumulation such as free-proline and soluble sugars (Shang et al. 2006). In another mode of application, soaking of seed in 5 μM L^{-1} BL significantly increased the activities of POD, SOD, and CAT under salt stress conditions (Zhang et al. 2007). Treatment of *Cucumis sativus* grown under saline conditions with 24-EpiBL significantly increased the level of SOD, POD, CAT enzymes (Lu and Yang 2013) to restore the damage caused by salt stress. Exogenous application of 24-EpiBL up-regulated the expression of osBRI1 and OsDWF4 responsible for the activities of antioxidant enzymes (Sharma et al. 2013). Treatment of eggplant seedlings grown under saline conditions with 24-EpiBL exhibited decreased superoxide production, MDA, H_2O_2 due to the increased activities of SOD, GPX, CAT and APX enzymes and the contents of non-enzymatic antioxidants such as AsA and GSH (Ding et al. 2012). Two varieties of pepper grown under stressful conditions showed remarkable increase in the activities of antioxidative enzymes and proline accumulation, total anthocyanins and minerals in the presence of 24-EpiBL (Abbas et al. 2013). Supplementation of *Vigna radiata* plants with 28-HomoBL detoxified the stress generated by NaCl by elevating the activities of antioxidative enzymes and the

proline content (Hayat et al. 2010). In a similar study, Rady (2011) reported that spraying of 5 μM of 24-epiBL to NaCl-exposed *Phaseolus vulgaris* showed significant elevations in the activities of antioxidative enzymes and proline content. Treatment of *Cucumis sativus* cultivars with 24-EpiBL grown under combination of two abiotic stress i.e. Cu and NaCl, enhanced the activities of antioxidant enzymes and restored the damage caused by two stress (Fariduddin et al. 2013).

Extensive reports are available on the role of BRs and its analogues in plant drought tolerance. Exogenous application of 24-EpiBL increased the activities of AsA and GSH in drought stressed *Chorispora bungeana* that increased the resistance against drought (Li et al. 2012). Treatment of *Zea mays* with brassinolide alleviated the ill effects of drought by enhancing the activities of antioxidant enzymes and proline accumulation (Anjum et al. 2011). Farooq et al. (2010) reported that exogenous application of 24-EpiBL improved the drought tolerance in rice with increased synthesis of metabolites and enhanced capacity of antioxidant system. 30-days old seedling of drought stressed *Brassica juncea* sprayed with 28-HomoBL improved the activities of CAT, POD, and SOD along with the proline accumulation (Fariduddin et al. 2009). Yuan et al. (2010) reported that 24-EpiBL successfully countered the damage caused by the drought stress by increased level of antioxidant enzymes and decreased levels of H_2O_2 and MDA in two genotypes of *Lycopersicon esculentum*. Two analogues of BR, 24-EpiBL and 28-HomoBL mediated alteration in the activities of antioxidant enzymes and proline accumulation lead to the reduction in the inhibitory effect caused by water stress in *Raphanus sativus* (Mahesh et al. 2013). In addition to this, BL also increased the activities of antioxidant enzymes under field conditions of 1-year-old *Robinia pseudoacacia* grown under drought stress (Li et al. 2008).

Out of various threat to agricultural soil, heavy metal stress has become a critical environmental concern due to their acute and chronic toxic effects on plants grown on such soils. In the recent past, researchers revealed that brassinosteroids play pivotal role in overcoming the heavy metal stress mediated loss of crop productivity through enhanced antioxidant enzymes and metabolites. In a study conducted by Hayat et al. (2007) showed that foliar application of 28-HomoBL improved the Cd tolerance capacity of *Brassica juncea* through enhanced activities of antioxidant enzymes. In *Phaseolus vulgaris*, 24-EpiBL treatment improved the Cd tolerance with increased proline accumulation and antioxidant enzymes (Rady 2011). Restoration of the Cd induced damages in tomato cultivars as a result of 28-HomoBL/24-EpiBL (10^{-8} M) mediated improvement in antioxidant defense system (Hasan et al. 2011). 24-EpiBL lessened the oxidative stress in *Raphanus sativus* through increased activity of GST and PPO enzymes (Sharma et al. 2012). Exogenous application of 24-EpiBL ameliorated Ni induced stress in *Brassica juncea* mainly by enhancing the activity of antioxidant enzymes (Kanwar et al. 2013). Foliage application of 28-HmoBL (0.01 μM) to five different cultivars of *Triticum aestivum* showed elevated level of CAT, POD, and SOD activities under Ni stress (Yusuf et al. 2011). In an another study conducted by Yusuf et al. (2012) revealed that application of 24-EpiBL as shotgun approach to two contrasting cultivar of *Vigna radiata* improved the activity of CAT, POX and SOD and proline

accumulation lead to the improvement of nitrogen metabolism (Yusuf et al. 2014). BRs successfully mitigate the Cu induced toxicity through elevated activity of CAT, POX and SOD and proline accumulation (Fariduddin et al. 2009). *Raphanus sativus* seedling exposed to 24-EpiBL reduced Pb and Hg toxicity (Anuradha and Rao 2007; Kapoor et al. 2014) by modulating the CAT, APX, GPX, SOD and POD activity (Rady and Osman,2012). Supplementation of 28-homoBL to *Raphanus sativus* seedlings help the plant to tolerate Zn toxicity by enhancing antioxidative enzyme activities, strengthening GSH metabolism and redox status, and also improved the contents of non-enzymatic antioxidants and proteins (Ramakrishna and Rao 2013). Raghu et al. (2014) reported exogenously sourced BR improved As-tolerance in *Raphanus sativus* due to the increased activity of SOD and CAT.

Plentiful of documents are available pertaining to the ameliorative role BR and its analogues under low and high temperature stress in various plant species. Two Indian rice cultivars differing in heat sensitivity when grown under high temperature stress and exposed to exogenous application of BL showed significant increment in the activities of POD and SOD isozyme expression and reduction in MDA levels (Cao and Zhao 2007). Exposure of *Vigna radiata* to 28-HomoBL increased the activities of antioxidant enzymes and detoxify the stress generated by high temperature stress (Hayat et al. 2010). Pre-treatment with BR to the col. stressed rape plants showed reduced ion leakage (Janeczko et al. 2007) and also increased the antioxidant defense mechanism along with osmoregulation of chilling stressed grapevines (Xi et al. 2013). *Brassica juncea* grown under low temperature (4 °C) showed excess accumulation of H_2O_2 which were nullified by the exogenous application of 24-EpiBL through enhanced level of antioxidant enzymes (Kumar et al. 2010). Pre-treatment of cucumber grown under low temperature stress with 24-EpiBL showed increased activities of enzyme related to first line of defense in plants i.e. antioxidant system (Fariduddin et al. 2011; Hu et al. 2013). In the same line, treatment of BRs significantly overcome the chilling injury of pepper fruit stored at 3 °C for 18 days by reducing the electrolyte leakage, MDA content; increasing the activities of antioxidant enzymes including CAT, POD, APX, and GR (Wang et al. 2012). The enhanced activities of the antioxidative enzymes as a result of BRs applications (Khan et al. 2015) could be due to increased de novo synthesis or activation of the enzymes, which is mediated through transcription and/or translation of specific genes to gain tolerance (Bajguz 2000). BRs have been implicated in a wide range of physiological and molecular responses in plants, like cell elongation and cell division in stem, and inhibit ions of root growth, promotion of xylem differentiation and abscission of plant organs (Nemhauser et al. 2004). Recently, research work of Aghdam and Mohammadkhani (2014) reported that exposure of tomato fruits to BRs inhibit the activities of phospholipase D (PLD) and lipoxygenase (LOX), major causes of chilling injury induction in tomato fruits. In addition to this, BRs protected the photosynthetic apparatus from cold-induced damage in Cucumis sativus plants by activating the enzymes of Calvin cycle and increasing the antioxidant capacity, which in turn mitigated the photo-oxidative stress and plant growth inhibition during the recovery of chilling injury (Jiang et al. 2013).

4 Mechanism Associated with Brassinosteroids Mediated Change in Antioxidant System Under Abiotic Stress

Unlike animals, plants are not able to cope with stressful environments by moving from one place to other place. To overcome this disability, plants have well-formulated mechanisms at various levels such as physiological, biochemical and molecular to combat with environment cues to enhance agricultural production. It well documented that compatible solutes such as proline, soluble sugars, proteins and organic acids are important traits of stress tolerant plants. Studies have reported that deleterious production of reactive oxygen species (ROS) during normal respiration, photosynthesis, and nitrogen fixation (Mittler et al. 2011) causes damage to the plants and loss of gross productivity. Moreover, plants subjected to abiotic stresses, series of ROS are generated, superoxide radical, hydroxyl radical, and hydrogen peroxide. ROS undergo a sequence of oxidation/reduction reactions known as the Halliwell-Asada pathway (Gratao et al. 2006). To protect themselves, plants contain antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), guaicol peroxidase (POX), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and glutathione peroxidase (GPX) (Ruley et al. 2004; Simonovicova et al. 2004), and non-enzymatic antioxidants, namely ascorbate, gluthathione, α-tocopherol, and carotenoids (Vardhini and Rao 2003; Ozdemir et al. 2004; Sharma and Dubey 2005). The activation of antioxidant enzymes keeps check and balances in the production and scavenging of the ROS for attaining degree of tolerance.

Since two decade brassinosteroids have been recognized as natural stress alleviator of plant grown under various stressful environments. Recent findings have revealed BRs modifies enzymatic and non-enzymatic antioxidants under various abiotic stresses (Fariduddin et al. 2014). Goda et al. (2002) demonstrated that *ATPA-2* and *ATP-24* gene encoding peroxidases are constitutively up-regulated in the *det-2 Arabidopsis* mutant during the biosynthesis pathway of BR. Additionally, oxidative stress-related gene encoding MDHAR and thioredoxin, cold and drought stress response genes *COR-47* and *COR-78*, and heat stress-related genes *hsp83*, *hsp70*, *hsf3*, *hsc70-3*, and *hsc70-G7* have been identified by a microarray analysis of either BR-deficient or BR-treated plants (Mussig et al. 2002). The enhanced oxidative stress resistance in *det-2* plants correlates with a constitutive increase in the SOD activity and increased transcription of the CAT gene. Moreover, BRs overcome the deleterious effect of abiotic stresses through upregulation of stress-related genes such as *WRKY3*, *WRKY6*, *HSP70*, and *MYB* and activation of antioxidant system (Nawaz et al. 2017). It is reported that during AsA-GSH Cycle/Asada Haliwell pathway, ascorbate play pivotal role in ROS scavenging and stability of its cellular pool maintained by dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) with NADPH as the reducing power. Mittler (2002) believed that under stress condition alteration of redox potential of cells, and destabilization of membrane take place. It is believed that BRs could maintain the modified cell redox by modulating activities of SOD, CAT, APOX, GR, DHAR and MDHAR. Moreover, application of BRs stabilizes the redox potential through reduc-

ing phospholipid peroxidation in cell membranes (Rajewska et al. 2016) or by the accumulation of proline under abiotic stress conditions (Fariduddin et al. 2014). Additionally, application of BRs under stress conditions modifies the activity of protein and related enzymes in membrane either influencing protein folding conformation or protein activity by the direct interaction of protein and sterols (Lindsey et al. 2003). It is very well documented that BRs is a protein complex of leucine-rich repeat receptor-like kinase enodes by BRI1 and this BRI1 receive peptide signals and therefore served as protective role (Wang et al. 2014). At onset of abiotic stresses, these signals modify defense responses. Various BRs-regulated genes play pivotal role in the regulation of stress responses that includes, osmolytes, organic acids, metallothioneins, and stress protective proteins such as heat-shock proteins (Gendron and Wang 2007). It was the report of Jiang et al. (2013) that BRs recover the loss of photosynthetic efficiency of plants under low temperature stress condition by stimulating the antioxidant defense system and enzymes of Calvin cycle. The probable reason is that BR application enhances AOX (ALTERNATIVE OXIDASE) activity in a RBOH manner and this enhances AOX then balances the chloroplast-to-mitochondria electron transfer by dissipation of excess reductant and leads to the decrease accumulation of ROS and therefore increased protection of photosystems (Deng et al. 2015). It is believed that BR mediated stress tolerance is associated with increased accumulation of ROS because Jian et al. (2012) reported that exogenous application of BR increased the production of ROS and also increased the activities of antioxidant enzymes. Moreover, accumulation of H_2O_2 in response to BRs could behave as signaling molecule in response to various environmental cues which in turn activates the MAPK also induces NADPH oxidase to upregulation of cellular H_2O_2. Increased level of H_2O_2 activates the antioxidant enzymes, dehydrines, transcription factors, heat shock proteins induced by various abiotic stresses to scavenge ROS, leading to suppression of ROS levels (Xia et al. 2009; Zhang et al. 2010; Cui et al. 2011; Zhu et al. 2013). However, BRs induce systematic stress tolerance by increasing the H_2O_2 production (Xia et al. 2011). In tomato, BRs mediated abiotic stress tolerance is due to increased apoplastic H_2O_2 and activation of MPK1/2 which was restricted in RBOH1-, MPK1/2- and MPK2- silenced plants but not in MPK1 silenced plants revealing a relatively more important role of MPK2 than MPK1 in BR-induced apoplastic H_2O_2 accumulation (Nie et al. 2013). In the same line, *Nicotiana benthamiana*, silencing of RBOH compromised the BR induced AOX activity and hence reduced ROS scavanging making the plant more susceptible to abiotic stresses (Deng et al. 2015). In a recent study, BR treatment was unable to elicit antioxidant defense in the rice and maize CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE (CCaMK) mutants. It was found that BR application results in increase in cytosolic Ca^{2+} concentration followed by increase in activity of CCaMK which further enhanced the BR-induced increase in cytosolic Ca^{2+} concentration thus forming a positive feedback loop of Ca^{2+} and CCaMK in BR signaling (Yan et al. 2015). However, a detail interpretation of the regulatory role of endogenous BR, as well as its comprehensive signalling mechanism, would be helpful in improving our understanding of BR-mediated abiotic stress tolerance (Fig. 11.2).

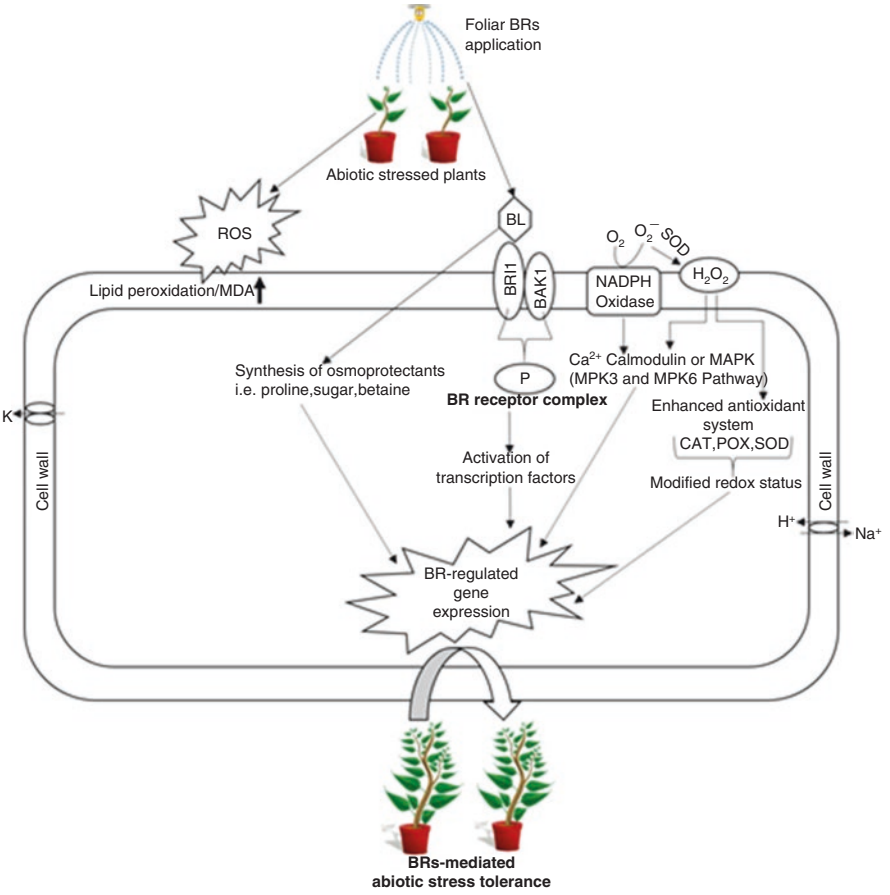

Fig. 11.2 Brassinosteroids mediated abiotic stress tolerance mechanism in plants

5 Cross-Talk of Brassinosteroids with Other Signals

Brassinosteroids showed diverse physiological, biochemical, and molecular responses due to its communication with other plant hormones under stress and stress free conditions. Plant growth and development are significantly controlled by environmental cues and/or various plant hormones. It is well documented that brassinosteroids interact with different phytohormone such as auxin, cytokinin, abscisic acid, ethylene, gibberellic acid, jasmonic acid, polyamines, salicylic acid to elicit various responses of plant metabolism and its growth and development (Saini et al. 2015).

The relationship between BR and auxin is not very profound however, they interact with each other and showed involvement in various physiological processes such as root development and hypocotyl elongation. Nemhauser et al. (2004)

believed that auxin and BR interact synergistically to enhance plant growth and gene expression. Recently, Lanza et al. (2012) reported that BR enhances the auxin signaling through regulation of the cytoskeleton and control of polar PIN2 location. Subsequently, ROP-GTpases shown to regulate cytoskeleton and PIN2 localization (Xu et al. 2002; Lin et al. 2012) and shows involvement in the regulation of NADPH oxidases (Duan et al. 2010). Moreover, BR activates ROP proteins that also regulate NADPH oxidase. With the prominent role of BR in regulation of cytoskeleton and ROS, BR significantly up-express a gene associated with microtubule protein and interact with a MAPK which subsequently shows involvement in regulation of NADPH oxidase genes (Zhu et al. 2013). It is believed that MAPK cascades control ROS production under abiotic stress in the BR-treated plants (Zhang et al. 2010) through enhanced antioxidant system (Fariduddin et al. 2014). Moreover, loss of MKK4 function lead to the reduced BR signalling and an inhibition of cell proliferation in rice (Duan et al. 2014) and these result suggested that ROS form an integration node for the BR signalling pathway with other development and/or hormonal signalling pathway.

It is very well documented that cytokinins play significant role in plant development under stress and stress free conditions (Werner et al. 2010; Nishiyama 2012). Several researchers have revealed the relationship between BR and cytokinin in various biological processes (Choudhary et al. 2012). Findings of Vercruyssen et al. (2011) suggested involvement of BR and cytokinins in the regulation of plant growth and development as BR treatment enhances the lateral root and leaf length in P10-CKX3 plants under over expression of *CKX3* and *BRI1* gene in *Arabidopsis* root (Saini et al. 2015). Moreover, (Yuldashev et al. 2012) also reported the involvement of BR in regulation of cytokinin level in wheat seedlings. In another study, a transgenic plant with delayed response to drought stress showed overexpression of *isopentyl transferase (IPT)* gene which lead to enhanced level of cytokinin before the onset of senescence leading to drought tolerance. This increase of cytokinins concentration also upregulate various BR-related biosynthesis (*DWF4*, *DWF5, HYD1*) and signalling gene (*BRI1, BZR1, BAK1, SERK1, BRH1*; Saini et al. 2015). This establish the relationship between cytokinins and brassinosteroids in conferring tolerance against the abiotic stress in plants. In addition to this, cytokinin treatment enhanced the endogenous accumulation of brassinosteroids in *Chlorella vulgaris* that proved the synergistic relationship between brassinosteroids and cytokinin (Bajguz and Piotrowska-Niczyporuk 2014). Brassinosteroids and cytokinin interact with each other post-transcriptionally to continuously adjust ethylene biosynthesis under various environmental cues (Hansen et al. 2009). Most recently the study of Yuan et al. (2015) reported that BR enhances cytokinin mediated anthocyanin biosynthesis in *Arabidopsis*.

ABA is antagonistically related to BR as AB inhibits seed germination and promotes seed dormancy whereas, BR stimulates seed germination (Steber and McCourt 2001). In an another study, Zhang et al. (2010) reported that treatment of BR induced NO production that leads to the ABA biosynthesis and this confer tolerance against the oxidative stress caused by drought stress. However, extensive research on the molecular mechanism of ABA mediated BR responses and its role

in the abiotic stress tolerance is a need of hour to establish more significant relation between ABA and BR.

Exogenous application of BR speed up the ethylene biosynthesis in *Arabidopsis* seedlings (Hansen et al. 2009) through enhanced expression of *1-aminocyclopropane-1-carboxylate synthase(ACS)*, gene responsible for the ethylene production (Muday et al. 2012). In addition to this, BR presence also act post-transcriptionally and stabilize the ACS proteins (ACS5, ACS6 and ASC9) by preventing its ubiquitination by 26S proteasome (Saini et al. 2015). Therefore, under different environmental cues, BR continuously regulate the expression of *ACS* to maintain the ethylene biosynthesis in different tissues (Hansen et al. 2009). In addition to this, crosstalk of ethylene with BR enhanced abiotic stress tolerance. Study of Wu et al. (2008) suggested that ethylene response factor protein (JERF3) activates the expression of oxidative genes which subsequently decreased the ROS accumulation and enhance abiotic stress tolerance. This finding indicates that ethylene and BR interact to sequestration of ROS during stress conditions.

Recent published work shows significant interaction between BRs and GAs for various biological processes of plant grown under stress and stress-free conditions (Wang et al. 2009; Vleesschauwer et al. 2012). BRs suppressed the *OsGSR1*, a member of *GAST* family which is critically involve in GA signalling and at the same time GA induced its expression in rice plant, therefore, both BRs and GA are antagonist to each other (Wang et al. 2009). Exogenous application rescued the dwarf phenotype of RNAi plants with reduced *OsGSR1* expression with reduced level of endogenous BRs. With the involvement of OsGSR1 in BR biosynthesis through direct interaction with DWF1 revealed that *OsGSR1* is a connecting point between GA and BR signalling pathways (Choudhary et al. 2012). Moreover, exogenous application of BR in cotton plant initiates the downregulation of four *DELLA* genes in cotton fiber cells, including GhGAI1 which is engaged in fiber cell initiation (Hu et al. 2011). To dissect out the pathway of GA and BR crosstalk, more intense research is needed to uncover various junction between GA and BR.

It is very well documented that interaction of BR and JA played significant role in plant growth and development under stressful environments. Kitanaga et al. (2006) reported that BR enhance the JA concentration in rice under stressful conditions which shows the higher antimicrobial activities. Moreover, Exogenous application of BR partially restore the JA sensitivity through modification in *psc1* in *coil-2* whereas, hypersensitivity of JA for *psc1* in wild type *coil-1* has been eliminated (Ren et al. 2009). Additionally, in the wild type plants JA mediated inhibition of root growth was observed on under BR treatment (Saini et al. 2015). When there is low BR concentration, genes related to the transcript levels of JA biosynthesis quality showed down-regulation. On the other hand, with the increase of higher concentration of BR, genes related to the transcript levels of JA biosynthesis and signaling gene were over-expressed. These results authenticate relationship between JA and BR biosynthesis and signalling gene, OsDWF4 and OsBRI1 (Nahar et al. 2013). Researcher should focus more on the interaction between BR and JA under abiotic stress conditions as more reports are published related to biotic stress conditions.

In the recent year, a lot of progress has been made regarding the significant research related to the crosstalk of BR and PA. However, researcher focused more on the crosstalk of BR and PA in relation to the abiotic stress tolerance in plants. Choudhary et al. (2012) revealed that 7-day old *Raphanus sativus* Cu-stressed plant exposed to exogenous BR had higher PA accumulation and antioxidant enzymes and suggested that exogenous application of BR ameliorates the Cu mediated oxidative stress are due to free accumulation of PA and antioxidant enzymes (Liu and Moriguchi 2007). Moreover, BR treatment also maintain the optimum concentration of spermidine which play pivotal role for normal growth and development of plant and at the same it also enhances the concentration of putrescine which have significant role in countering oxidative stress generated by heavy metal stress (Takahashi and Kakehi 2010). In another study by conducted by Fariduddin et al. (2014) showed that combine application of PA and 24-EpiBL successfully counter the salinity induced oxidative stress through modulation of proline accumulation and antioxidant system. Moreover, co-application of BR and spermidine to plants grown under excess Cu showed expression of gene involved in Cu homeostasis and tolerance mechanism. These reports showed significant responses for the abiotic stress tolerance for various plants and in the coming years researcher can utilize the combination of PA and BR for sustainable agricultural practices and dissect out BR and PA mediated signalling pathway and translation factors.

It is well documented that crosstalk of SA and BR play significant role in conferring tolerance against various environmental cues. It has been reported that interaction of SA and BR conferred tolerance against salt induced stress through the modulation of stress hormones (Divi et al. 2010). Moreover, functional NPR1 for the expression of BR effect through controlling BR signalling components (Divi et al. 2010). However, crosstalk of BR and SA induce signaling pathway for conferring tolerance but it acts individually (Nakashita et al. 2003). There is some contradictory report for being biotic stress tolerance. In one study conducted by Vleesschauwer et al. 2012 in rice plants *Pythium graminicola* utilize BR as virulence factor and hijack the rice BR machinery to cause biotic stress. Therefore, an attempt should be made to dissect out the BR and SA interaction pathway and its mechanism for tolerance as well as sensitivity to biotic and abiotic stresses.

6 Conclusions and Future Perspectives

Brassinosteroids emerged as the natural master regulator of steroidal nature for plant growth and development under both stress and stress-free environments. BRs play pivotal role in modulating key physiological traits such as electrolyte leakage, lipid peroxidation, proline, and various enzymes of antioxidant system under stressful environments. Moreover, different mode of BRs application successfully counter the damage caused by the excess generation of ROS under abiotic stress conditions through increased activities of CAT, POX, and SOD along with the up-regulation of stress related genes in various tested plants. However, the effect of

exogenous application of BRs is time and dose dependent and also varies with different plant species. On the other hand, BRs application in combination with other phytohormones such as auxin, cytokinin, abscisic acid, ethylene, gibberellic acid, jasmonic acid, polyamines, salicylic acid showed significant interplay to improve the metabolism of plant and also enhance the crop productivity under stress conditions. Moreover, dissecting the basic mechanism associated with BR homeostasis and its crosstalk with other signals will significantly add new vistas in BR investigation. With the recent advancement in BRs research, available literature revealed that transgenic plants with altered BR activity have been tested in field under very few abiotic stress conditions, however, there is no report about the nutritional quality of the grain and seed derived from BR altered transgenic plant. It is the very demand of time that more and more transgenic plants with altered BR should be tested in field under various abiotic stress either individually or in combination of stresses to unravel potential of BRs in enhancing crop productivity under various combination of abiotic stresses.

In the recent past with the advancement of BRs research, many debatable questions need to be answered by the researcher such as (i) how BR levels affected in plant tissues and organs under various abiotic stress conditions? (ii) to dissect out the mechanism behind the interplay of BRs with other signalling pathways that influences the plant growth, development and metabolism under stress and stress-free conditions. In the near future significant research related to these questions would progress our knowledge about the BRs mediated regulation of plant growth and development under stressful environments and also help to deduce the traits related to the stress-related defense pathways.

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Chapter 12
Brassinosteroids: The Promising Plant Growth Regulators in Horticulture

Barket Ali

Abstract Brassinosteroids (BS), a class of polyhydroxylated steroidal plant hormones were collectively named as 'Brassins' after their initial discovery from the pollen grains of *Brassica napus*. They occur in whole plant kingdom and almost all plant parts. Pollen and immature seeds are the richest sources of BS. A spectrum of physiological, biochemical and molecular responses in plants have been attributed to BS, which include shoot and root growth, fertility and seed germination, cell elongation, vascular differentiation, xylem formation in epicotyls, and also in the regulation of expression of several genes involved in xylem development. They also affect cotyledon growth, root elongation, leaf formation and growth, and plant biomass. Ethylene production is another important physiological response in plant that has been attributed to BS activity. They have also been found to protect plants from various abiotic and biotic stress factors, such as salt, temperature, water, heavy metals and pathogens. BS also enhance the yield of several cereals, legumes, oilseed crops and crops of horticultural importance. In horticultural crops, they favour fruit production and quality of the fruits. This chapter describes various studies wherein BS have been exploited to enhance the productivity of different horticultural crops. Most importantly, they are naturally occurring and eco-friendly, thus they can easily replace the hazardous chemicals.

Keywords Brassinolide · Brassinosteroids · 24-Epibrassinolide · Ethylene · Flowering · Fruits quality · 28-Homobrassinolide · Horticulture

1 Introduction

Brassinosteroids (BS), a recently recognized new class of plant hormones (Clouse and Sasse 1998; Khripach et al. 1999) is also called "polyhydroxylated steroidal plant hormone" (Fariduddin et al. 2014) and a new and unique class of plant growth regulators (Sirhindi 2013). Their occurrence was first noted in the pollen grains of

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Brassica napus. They were subsequently called "Brassinosteroids", although they were initially named as "Brassins". They occur in whole plant kingdom as well as all plant parts. However, their quantity varies from one part to another. Their quantity is higher in young growing tissues than mature tissues. Highest levels (1–100 μg kg^{-1} fresh tissue) are found in pollen and immature seeds, whereas shoots and leaves usually, possess lower amounts i.e. 0.01–0.1 μg kg^{-1} (fresh tissue).

Initially they were found associated with reproductive development of a plant (Clouse and Sasse 1998). However, later research broadened their role in a wide spectrum of growth and developmental events. The developmental processes affected by BS include cell division and cell elongation in stems and roots, photomorphogenesis, reproductive development, leaf senescence, and also in stress responses (Ali et al. 2007; Sirhindi 2013; Fariduddin et al. 2014). Their essentiality in normal growth and development was proved beyond doubt by some eminent worker such as Clouse and Sasse (1998) and Sasse (2003). The essentiality of BS in plant growth and development has been proved in different studies wherein "brassinazole" an inhibitor of BS biosynthesis has been used. The other processes influenced by BS include shoot and root growth, fertility and seed germination, cell elongation, vascular differentiation, xylem formation in epicotyls, and also in the regulation of expression of several genes involved in xylem development (Clouse and Sasse 1998; Taiz and Zeiger 2004). They also affect cotyledon growth, root elongation, leaf formation and growth, and plant biomass. Exogenous application of BS also improves the activities of different enzymes such as carbonic anhydrase, nitrate reductase (Ali et al. 2006; Alam et al. 2007), rubisco (Yu et al. 2004) and those involved in Calvin cycle (Fedina et al. 2008). In addition to this, BS have a great potential to confer resistance to plants against various biotic and abiotic stresses, such as salinity (Ali et al. 2007), water stress (Vardhini and Rao 2002), temperature extremes (Sirhindi 2013), and heavy metals (Hayat et al. 2007; Ali et al. 2008a, b; Yusuf et al. 2012). Besides these key roles, BS have also been found to affect whole physiology of the plant, starting from seed germination to harvest or seed maturation. Application of BS has been found to enhance the seed germination in chickpea (Ali et al. 2005), Indian mutard (Sirhindi 2013) and tobacco (Lubner-Metzger 2001). Furthermore, the exogenous application of BS has been found to enhance the yield of a number of crop plants such as *Brassica juncea*, *Arachis hypogeal, Vigna radiata* (Vardhini and Rao 2002), *Lycopersicon esculentum* (Ali et al. 2006) and *Cicer arietinum* (Ali et al. 2007), both under stress and stress free conditions. However, few treatments have been performed in the field, under real growing condition. Most of the studies have been conducted with plants grown under controlled environmental conditions in the laboratory. Many BS and BS-analogues that showed high biological activity in bioassays or controlled-environment experiments failed to stimulate plants grown under field conditions (Hola et al. 2010). This can be explained by various reasons such as the timing of BS application (Nunez et al. 2003), duration of exposure and the BS treatment, frequency of BS treatment and the dose, type and mode of BS can also substantially affect the growth/yield promoting activity of these compounds (Hola et al. 2010). However, more accurate studies on dosage, mode and time of application, fit

brassinosteroid suitability for the plant or cultivar, and association with other phytohormones are needed.

BS increase crop yield and show anti-stress effects on several plants at very low doses. Besides this, they are easily metabolized (Adam and Schneider 1999; Schneider 2002) and are also eco-friendly (Kang and Guo 2011) with a huge potential of increasing agricultural and horticultural productivity. In order to make them cost-effective many types of BS analogues have been prepared (Zullo and Adam 2002). The analogues include BB6 and MH5, DI-31 (BB16) and DI-100.

The phenomenon of plant growth, development and productivity is determined both by exogenous and endogenous factors. Phytohormones play very a critical role among the endogenous factors. Therefore, they are extensively exploited in order to improve crop performance/yield (Montoya et al. 2005). Although, it is well established that BS have a beneficial effect on the growth and productivity of many agricultural and horticultural crops. However, these are very costly and cannot be afford by the farmers of developing countries. To make them cost effective, some commercial analogues of many BS have been synthesised and are used in many countries.

2 Occurrence

Brassinosteroid (BS) analogues, brassinolide (BL) and castasterone (CS) occur in whole plant kingdom. The brassinosteroids has been isolated and characterised almost from every plant part, which includes pollen grains, flower buds, fruits, seeds, vascular cambium, leaves, shoots and roots. They occur both in free as well as conjugated form (specifically with sugars and fatty acids). Sixty-nine BS analogues have been isolated from different plants/parts so far (Bajguz and Tretyn 2003). They also occur in galls of *Castanea crenata, Distylium racemosum* and *Catharanthus roseus*. Their quantity is higher in young growing tissues than mature tissues. Highest levels (range of 1–100 μg kg^{-1} fresh tissue) are found in pollen and immature seeds, whereas shoots and leaves usually possess lower amounts of BS i.e. 0.01–0.1 μg kg^{-1} (fresh tissue). The group wise number of plants which possess at least one BS include 53 angiosperms (12 monocotyledons and 41 dicotyledons), 6 gymnosperms, 1 pteridophyte (*Equisetum arvense*), 1 bryophyte (*Marchantia polymorpha*) and 3 algae (*Chlorella vulgaris, Cystoseira myrica and Hydrodictyon reticulatum*) (Bajguz and Tretyn 2003).

3 Structure

Plant sterols are converted to BL via teasterone, typhasterol and castasterone, are synthesised by an isoprenoid biosynthetic pathway, including acetyl CoA, mevalonate, isopentenyl pyrophosphate, geranyl pyrophosphate and farnesyl

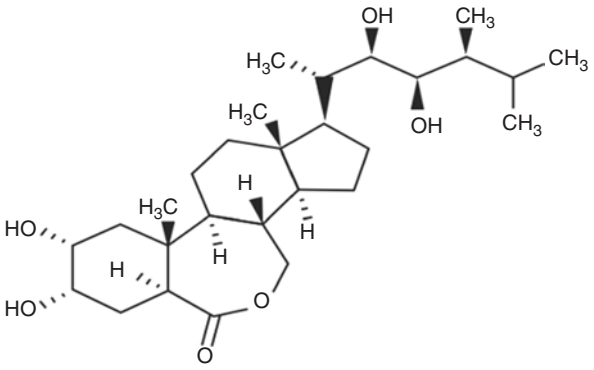

Fig. 12.1 Structure of Brassinolide (BL)

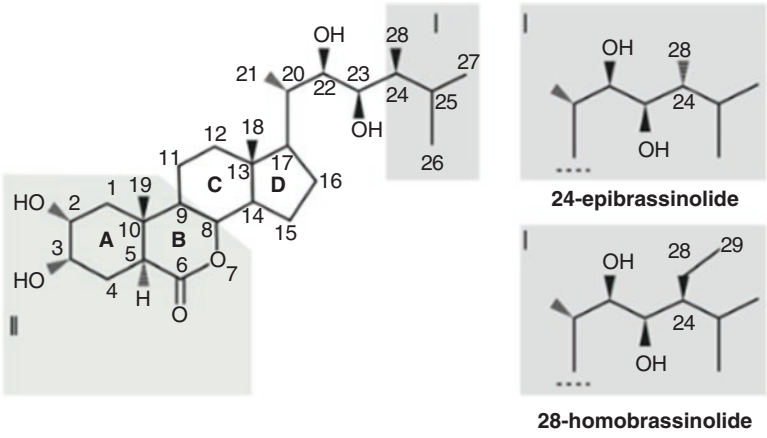

Fig. 12.2 Sturcture of Steroid, 24-Epibrassinolide (EBL) and 28-Homobrassinolide (HBL)

pyrophosphate (Clouse and Sasse 1998; Symons et al. 2008). Brassinosteroids are polyhydroxy steroid lactone with the structure of brassinolide (BS) (Fig. 12.1) and the structure of steroids (Fig. 12.2) having the same carbon skeleton of animal steroids as cholestane, ergostane, and stigmastane. However, the chemists and plant physiologists used an approach in which the most active and first identified representative of this class of compounds, i.e., brassinolide (BL), is taken as the basic structure of the system. A great diversity in the basic structure at cyclic and side chain is found which is responsible for important metabolic transformations to form two other highly active analogues of BS namely 24-Epibrassinolide (EBL) and 28-Homobrassinolide (HBL) (Fig. 12.2). Furthermore, BS are nontoxic (Esposito et al. 2011) and environmental friendly hormones (Kang and Guo 2011).

4 Outline Biosynthesis Pathway (Fig. 12.3)

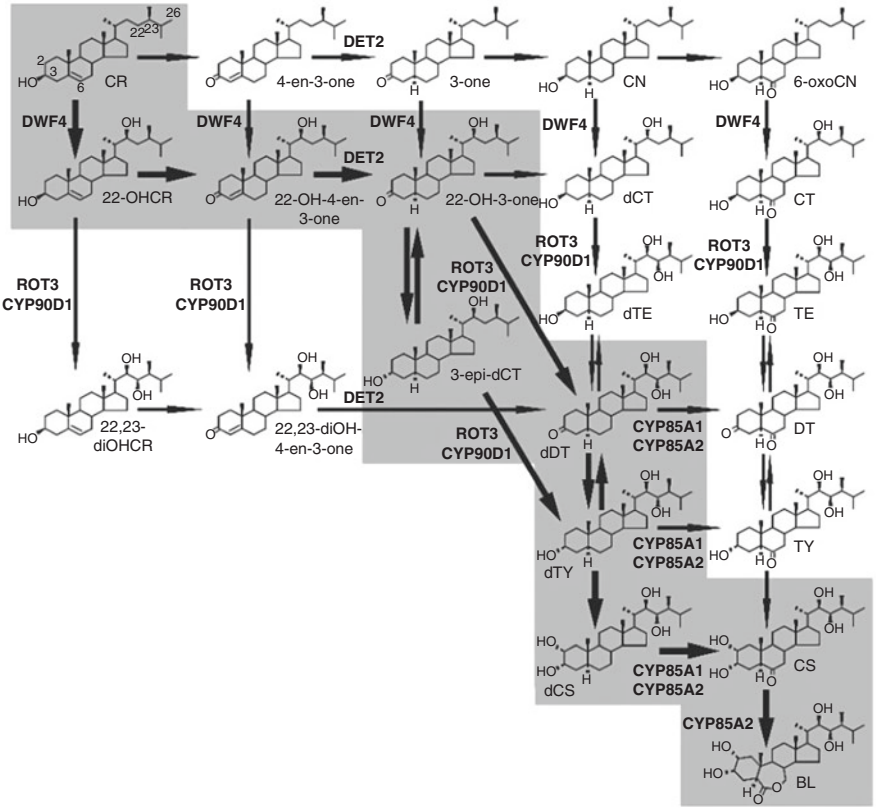

Fig. 12.3 The schematic pathways of BS biosynthesis. Arrows correspond to conversion steps, fat arrows denote reactions which, based on enzymological data, constitute the main synthesis routes (highlighted by gray background). The numbering of the important substituted carbon atoms is shown in the structure of campesterol (CR). Other steroid compounds are: 22-hydroxycampesterol (22-OHCR), 22,23-dihydroxycampesterol (22,23-diOHCR), (22S,24R)-hydroxyergost-4-en-3-one (4-en-3-one), (22S,24R)-22-hydroxyergost-4-en-3-one (22-OH-4-en-3-one), (22S,24R)-22,23-dihydroxyergost-4-en-3-one (22,23-diOH-4-en-3-one), (22S,24R)-hydroxyergost-3-one (3-one), (22S,24R)-22-hydroxyergost-3-one (22-OH-3-one), 3-epi-6-deoxocathasterone (3-epi-dCT), campestanol (CN), 6-oxocampestanol (6-oxoCN), 6-deoxocathasterone (dCT), cathasterone (CT), 6-deoxoteasterone (dTE), teasterone (TE), 3-dehydro-6-deoxoteasterone (dDT), 3-dehydroteasterone (DT), 6-deoxotyphasterol (dTY), typhasterol (TY), 6-deoxocastasterone (dCS), castasterone (CS), brassinolide (BL). The Arabidopsis enzymes with in vitro confirmed functions are the C-22 hydroxylase DWARF 4 (DWF4)/CYP90B1 (At3g50660), the C-23 hydroxylases ROTUNDIFOLIA 3 (ROT3)/CYP90C1 (At4g36380) and CYP90D1 (At3g13730), the steroid 5α-reductase DE-ETIOLATED 2 (DET2; At2g38050), the C-6 oxidase CYP85A1 (At5g38970), and the C-6-oxidase, BL synthase CYP85A2 (At3g30180). (Figure adopted from Hategan et al. 2010)

5 Brassinosteroid Signalling (Fig. 12.4)

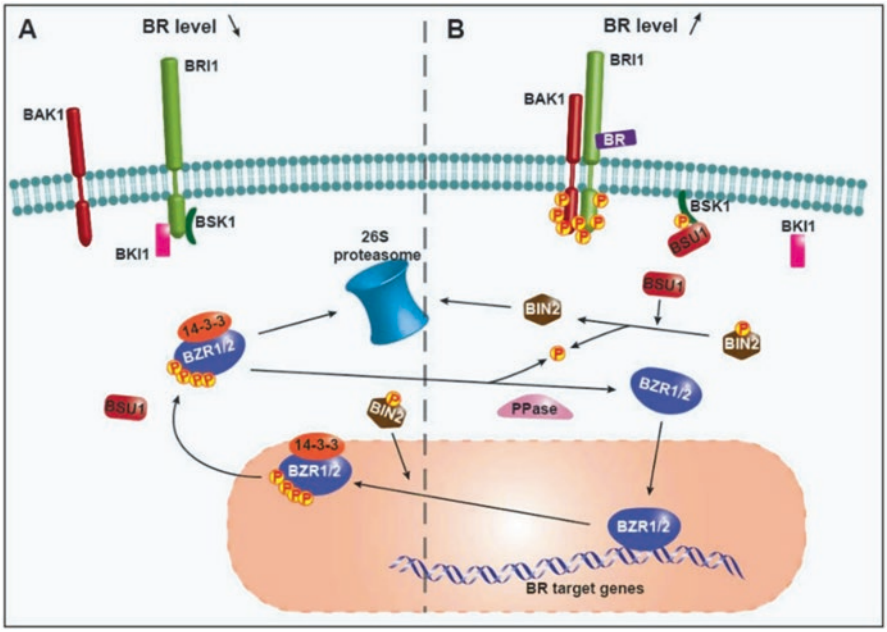

Fig. 12.4 The BR (=BS) signal transduction pathway in Arabidopsis. (**a**) Inactive BR pathway. In the absence of BRs, BRI1 is inactive and associates with BKI1. The BRI1-bound BSK1 and BSU1 are inactive, and consequently BIN2 is active. BIN2 phosphorylates BZR1 and BZR2/BES1, which cannot bind DNA and are retained in the cytoplasm by the 14-3-3 proteins to finally be degraded by the proteasome. (**b**) Active BR pathway. In the presence of BRs, BRI1 is activated through dissociation of BKI1 andoligomerization/transphosphorylation with BAK1. Activated BRI1 phosphorylates BSK1, which activates BSU1. The activated BSU1 inhibits BIN2 through dephosphorylation and, hence, BZR1 and BZR2/BES1 are dephosphorylated, possibly with the help of an unknown phosphatase (PPase). The unphosphorylated BZR1and BZR2 accumulate in the nucleus and regulate the BR responses. (Picture adapted from Tang et al. 2010)

6 Application of BS in Horticulture

Horticultural plants are the garden crops such as fruits, nuts, vegetables, culinary herbs and spices, beverage crops and medicinal as well as ornamental plants. The edible horticultural crops are used entirely as human food and are often used in the living state, are highly processed, are often used as animal feed and usually contain high percentage of dry matter. Show great diversity with respect to flower or fruit colour, shape and value. This is well established fact that the production of horticultural plants requires intense management, high management cost, environmental control, significant technology use and high risk. Different ways

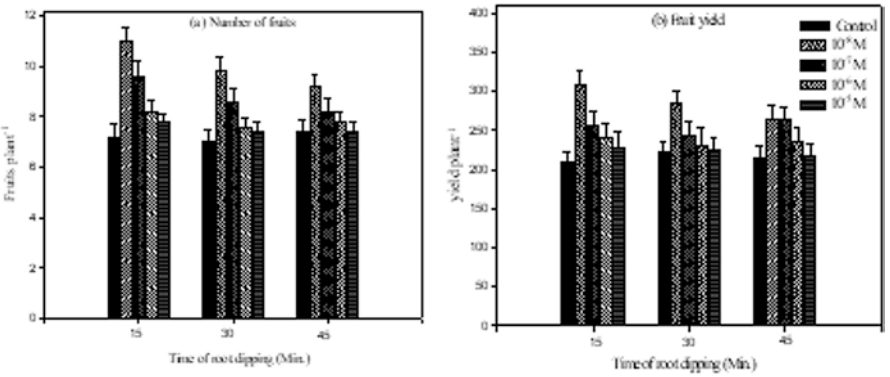

Fig. 12.5 Effect HBL on the number of fruits (**a**) and fruit yield (**b**) in *Lycopersicon esculentum* Mill. (Ali et al. 2006)

such as cultivation of high yielding cultivars/hybrids, fertilizers, pesticides, insecticides and other chemicals have been exploited to enhance the productivity of the horticultural crops to feed the ever increasing human population. However, repeated and excessive use of these chemicals deteriorates the environment and the ecosystem. On the other hand, application of the plant growth regulators in general and BS in particular are highly beneficial for plant productivity and eco-friendly too.

6.1 Effects on Tomato

Brassinosteroids have been applied to tomato plants at different stages and through different modes such as pre-sowing seed soaking, root dipping and foliar spray. Pre-sowing seed soaking treatment of tomato for 4 h in 1 ppm solution enhanced the yield of tomato plants under greenhouse conditions (Takematsu and Izumi 1985). The application of 22,23,24-triepibrassinolide and 28-homobrassinolide increased tomato fruit setting by 43–111%, whereas in response to 28-homobrassinolide, this increase was of the magnitude of 118–129% (Mori et al. 1986). Likewise, tomato sprayed with EBL exhibited an increase of 10–18% in their fruit yield (Savelieva et al. 1997). In some other studies, the treatment of tomato plants with BS, at flowering stage led to the enhancement of the number and weight of tomato (Balmush et al. 1995). The highest crop enhancement, in field conditions was obtained when tomato and cucumber plants were treated with EBL twice, first the seed soaking followed by spraying at flowering stage (Churikova and Derevshchukov 1997). Supplementation of tomato plantlets through root dipping, at the time of transplantation, with varied concentrations of HBL for 15, 30 and 45 min caused an increase in the number, size and weight of the fruits (Fig. 12.5a, b) and the

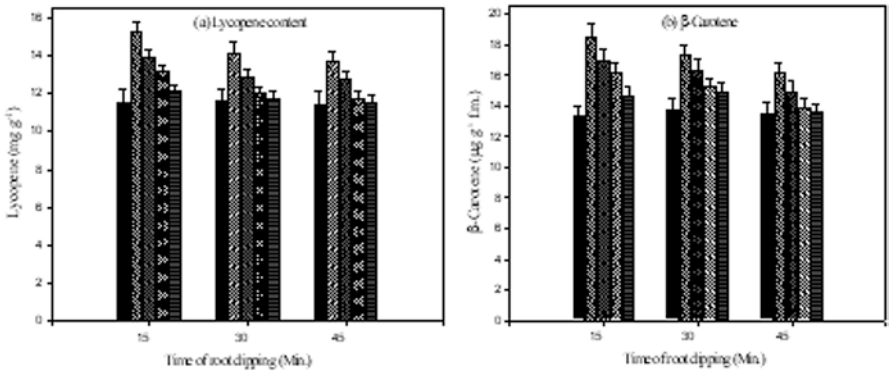

Fig. 12.6 Effect HBL on the lycopene content (**a**) and β-carotene content (**b**) of ripe fruits of *Lycopersicon esculentum* Mill. (Ali et al. 2006)

improvement in the quality characters like lycopene and β-carotene content were also reported (Fig. 12.6a, b; Ali et al. 2006).

Vardhini and Rao (2002) also observed that BS application increased the lycopene and carbohydrate levels and ethylene production, whereas the level of chlorophyll and ascorbic acid decreased, which was consistent with accelerated ripening, mediated by the ethylene production. In a different study, Montoya et al. (2005) also found that biosynthesis of BS was enhanced in the developing fruits of tomato.

The expression of different genes is also altered by BS application. These genes include golden 2-like (LeGLK2), phytoene synthase 1 (LePSY1), ripening-related ACC synthase 2 (LeACS2), ripening-related ACC synthase 4 (LeACS4), 1-aminocyclopropane-1-carboxylate oxidase 1 (LeACO1) and 1-aminocyclopropane-1-carboxylate oxidase 4 (LeACO4) involved in lycopene and ascorbic acid biosynthesis which showed a declining trend. Moreover, the expression of LeACS2, LeACS4, LeACO1, LeACO4 and LePSY1 was increased by a followup treatment with brassinolide treatment, while the expression of LeGLK2 was reduced. However, fruit treated with brassinazole showed the opposite effects, where tomato fruit ripening was delayed. These findings suggest that brassinosteroids are involved in the development of fruit quality attributes and ethylene-mediated fruit ripening of tomato. These authors concluded that postharvest application of brassinolide significantly promoted lycopene synthesis but suppressed chlorophyll synthesis via regulating transcript levels of LePSY1 and LeGLK2. Moreover, ethylene production was obviously increased by brassinolide treatment through inducing the expression of ethylene biosynthesis related genes, including LeACS2, LeACS4, LeACO1 and LeACO4. This effect of brassinosteroids might be due to the promotion of ethylene synthesis to some extent, which contributed to LePSY1 and LeGLK2 changing (Lisso et al. 2006; Zhu et al. 2010; Liu et al. 2014). The application of BS also reduce the electrolyte leakage and malanaldehyde content and enhance phenol

and proline content, thereby preventing the fruit damage caused by oxidative stress, thus enhance the shelf life of the fruits (Aghdam et al. 2012).

6.2 Effects on Pepper, Spinach, Sugarbeet and Cabbage

24-epibrassinolide (10^{-6} M) application at different stages (vegetative, buds formation and early fruiting) improved flower number, fruit number and yield per plant, but was without affecting fruit mass and size. The response was proportionate to the growth stage of the plant and the application frequency of the hormone (Samira et al. 2012). Similarly, other BS analogues such as s DI-31 and DI-100 at the rate of 4, 8 and 12 ppm concentration together with a seaweed extract and amino acid mixture called Tomex Amin (2.5 l/ha) also enhanced the pepper quality, such as fresh weight, height/diameter (h/d) ratio, lobe number/fruit, firmness, colour and ripening index. Moreover, antioxidant activity and phenolic content was higher in pepper treated plants than control (Serna et al. 2012).

Epibrassinolide (EBL, 10^{-2} ppm) treatment of spinach for 8 h enhanced its germination from 54% to 72% (Ikekawa and Akutsu 1987) and crop yield of cabbage (Asatova 1991). Genma (1987) observed 18% increase in the yield of sugarbeet by BS treatment and Vedeneev et al. (1995) observed an increase of 26–33%. In a different study, Kurganskii (1993) observed an increase of 10–13% in crop yield and the sugar content in sugarbeet under stress free condition and Schilling et al. (1991) observed 8% increase under stress conditions, in response to BS treatment.

6.3 Effects on Potato

Treating potato tubers with EBL solution induced/prolonged their dormancy and inhibited sprouting, by increasing production of ethylene and ABA (Korableva et al. 2002). Genma (1987) also observed that 0.3 g ha^{-1} BS application enhanced the tuber fraction by 24%. Similarly, Savelieva et al. (1997) also observed an increase in the size of potato tubers in response to BS application. In a different study, spraying the potato plants with BL (10^{-2}–10^{-4} ppm) three times, at the interval of 1 week increased the mean tuber weight from 100 to 145 g mediated by changes in abscisic acid and ethylene level in the treated tubers (Korableva et al. 1998). It was noted that brassinolide promoted potato tuber development, inhibited its germination during storage and increased resistance to infections by *Phytophthora infestans* and *Fusarium sulfureum* (Kazakova et al. 1991).

6.4 *Effects on Cucumber*

Horticultural crop productivity largely depends on the number of the female flowers and successful pollination. EBL treatment of cucumber plants increased female flower production, mediated by BS-induced ethylene production. Comparing the response of cucumber, melon and zucchini to the exogenous treatment of BS, cucumber was more sensitive than zucchini, which was reflected as reduction in the number of male flowers in the initial phase of development and promoting the initiation of the female flower in the main shoot (Papadopoulou and Grumet 2005). BS also play an important role during early fruit development which was demonstrated by using cucumber cultivars with different parthenocarpic capacities (Fu et al. 2008). BS triggered active cell division together with increased transcripts of cell cycle-related genes, especially that of cyclin D3 genes. These results strongly suggest that BS play an import role during early fruit development in cucumber (Fu et al. 2008).

6.5 *Effects on Watermelon, Strawberry, Cranberry, Gooseberry, Apple, Cherry, Citrus and Peach*

Productivity of a fruit crop is greatly influenced by fruit setting. EBL treatment of melon enhanced the fruit yield by 10–20% (Ikekawa and Nagai 1987; Wang et al. 1994). The improved yield was mediated by an increase in the fruit setting, number of flowers and delayed senescence. Khripach et al. (1999) also attributed the BS mediated improvement in the quality and yield of strawberries, cranberry, gooseberry, apple, cherry, citrus and peach the increase in the fruit set, prevention of the premature fall of young fruits, delayed senescence and other factors involved in the fruit yield and quality. Moreover, molecular biotechnology has also proved the involvement of BS in the strawberry fruit ripening (Bombarely et al. 2010). Chai et al. (2013) also explained its possible mechanism of action. They analysed BS content and BS receptor gene FaBRI1 expression during ‘Akihime’ strawberry fruit development. It was found that BS levels increased during the later developmental stages, and the mRNA expression levels of FaBRI1increased rapidly from white to initial red stages, suggesting that BS is associated with fruit ripening. This was further confirmed by exogenous application of BS and its inhibitor brassinazole (BZ) to big-green fruit, which significantly promoted and inhibited strawberry fruit ripening, respectively. More importantly, down-regulation of FaBRI1 expression in de-greening fruit markedly retarded strawberry red-colouring.

6.6 Effects on Grape, Berry and Mango

There are intriguing evidences which suggest that increase in endogenous BS levels are associated with ripening in grapes. Exogenous application of EBL to grape berries, significantly promoted their ripening, while brassinazole (Brz), an inhibitor of BS biosynthesis, significantly delayed fruit ripening (Symons et al. 2006; Lisso et al. 2006). A significant increase in endogenous BS levels in grapes stimulates BS receptor gene brassinosteroid insensitive 1 expression that was consistent with observed at the onset of fruit ripening. Symons et al. (2006) also observed an increase in the expression of BS biosynthesis enzyme gene, brassinosteroid-6-oxidase demonstrating that BRs are involved in grape berry ripening.

Zaharah et al. (2012) demonstrated that the exogenous application of EBL promoted fruit ripening in mango. There was a marked accumulation of BS analogues, castasterone and brassinolide. However, the castasterone level was slightly higher than that of BL, on day 8 of the study (0.13 ng g^{-1} FW). Moreover, the exogenous application of EBL treatments (45 and 60 ng g^{-1} FW) significantly advanced the onset of the climacteric peak of ethylene production and respiration rate by 2 and 1 day(s), respectively. Both of these treatments also had a higher climacteric ethylene production peak (4.81 and 5.74 nmol $C_2H_4 kg^{-1}$ h^{-1}) and respiration rate (4.87 and 5.06 mmol $CO_2 kg^{-1}$ h^{-1}) compared with the control. Furthermore, the exogenous applications of EBL also promoted fruit softening, particularly between days 3 and 7 of the ripening.

6.7 Effects on Passion Fruit

The application of BB-16, a BS analogue 3 weeks after flowering, increased in the estimated yield of the passion (*Passiflora edulis flavicarpa*) fruit by 65%. The yield parameters were number of fruits $plant^{-1}$ and the mean mass of each fruit, corresponding to an estimated production of 20.1 t ha^{-1}, compared to that of the control (12.6 tons ha^{-1}). The BS analogue was considered more efficient when applied for three consecutive weeks after the appearance of the first flower due to the great increase in yield and soluble solids contents in passion fruit (Gomes et al. 2006).

6.8 Effects on Orange

Brassinolide (BL) treatment of orange trees during flowering increased their fruit setting. However, when applied during fruit growth it decreased the physiological drop of fruits, causing an increased number of fruits per plant, accompanied by an increase in the average fruit weight. BS treatment also enhanced juice production in

Citrus unshiu together with a higher brix/acidity ratio (Kuraishi et al. 1991) and also prevented fruit abscission in *Citrus madurensis* Lour. (Iwahori et al. 1990).

6.9 Effects on Litchi, Passiflora and Jujube Fruits

BS treatment improved fruit yield and quality in litchi in terms of increased the activities of pectin methyesterase and polygalacturonase and the content of water-soluble pectin, protopectin and calcium in the fruit pericarp, and reduced fruit cracking rate thereby increasing the commercial value of the fruit (Peng et al. 2004). Likewise, BS treatment also reduced postharvest decay caused by *Penicillium expansum* in jujube fruit. Besides this, BS application also delayed fruit senescence thereby increasing the life span of the fruit.

6.10 Effects on Tea and Coffee

Foliar spray of summer tea plants with 24-epibrassinolide (EBL), a bioactive BS analogue, promoted photosynthesis in a concentration-dependent manner. EBL also increased concentrations of tea polyphenols and amino acids. Furthermore, concentrations of catechins and theanine increased, while that of caffeine remained unaltered following treatment with the BS. EBL also improved activity of phenylalanine ammonia-lyase (PAL) and glutamine: 2-oxoglutarate aminotransferase (GOGAT) enzymes involved in catechins and theanine biosynthesis, respectively. These favourable metabolic changes consequently improved the quality of summer tea (Li et al. 2016). Mazzafera and Zullo (1990) demonstrated that EBL or 24-epicastasterone treatment of coffee showed no significant effect on seed setting, seed size or yield. However, *Coffea stenophyllacalli* grew up to 237% between 60 and 130 days of culturing in the presence of 24-epibrassinolide (Ramos et al. 1987).

6.11 Reproductive Growth

Horticultural crop productivity primarily depends upon the successful pollination and subsequent fertilization. Relatively higher levels of BS in pollen and seed reflect a critical role of BS in reproduction. BS also influence branching and flower formation via modulating metabolic pathways and relative nutrient allocation or interacting with other signalling pathways. BS also affect fertilization via the stimulation of filament and pollen growth, and modify pollen properties (Mussig 2005). Cuttings grown in a nutrient medium containing BL, EBL and HBL analogues of BS increased the yield of cuttings suitable for planting by 25–50% depending on the cultivar and finally increased crop yield up to 50% (Bobrick 1995).

6.12 Effects on Flowering

Flower formation, survival and maturation of the flower, pollination and the subsequent fruit setting and maturation are the main factors that determine the fruit productivity of a horticultural crop. Application of BB-6 and BB-16 (BS analogues) to the foliage of the *Cactus* pear hastened vegetative buds formation both under greenhouse and field conditions (Aristeo-Cortes et al. 2003). However, a concentration and the method of BS application dependent decrease in the number of flowers was observed in *Pharbitis nil* in response to BL and castasterone (CS) treatment where 1 and 10 μM of BL caused a complete inhibition of flower formation (Kesy et al. 2003). Contrary to this, BS treatment increased the number of flowers in strawberry and grape fruits (Vardhini and Rao 2002).

6.13 Effects on Micropropagation of Horticultural Plants

Potential of regulation of growth and development in plants by BS has also been efficiently utilised in the micropropagation of various plants of horticultural importance such as cassava (*Manihot esculenta* Crantz), yam (*Dioscorea alata* L.) and pineapple (*Ananas comosus* L. Merril). BS analogues, 28-homocastasterone or 3β-acetyl-28-homoteasterone have been used successfully for this purpose (Bieberach et al. 2000). 5α-fluoro-28-homocastasterone (5F-HCTS), another BS analogue facilitated the apple rootstock multiplication rate up to 112% mediated by an increase in the number of primary and secondary lateral branches (Schaefer et al. 2002). 5F-HCTS also stimulated branch elongation in *in vitro*-grown shoots of *Malus prunifolia* that was mediated by the manipulation of endogenous BS levels (Pereira-Netto et al. 2006; Kang and Guo 2011). BS in culture medium also stimulated adventitious bud formation in cauliflower and coconut. In coconut, plumule explants efficiently formed initial callus, embryogenic callus and somatic embryos in presence of BS in culture medium (Azpeitia et al. 2003).

7 Conclusion and Future Prospects

1. Brassinosteroids is a group of naturally occurring steroidal plant hormones which is represented by several analogues. Out of them, the stable ones are epibrassinolide, homobrassinolide and brassinolide. Besides their stability, they are also non-toxic and eco-friendly.
2. Their exogenous application can enhance the productivity of tomato, potato, mango, straw berry, litchi, passiflora, grapes, watermelon etc. However, the studies are very less and the field is very vast which has remained almost untouched. Keeping in view, the great potential of BS, they can be exploited in the enhancement of vegetables productivity.

3. Since BS are also known for their role in protection of plants from different stress situations including biotic stress such as the attack of different pathogens. Therefore, it can easily and efficiently replace different pesticides and fungicides, which otherwise have health hazards and also degrade environment.
4. Micropropagation is an unconventional method of plant propagation wherein a large number of plantlets are generated from a small explant. BS have been exploited in a limited number of studies. This field is still open and potential of BS can also be exploited to fulfill the food requirements of increasing population reducing both time and labour.

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Chapter 13
A Current Scenario on Role of Brassinosteroids in Plant Defense Triggered in Response to Biotic Challenges

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Abstract Various biotic stresses induced by microbes/pathogens affect growth, yield and production in plants. Plants sequester a broad spectrum of receptor armory to instigate innate immune approaches which are unbeatable by pathogens. Several phytohormones, interact in multifaceted interconnected signaling networks. Recent studies have elucidated direct or indirect regulation of plant defense responses by phytohormones. Brassinosteroids (BRs), a growth-promoting hormone is also an imperative plant defense regulator. They have been recently observed as a modulator of plant defense response to pathogen attack. They enhance plants resistance to a wide array of plant diseases. BRs increase the efficacy of Pathogen Assisted Molecular Patterns (PAMP) triggered immunity. They also mediate crosstalk between different defense-signaling cascades including phytohormones signaling, DELLA proteins, Pattern-Recognition Receptors Triggered Innate Immunity (PTI) and plant pathogen interaction. Furthermore, BRs also regulate sulfur metabolism and production of nitric oxide and consequently affect plants immune responses.

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1 Introduction

Phytohormones regulate plant defense in addition to routine growth and development in plants (Denance et al. 2013; De Vleesschauwer et al. 2013). Among plant hormones, salicylic acid (SA), jasmonic acid (JA), gibberellins (GA), auxins, brassinosteroids (BRs) are known to show modulatory effects upon growth and immunity (Huot et al. 2014). BRs are the primary regulator of plant growth and they play pivotal roles in almost all the stages of plant life i.e. seed germination, vegetative growth and reproductive development (Zhu et al. 2013; Lozano-Duran and Zipfel 2015). BRs are also recognized as stress managers. Steroidal hormones are prerequisite for stressed plant tissues, as they transduce quicker response than other phytohormones. Due to their lipophilic nature, they pass through membranes directly and act at nuclear level (Marcinkowska and Wiedlocha 2002). Current advancement in understanding of underlying molecular contrivance has led to identification and characterization of BRs induced signalling cascades (Li 2003). Their participation is imperative at the time of biotic challenges caused by fungal, bacterial and viral infestation in plants (Jager et al. 2008). Various plants extracts containing BRs and commercially accessible BR-analogues have been proven to provide resistance against biotic stresses (Friebe 2006; Jaillais et al. 2011; Shi et al. 2013). The basic defense and disease resistance alluded by BRs includes enhanced expression of pathogenesis related proteins, BAK 1 and BKK 1 regulators, antioxidative defense components and mitogen activated proteins.

Plants have efficiently developed multiple signaling mechanisms to balance between growth and immunity within them (Vert and Chory 2011). One such mechanism is pathogen-associated molecular patterns (PAMPs) present on the surface of plant cells. The binding of PAMPs to the cell surface leads to activation of plant-recognition receptors (PRRs). This consequently results in initiation of PRR-triggered immunity (PTI) (Boller and Felix 2009). The crosstalk among BR signalling and innate immunity along with phytohormones under stressed conditions have been postulated a long time ago (Choudhary et al. 2012). BRs can interact with number of hormones such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), gibberellins, auxins etc. and course of their action depends upon the conditions and regulatory mechanisms of plants which adapt to balance the defense mechanisms under different stresses (De Vleesschauwer et al. 2012). Also, BRs are involved in plant response to pathogen attack. They have positive or negative impact on plant resistance to various pathogens (Sahni et al. 2016; Deng et al. 2016). Furthermore, exogenous and endogenous levels of BRs induce alteration in sulfur and nitric oxide metabolism.

In this book chapter, we elucidate current advancement in understanding the role of BRs in plant defense, with specific focus on BR signaling under biotic stress, basic defense alluded by BRs, role of PAMP-triggered immunity and BRs interplay with other defense signaling cascades.

2 BRS Signaling Under Biotic Stress

Molecular and genetic studies carried out in the recent past employing *Arabidopsis thaliana* and rice plants have led to identification of various genes encoding BR biosynthetic pathway and gene regulation (De Bruyne et al. 2014). BRs is recognized in plant cells by BRI1 (BR-insensitive 1) and further competes with immune response for BAK1 (membrane receptor) whereas BR can also stimulate immune response in a BAK1-independent manner (Shiu and Bleecker 2001; He et al. 2007; Segonzac and Zipfel 2011). BR signal transduction may trigger the transcript level of gene encoding nicotinamide adenine dinucleotide phosphate oxidase to mediate generation of H_2O_2 and regulation of oxidative stress tolerance responses. Active BR signaling may also enhance nitric oxide generation, which stimulates the biosynthesis of abscisic acid consequently leading to enhanced tolerance to stress. BZR1 and BES1 transcription factors play an imperative role in BR-mediated responses against disease caused by pathogens in plants. BES1 binds to AtMYB30, an important defense regulator and together they are associated with inducing BR target gene expression (Mora-García et al. 2004). BR-regulated stress tolerance responses in Arabidopsis shows interplay with ethylene, ABA and SA. BZR1 was reported to directly regulate genes, associated with various phytohormones signaling or biosynthetic pathways (Achard et al. 2006). Activated form of BRI1 commences a cascade of phosphorylation steps of its downstream BR signaling kinases, plasma membrane bound receptor like cytoplasmic kinases and constitutive differential growth 1 to mediate signals from membrane receptors to cytoplasmic regulators (Tang et al. 2008; Kim et al. 2011; Sreeramulu et al. 2013). Mass-spectrometric studies revealed that BRI1 phosphorylates constitutive differential growth 1 at Ser-234 and BR signaling Kinase 1 at Ser-230 and consequently activate and phosphorylate BRI1-suppressor 1 (BSU1). It has been observed that BSU1 might be activated by BRI1 either by phosphorylates constitutive differential growth 1 or BR signaling kinase 1. Furthermore, BR signaling kinase 1 stimulated activation requires BRI1 kinase activity while constitutive differential growth 1 stimulated activation does not require BRI1 but is increased by BRI1. Either of one set of interplay partners, BSU1-constitutive differential growth 1 or BSU-BR signaling kinase 1 is the minimal set of components for transferring signal from receptor kinase BRI1 to the GSK3-like kinase BIN2 (Kim et al. 2011). BSU1 dephosphorylates BIN2 to suppress its function and alleviate inhibitory effect on two major transcription factors such as BZR1 and BZR2 also recognized as BRI1-EMS suppressor 1 (BES1) involved in BR signaling pathway (Kim and Wang 2010). BZR1 and BES1 are dephosphorylated through protein phosphate 2A and liberated from 14-3-3 proteins which lead to their nuclear localization to bind with promoter region of their respective target genes to mediate their gene expression (Tang et al. 2011; Yu et al. 2011; Wang 2012b).

3 Basic Defense and Disease Resistance Alluded by BRS

Apart from essential role in growth and physiological processes, BRs are also involved in plant defense responses against biotic stress (Fig. 13.1). Treatment of 24-epibrassinolide (EBL) declined the damage of *Fusarium* head blight disease caused by pathogen *Fusarium culmorum* in barley (Ali et al. 2013). Application of BR increased resistance against *Tobacco mosaic virus*, *Oidium* spp. and *Pseudomonas syrinage* pv. tabaci in tobacco. It also provided protection in rice against *Xanthomonas oryzae* pv. *oryzae* and *Magnaporthe grisea* (Nakashita et al. 2003). A study conducted by Roth et al. (2000), reported that application of BR to aqueous extracts from seeds of *Lychnis viscaria* in range of 0.5–10 mg L^{-1} increased the resistance of cucumber, tobacco and tomato against fungal and viral pathogens in comparison to water treated control seeds. Stimulation of plant defense responses was associated with induction of pathogenesis-related proteins, which are molecular markers of systemic acquired resistance (Roth et al. 2000). Expression of pathogenesis related proteins (PR1, PR2 and PR5) was found to be declined in BR biosynthetic *Arabidopsis* mutant (*cpd*). The expression of *CPD* was overexpressed in transgenic plants, there was significant stimulation of these pathogenesis related proteins (Szekeres et al. 1996). In *Arabidopsis thaliana*, BAK1 and BKK1 (positive regulators in BR signaling) are the important constituents which reduce Turnip crinkle virus infection (Yang et al. 2010). A study conducted by Zhang et al. (2015), reported that BR treatment enhanced the activity of antioxidative enzymes, modulated the expression of defense related genes and decreased photosystem damage in cucumber mosaic virus (CMV) infected *Arabidopsis thaliana* plants. Application of BRs enhanced tolerance against Tobacco mosaic virus by regulating MEK2 (MAPKK)-SIPK (salicyclic induced protein kinase) and respiratory burst oxidase homolog protein B. BES1/BZR1 repressed respiratory burst oxidase homolog protein B- dependent Reactive Oxygen Species (ROS) production and act as a crucial regulator in BR signal transduction between growth and immune responses. If active form of BES1/BZR1 (positive regulator of BR signaling) is in small quantity, the respiratory burst homolog protein B-dependent ROS production is mediated by MEK2-SIPK cascade which may further provide tolerance against Tobacco mosaic virus. The active form of BES1/BZR1 (high concentration) increased BR signaling may suppress respiratory burst oxidase homolog protein B dependent ROS production by BES1/BZR1 and induce plant growth (Deng et al. 2016). Treatment of 28-homobrassinolide reduced the oxidative stress caused by nematodes and boosting the tolerance capacity of tomato plants by improving growth and activity of antioxidative defense system (Kaur et al. 2013). Application of EBL at low concentrations stimulated susceptibility in the roots while high concentrations of EBL induced systemic defense against nematode stress (Nahar et al. 2013).

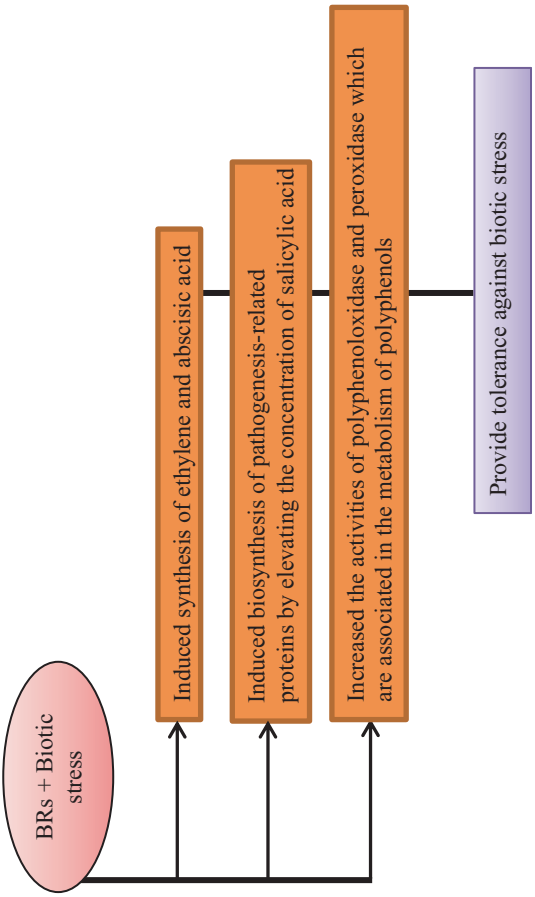

Fig. 13.1 BR induced biotic stress tolerance in plants

4 BRS Regulates PAMP-Triggered Immunity

The initial response of plants to stress depends upon the recognition of PAMPs on the surface of cell via PRRs. After binding of PAMPs, activation of PRRs occurs that further initialize signaling cascade and leads to the PRR-triggered immunity (PTI) (Boller and Felix 2009).

The molecular mechanisms of underlying role of BRs in regulating growth and immunity can be studied indirectly through series of transcription factors. Alteration in the expression of transcription factors BRASSINAZOLE-RESISTANT 1 (*BZR1*) and bHLH HOMOLOG OF BR ENHANCED EXPRESSION2 INTERACTING WITH INCREASED LEAF INCLINATION1 BINDING bHLH1 (*HBI1*) isolated from *Arabidopsis thaliana* plants is a new evidence of crosstalk among BRs and PAMP-triggered immunity that represses immunity under BR recognition (Lozano-Duran and Zipfel 2015). *BZR1* and *HBI1* together forms a regulatory network which regulates effective communication between growth and immunity. Taking this into consideration, it was postulated by Lozano- Duran and Zipfel (2015) that BR leads to enhance plant's immunity via BRI1-ASSOCIATED RECEPTOR KINASE 1 (*BAK1*) activation. It was also suggested that exogenously applied BRs have no effect upon PTI in *Arabidopsis thaliana* but effectively alters LRR-RK FLAGELLIN SENSING 2 (FLS2)-mediated immune signaling (Albrecht et al. 2012; Belkhadir et al. 2012). Furthermore, BR-mediated inhibition occurs downstream and independent of *BAK1* expression (Albrecht et al. 2012). Apart from this, null *bak1* mutants expressing $bak1^{elg}$ allele up-regulated BR signalling but is unable to provide resistance to *Pseudomonas syringae* pv. *tomato (Pto)* DC3000 upon application of flagellin epitope *flg22* (bacterial flagellin) (Jaillais et al. 2011). The generalised model representing the interactions among BRs and PTI signalling in *Arabidopsis* and their impact upon growth and immunity has been explained in Fig. 13.2.

Also, mutation in BAK1 (*bak1-5)* did not have any negative effect upon BR signaling, thereby compromised PTI signaling by providing susceptibility to virulant *Pto* DC3000 mutant deficient in phytotoxin coronatine production takes place (Schwessinger et al. 2011). This shows that BAK1 pathway is regulated by interactions among receptor and co-receptor and differential phosphorylation (Jaillais et al. 2011; Schwessinger et al. 2011). At the same time, it was found that BRs can act antagonistically as well as synergistically towards PTI responses in *Arabidopsis thaliana*.

This suggested that excess or loss of BR content as well as enhanced BR signalling stimulated by BRI1 over expression and impaired BAK1-controlled PTI, induces innate immunity. On the contrary, plants over expressing BRI^{sud1} allele, stimulated flg22- induced PTI signalling by BAK1 determinants (Belkhadir et al. 2012). In spite of these, triggered PTI-responses, BRI^{sud1} plants exhibited susceptibility against *Hyaloperonospora arabidopsidis*, a biotrophic pathogen, along with the enhanced resistance in *bak1* mutant through altered BR signaling. Therefore, it suggests that BAK1 not only acts as the controller of synergistic activities but also their role in plant defense is independent of BAK1 (Belkhadir et al. 2012).

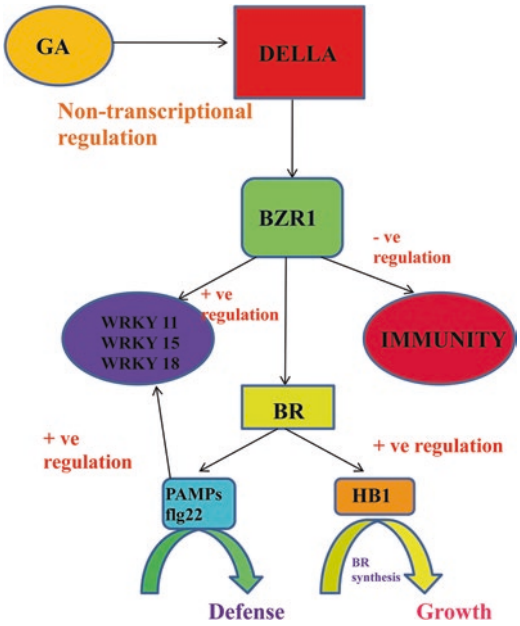

Fig. 13.2 Schematic representation of BR and PTI signalling in *Arabidopsis* during immune responses (Abbreviations: *GA* gibberellin, *DELLA* DELLA (Asp-Glu-Leu-Leu-Ala) domain repressor proteins, *BZR1* BRASSINAZOLERESISTANT 1, *WRKY* WRKY (Trp-Arg-Lys-Tyr) domain transcription factor, *HBI1* bHLH HOMOLOG OF BR ENHANCED EXPRESSION2 INTERACTING WITH INCREASED LEAF INCLINATION1 BINDING bHLH, *PAMPs* pathogen-associated molecular patterns, *flg22* bacterial flagellin)

Furthermore, other studies suggested that *BZR1* is essential transcription factor for PTI antagonism and inhibition of PAMP-induced signaling. It activates *WRKY* genes, which negatively regulates PAMP-controlled ROS production and gene expression independently of MAPK signalling. They also recommended a model explaining the role of *BZR1* and *WRKY40* in down regulation of defence- related genes (Lozano-Duran et al. 2013).

Studies have been found that FLAGELLIN SENSING2 (FLS2), an immune receptor in *Arabidopsis* plants forms a complex which leads to the activation of (PAMP)-mediated immunity (PTI) upon flagellin (bacterial protein) recognition. In flagellin signaling, normally with FLS-BIK1 complex dynamics, BR1 undergoes association along with BIK1 and is removed from BR1 receptor upon BR treatment. On the other hand, BAK1- controlled FLS2–BIK1 dissociation; BAK1 is non-essential for the dissociation of BRI1- BIK1. Furthermore, in case of FLS2 signalling, is totally dependent upon BAK1 to phosphorylate BIK1, BRI1 phosphorylates BIK1 directly in BR signaling. Consequently, BIK1 mediates signaling in plant immunity and BR- triggered growth through phosphorylating BAK1 and BRI1.

Another factor, i.e. cytoplasmic kinase BRASSINOSTEROID-SIGNALING KINASE1 (BSK1) along with FLS2 is crucial for PTI activation. It was analysed

through whole-genome phosphorylation that Mitogen-Activated Protein Kinase Kinase Kinase5 (MAPKKK5) acts as a primary substrate of BSK1 and further phosphorytates MAPKKK5 (Yan et al. 2018). Additionally, it was determined that wild type bsk1-1 mutant, did not lead to phosphorylation of Ser-289 residue of MAPKKK5 because of its wild nature. Similarly, MAPKKK5 mutant showed increased susceptibility to avirulent and virulent strains of *Pseudomonas syringae* and *Golovinomyces cichoracearum* in *Arabidopsis* plants. The phosphorylation of the Ser-289 is important for MAPKKK5- controlled resilience to these pathogens but play no role in MAPKKK5- mediated cell death. Altogether, it was concluded that BSK1 not only regulates plant immunity through phosphorylation of MAPKKK5, but plays a direct role in the signaling from immune complexes to MAPK pathway (Yan et al. 2018). Moreover, Shi et al. (2013) explained the role of BR1-related signaling kinase BSK1, where BR1 acts as substrate and positively regulates flg22-mediated ROS production and SA accumulation. However, BSK1 inhibition enhanced the susceptibility to virulent as well as avirulent pathogens. Therefore, negative BR-PTI leads to up-regulation of BIN2 as a result, competition among BR1 and FLS2 signaling leads to negative effect of BR on flg22 independently of BSK1 (Shi et al. 2013). Recently, another factor i.e. BIK1 (Botrytis-induced kinase 1) is known to regulate BR signalling negatively and FLS2-PTI positively where both their functions are mechanistically uncoupled (Lin et al. 2013).

FLS2 promotes hetero-dimerization among FLS2 and BAK1 along with trans-phosphorylation and activation of other domains (Schulze et al. 2010). This phenomenon undergoes in response to uncoupling of receptor like- cytoplasmic kinase (RLCK) BIK1 from FLS2 and BAK1 that initiates phosphorylation during signal transduction (Zhang et al. 2010; Kadota et al. 2014). For instance, membrane-bound NADPH oxidase RBOHD (respiratory burst oxidase homologue D) leads to the production of ROS after phosphorylation (Li et al. 2014). It also positively modulates calcium dependent protein kinases, which constitute the most important branch of signalling receptor complex that controls flg22- mediated transcriptional changes (Tena et al. 2011; Boudsocq et al. 2010).

5 BRS Interplay with PTI Signaling Cascades

The associations among BR and defense signaling (PTI signaling) was found to be unidirectional via initiation of BR signaling, stimulated either by hormonal treatment (exogenously) or by genetically over-expressing BRI1 or DWARF4 (BR biosynthetic enzyme) that impeded PTI responses in *Arabidopsis thaliana* plants (Albrecht et al. 2012; Belkhadir et al. 2012). Although, the studies supported negative interactions also, the molecular mechanisms involving BR-mediated alteration of immunity were particularly related to the participation of BAK1 (Wang et al. 2012a). To study the interactions among BR and PTI, supplementation of BR or flg22 was done to wild *Arabidopsis* plants alone as well as in combinations. It was

found that both these treatments did not affected BR responses and reduced the flg22 response, as a result of negative crosstalk (Albrecht et al. 2012). It was further revealed through immune-precipitation experiments that BR-dependent suppression of PTI was unrelated to BAK1 and concluded that BAK1 is not rate limiting step in these pathways. Along with this, BR application showed no effect upon phosphorylation of BIK1 and flg22-mediated FLS2, which indicated that the crosstalk occurs downstream to the receptor complex. In addition to this, BR also altered the fungal PAMP chitin induced responses, independently of BAK1 but the same was response enhanced on flg22 in *bak1-4* mutant (Ranf et al. 2011; Albrecht et al. 2012).

Moreover, BRs increased resistance to pathogens independently of SA but antagonise SA-induced defenses to *P. graminicola,* a root pathogen of rice (Nakashita et al. 2003; De Vleesschauwer et al. 2012). Additionally, various SA-marker genes are up-regulated upon (brassinolide) BL application in *Arabidopsis* raising the perspective of synergistic relationship between BR and SA (Divi et al. 2010). Furthermore, BRs show negative interactions with JA by modulating growth in *Arabidopsis* and impairing JA-induced resistance to *P. graminicola* (root knot nematode) in rice (Choudhary et al. 2012; Nahar et al. 2013). They speculated the inhibitory action of BR biosynthesis through qRT-PCR upon exogenous application of BL. Further, they reported that 12-oxo-phytodienoic acid the precursor of jasmonic acid gets accumulated in BR signaling pathways that acts antagonistically within rice roots demonstrating their role in effective *M. graminicola*- rice interactions (Nahar et al. 2013).

Moreover, BAK-1 has also been known to be involved in providing resistance against herbivory attack by *Manduca sexta* in *Nicotiana attenuata*. It was observed that *NaBAK1* gene expression was modulated in these plants in response to oral secretions of *Manduca sexta.* On silencing NaBAK1 gene in plants exposed to larval oral secretions (LOS), they showed higher levels of JA and isoleucine and SA, independent of MAPK activity. This upon application of JA in *NaBAK1* gene -silenced plants stimulated the levels of trypsin proteinase inhibitors, reduced *NaTD* transcription and up regulated *NaJAR4* and *NaJAR6* expression. Therefore, they induced the resistance in *Nicotiana attenuata* towards *Manduca sexta* through accumulation of JA as well as secondary metabolites (Yang et al. 2013). BRs also get associated with auxins in plant immune responses through SA/JA signaling or by BR-auxin network which contributes towards BRs in disease resistance (Choudhary et al. 2012; Pieterse et al. 2012). Studies have also been reported in which BRs interact with ET (ethylene) for providing defence responses against different biotic stresses (Bar et al. 2010). It was observed that Eix proteins (LeEix1 and LeEix2) are predominantly involved in providing defence responses in tomato plants by binding to BAK1 (Bar et al. 2010). In addition to this, BRs also interact with GAs. For instance, BRs diminish immune responses in rice infected by *P. graminicola* (root knot nematode) by hampering GA metabolism at multiple levels *OsGSR1*is a DELLA GA-signalling repressor which provides resistance to *P. graminicola* and it is also up-regulated under BR exposure. It suggests that BR- GA antagonism relationship stabilises DELLA proteins and GA signalling inhibitor (SLR1) in rice.

It occurs at biosynthetic level during signal transduction when BR inhibits GA biosynthesis and GA repressor genes (De Vleesshauwer et al. 2012). It was also suggested that BR- GA crosstalk is mediated by physical interaction between BZR1 (transcription factor) and DELLA proteins leads to GA suppression. Further research have supported the existence of crosstalk among SA and BRs that play pivotal role in providing resistance against *P. graminicola* in *Oryza sativa.* Supplementation of BRz (inhibitor of BR synthesis) reduced the susceptibility against *P. graminicola* (De Vleesshauwer et al. 2012). This indicates the role of BRs in negative control for providing immunity against different pathogens and exhibit the pathogen induced steroid homeostasis.

5.1 BRs, Gibberellins and DELLA Proteins

BRs and GA are plant growth regulators which interact with each other (Fridman and Savaldi-Goldstein 2013) and control host defense response against pathogen attack. BRs play significant role in growth and development by regulating immune system functions. They act as regulator of plant immunity with radiating outcomes (Hao et al. 2013). Exogenous application of BRs reported systemic as well as local defense response from various leaf pathogens in rice and tobacco (Nakashita et al. 2003). Similarly, GA is terpenoidal hormone which acts as key regulator of plant development during stress conditions (Colebrook et al. 2014). It has been observed that exogenous application of Gibberellic acid (GA_3) boosts photosynthetic machinery by enhancing Chl b content in bean plants. Similarly, significant enhancement in Chl a, b and total chlorophyll content has been recorded in lupin plant (Sharaf et al. 2009). During stress conditions, GA level increases with increase in ROS production while exogenous application of H_2O_2 not only increase the intracellular H_2O_2 but also enhances GA level (Liu et al. 2018).

BRs and GAs have overlapping functions and BRs are considered as master regulators of GA synthesis. Their signalling pathways interplay with each other at the transcriptional level through BZR1, BES1 (BRI1-EMS-SUPPRESSOR 1) and DELLA proteins. DELLA proteins inhibit the cell growth and enlargement. Hence, plant growth is promoted by inhibiting DELLA proteins through 26S proteosomal destruction. DELLA proteins inhibit transcription of BZR 1 (Fig. 13.3). Phytohormones like GA, auxin and ethylene promote DELLA inhibition (Alvey and Harberd 2005). On application of BRs, De Vleesschauwer et al. (2012) reported repression in root immunity to *Pythium graminicola* which leads to proliferation of infection and make it more susceptible. Phythium species itself lack machinery for sterol biosynthesis. They explain that *P. graminicola* hijacks the plant BR biosynthesis and signaling pathway as a boosting strategy to infest in host which clearly indicates *P. graminicola* strategy of hijacking plants BR machinery to support their growth and reproduction and also alteration of SA and GA defenses.

An investigation done by Tong et al. (2014) in rice plant, indicated role of BRs regulating the biosynthesis of GAs. GA bind to its receptor GIBBERELLIN

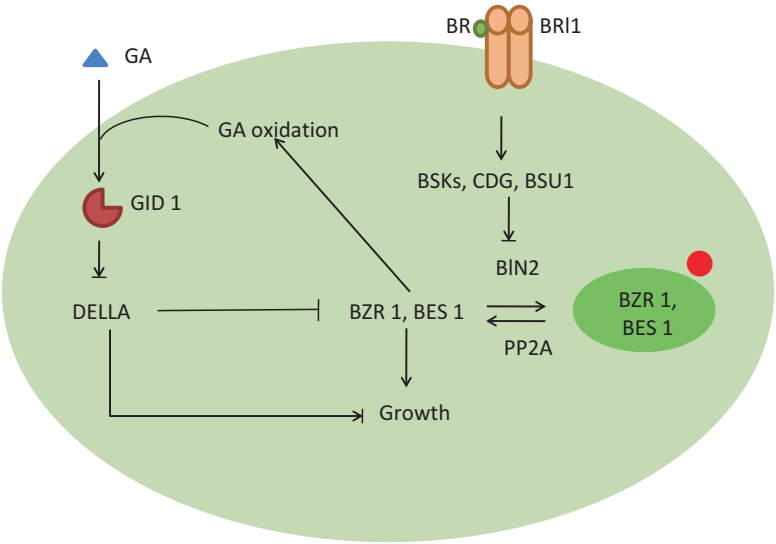

Fig. 13.3 Signalling model of BR-GA interaction (Abbreviations: *BRI1* is a Brassinosteroid Insensitive1, *BSKs* Brassinosteroid-Signalling Kinases, *CDG* Constitutive Differential Growth, *BSU1* Bri1-Suppressor1, *BZR1* Brassinazole Resistant 1, *BES1* BRI1-EMS-Suppressor 1, *PP2A* Protein Phosphatase 2A, *BIN2* Brassinosteroid Insensitive2, *GID1* Gibberellin Insensitive Dwarf1)

INSENSITIVE DWARF1 (GID1) resulting in activation of N terminal of the receptor to act as sticky agent and form trimeric complex by interacting with SLENDER RICE 1 (SLR1, a DELLA protein) which functions as negative regulator in resistance to *P. graminicola*. The key requirement of this complex is to degrade SLR1 which is targeted by polyubiquitination. Degradation of SLR1 is done by 26S proteosomal degradation in rice plant (Gao et al. 2011). DELLAs act as negative regulator of GA resulting in decrease in plant growth and development (Xu et al. 2014) whereas DELLAs protein activity is inhibited by GA in order to promote plant development (Sun 2010) (Fig. 13.4).

Therefore, GA induces the degradation of DELLAs which are nuclear proteins and growth inhibitors. BR-GA interaction shows DELLA proteins as negative regulator of GA signaling degraded by bioactive GA which then interacts with BZR1 leading to reduction in plant growth response (Ross and Quittenden 2016). In Arabidopsis, four DELLA-genes were studied and were found to be targets of BZR1 factor in BR signalling, which shows BR may control GA level by controlling DELLA synthesizing genes (Sun 2010). It was observed by Li et al. (2012) that DELLA genes were not altered by either BR application or by the *bzr1-1D* mutation that increase BR signaling. They also suggested role of BZR1 and DELLAs in communicating and coordinating BRs and GAs interaction in plant growth through protein-protein interaction.

A study conducted by Unterholzner et al. (2015) on BR mutated *Arabidopsis thaliana* shows regain in phenotypic characters of plant on exogeneous application

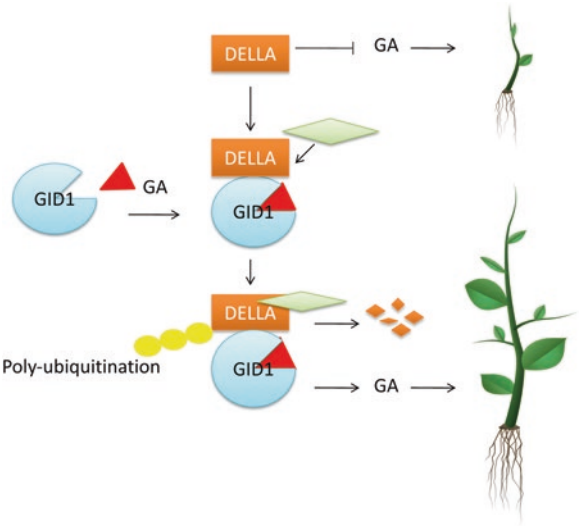

Fig. 13.4 DELLA as a regulator of GA responses in plants

of GA or by enhancing the expression of biosynthetic genes of GA. Similar result was found by Tong et al. (2014) in rice plant. GA synthesis theory is supported by both Tong et al. (2014) and Unterholzner et al. (2015) by altering BR levels. Change in level of BRs and GA has been recorded by Stewart Lilley et al. (2013). In BR mutants, GA wasn't able to regain the growth of plant during various developmental period of plant (Tong et al. 2014). Mutated rice plant with increased BR level showed five time increase in bioactive GA_1 with reduction in GA_{20} in comparison to wild type. Moreover, Oikawa et al. (2004) reported two to three times increase in GA_1 enhances plant growth. But other studies conducted by Jager et al. (2005) in pea and Kurepin et al. (2012) in sunflower suggested increase in level of GA_{20} and constant GA_1 in reduced BR levels or hindered signaling. The above studies showed significant effect of different levels of BR on GA but not with a constant pattern which clearly indicates unpredictable effect in different species.

5.2 Role of BRs in Plant Pathogen Interactions

A positive but variable effect of most active BR, i.e. BL on disease resistance are reported in tobacco and rice to different leaf pathogens (Nakashita et al. 2003). Brassinoloide (BL) activates resistance in plants against various pathogens. In tobacco plants it provide resistance against a viral pathogen i.e. *Tobacco mosaic virus*, the bacterial pathogen *Pseudomonas syringae* and a fungal pathogen i.e. *Oidium* sp. In rice plants, it provides resistance against *Magnaporthe grisea* and *Xanthomonas oryzae*, which are causative agents of rice blast and bacterial blight, respectively. In potato tuber tissues, BRs induce susceptibility by stimulating the mycelial growth and intensity of spore formation of *Phytophthora infestans.* It was

observed that the immune status of plant tissues was weakened for at least 4 months after the treatment of whole potato tubers with BR (Vasyukova et al. 1994). Moreover, it was recently investigated that exogenous application of BR also induces susceptibility to *Pythium graminicola* in rice roots. It was reported that the BR pathway suppressed plant innate immunity against *P. graminicola* through its antagonistic interaction with the SA and GA pathway (De Vleesschauwer et al. 2012). The role of BR in modulating interaction between rice and the root-knot nematode (RKN) *Meloidogyne graminicola* is also reported. BL pre-treatment renders rice hyper susceptible to the root pathogens *Meloidogyne graminicola* and act as potential negative regulator of plant immunity. The mechanism by which BR induce susceptibility or resistance against pathogens is dependent on the hormone concentration and timing and involves the activation or suppression of other hormone pathways (Nahar et al. 2013). Application of low concentrations of BR-containing (24-epicastasterone and 24-episecasterone) extract of *Lychnis viscaria* seeds resulted in increased resistance of tobacco to tobacco mosaic virus, cucumber to *Sphaerotheca fuliginea* and tomato to *Botrytis*. (Roth et al. 2000). Furthermore, BL enhanced resistance to the bacterial pathogen, *Pseudomonas syringae pv*. *tabaci* (*Pst*), and the fungal pathogen, *Oidium* sp. in tobacco plant.

Application of EBL (5–15 mg ha^{-1}) to barley plants significantly decreased the extent of leaf diseases induced by mixed fungal infection and also resulted in an increase in crop yield (Bajguz and Hayat 2009). It was also studied that *N. benthamiana* plants treated with brassinolide showed increased resistance against infection of TMV (Deng et al. 2016). Similar, application of EBL to heads of 'Lux' barley resulted in reduced severity of *Fusarium* head blight (FHB) caused by *Fusarium culmorum* by 86% and reduced the FHB-associated loss in grain weight by 33% (Ali et al. 2013). BRs are also effective against blue mould rot caused by *Penicillium expansum* on harvested jujube fruit. Five micrograms concentration of BRs inhibited growth of blue mould rot and improved the activities of defense-related enzymes, such as phenylalanine ammonia-lyase, polyphenoloxidase, catalase and superoxide dismutase (Zhu et al. 2016). It was suggested using the proteomic and virus-induced gene silencing methods that BRs signaling play essential roles in the cotton disease resistance to *Verticillium dahliae*. Researchers found that exogenous application of brassinolide on cotton enhanced disease resistance (Gao et al. 2013). Brassinosteroids are also reported to increase resistance against necrotrophic fungal pathogens *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* in transgenic *Brassica napus* plants (Sahni et al. 2016). Uzu barley lines have a mutation in a highly conserved residue (His-857 to Arg-857) in the kinase domain of the BR receptor protein BRI1. It was reported that introgression of the uzu mutation into barley showed improved resistance against many pathogens. Enhanced resistance was reported against leaf blast disease, take-all of roots, eyespot disease of stems and crown rot disease of the stem which is caused by *Magnaporthe grisea*, *Gaeumannomyces graminis var. tritici*, *Oculimacula* spp. and *Fusarium* fungi respectively (Goddard et al. 2014; Chen et al. 2014). Uzu derivatives of barley cvs. Akashinriki and Bowman were found to be more resistant to the obligate pathogen Barley Stripe Mosaic Virus (BSMV), the necrotrophic net blotch pathogen

Pyrenophora teres and the toxigenic hemibiotrophic fungus *Fusarium culmorum* that causes Fusarium head blight (FHB, also known as scab disease of cereals) (Ali et al. 2014).

EBL was also effective against stress induced by *Verticillium dahliae* toxin in cotton callus. Treatment of cotton callus with EBL ameliorated the effects of *Verticillium dahliae* toxin and resulted in increased contents of chl a and b, carotenoids, total phenols, flavonoids, soluble sugars, and proteins and increased the activity of enzymes involved in secondary metabolism like polyphenol oxidase (PPO), phenylalanine ammonialyase (PAL), cinnamyl alchol dehydrogenase (CAD), and shikimate dehydrogenase. Also, Citrus Huanglongbing (HLB), also known as citrus greening, disease of citrus is caused by the fastidious gram negative α-proteobacteria *Candidatus* Liberibacter asiaticus. The bacterium propagates within the phloem of citrus plants producing die-back, yellow shoots, blotchy mottles on leaves and off-tasting and malformed fruit (Bove 2006). Foliar spray of EBL to citrus plants which were infected with *Candidatus* Liberibacter asiaticus resulted in reduced bacterial titres (Canales et al. 2016). The role of BRs in *Cucumber mosaic virus* (CMV) tolerance in *Arabidopsis thaliana* leaves was investigated by pretreating the plant with BL and water. The results revealed that accumulation level of CMV was less and detectable earlier in BL-pretreated plants as compared with water pretreated plants (Zhang et al. 2014). Similarly, it was found that the application of EBL to rice resulted in *Rice black-streaked dwarf virus* (RBSDV) infection. However, application of a mixture of methyl jasmonate (MeJA) and BL resulted in a significant reduction in RBSDV infection as compared with a single BL treatment (He et al. 2017).

5.3 BRs and Sulfur Metabolism

Sulfur is the important element for all organisms because of its role in various processes. It is an essential constituent of proteins and active component of many coenzymes and prosthetic groups (Kopriva et al. 2015). The uptake and assimilation of sulfur plays an important function in controlling growth and development of plants and is an essential component of various essential compounds that decipher growth and vigor of plants under favorable and stressed conditions (Nazar et al. 2014). Glutathione is a sulfur containing compound consisting of three amino acids and is considered as a key non-protein thiol in various organisms. Glutathione has multiple roles to play in plant system but mainly induces redox homeostasis buffering. Grade of glutahione is altered by oxidants as well as nutritional and other factors that control the structure and activity of proteins by alterations in thiol-disulfide equilibrium. Due to these facts, glutathione is believed to be transducer that combines environmental information into the cellular network (Noctor et al. 2011).

Jiang et al. (2012) studied the effects of BR-caused enhancement in CO_2 assimilation in *Cucumis sativus* and reported that exogenous application of BRs enhanced the ratio of reduced to oxidized glutathione (GSH:GSSG). These findings indicated

that BR-mediated photosynthesis requires hydrogen peroxide induced enhancement in the GSH:GSSG ratio, which may control the synthesis and activation of redox-sensitive enzymes in carbon fixation. Many genes with stress-linked roles were identified and these include thiol oxidoreductase genes such as glutathione-S-transferases (GSTs) and glutaredoxins (GRX) which present reducing power to a array of stress associated enzymes (Zagorchev et al. 2013). Similarly, Kaur et al. (2014), reported that activity of glutathione reductase (GR) was reduced with nematode inoculation and supplementation with 28-homobrassinolide, which enhanced their activity, whereas glutathione peroxidase (GPOD) activity was enhanced with both nematode inoculation and treatment of 28-homobrassinolide. The regulation of ROS linked genes encoding glutathione-S-transferace emphasized on the association of BRs and ROS. BR stimulates ROS production to activate the defense gene expression, cell wall stability and programmed cell death to reduce the extent of pathogen infestation. The recognition of various cell wall associated genes directly linked to BRs role in alteration of cell wall, a strategy which is being used to correlate with enhanced plant stress tolerance (Sahni et al. 2016).

5.4 BRs and Nitric Oxide

Studies carried out by Xia et al. (2009) and Cui et al. (2011) also observed positive interaction among BRs, H_2O_2 and NO, and all these molecules act as important cell signaling components under stress conditions. On the basis of this fact, Deng et al. (2016) proposed a hypothesis that H_2O_2 and NO are involved in providing systemic virus resistance to plants, mediated by BRs. Moreover, H_2O_2 has also been observed to act upstream of NO, which is involved in the modulation of stomatal function by BRs (Shi et al. 2015). They suggest that BRs regulate ethylene biosynthetic pathway and cause activation of G_α subunit, and then promote the production of H_2O_2, leads to the generation of NO in leaves and ultimately cause closing of stomata. In addition to BR-regulated growth of plants, BRI1 also have crucial involvement in the BR-regulated H_2O_2 and NO generation which ultimately provides virus resistance to plants (Deng et al. 2016). In *Lycopersicon esculentum,* a possible crosstalk between NO and BRs was observed which resulted in the regulation of antioxidative defense system as well as photosynthetic performance (Hayat et al. 2010). BRs are capable of inducing plant resistance against salt stress through NO signaling pathway (Zhu et al. 2016). It may be due to the regulation of the alternative oxidase enzyme (AOX) by BRs as well as NO. It is also essential for the BR-induced AOX activity and plays an important role in increasing the antioxidative potential of plants under stress conditions (Zhu et al. 2016). Crosstalk between BRs and NO is also involved in the activation of plant's immune system in response to virus infection (Zou et al. 2018).

BRs on their exogenous application cause a significant accumulation of nitric oxide (NO) in leaves of tea. This NO generation plays an important role in the BR-mediated biosynthesis of anthocyanins. Moreover, NO also alters the activity of

enzyme PAL, which ultimately plays a crucial role in the secondary metabolism mediated by BRs (Li et al. 2017). BR-mediated generation of NO in plants also regulates the biosynthesis of ABA under abiotic stress, indicating a possible cross-talk between BRs and NO (Choudhary et al. 2012; Ahmad et al. 2018). Biosynthesis of NO mediated by BRs, is also known to play an important role in the development of roots as well as maintaining their architecture (Tossi et al. 2013). In the mesophyll cells of maize plants, BRs are reported to enhance the NO concentration. The enhanced NO levels resulted in activation of the biosynthetic pathway of ABA, which plays an important role in BR-mediated enhanced stress tolerance in maize plants (Zhang et al. 2010). BR-mediated generation of H_2O_2 is also involved in the production of NO and it was supported by the fact that scavenging of H_2O_2 resulted in the reduction/blockage of BR-mediated NO production (Deng et al. 2016). Moreover, these researchers also suggested that BRI1 (BR insensitive 1) is also involved in this BR- H_2O_2-NO interaction.

6 Conclusion

Biotic challenges in combination to other adverse environmental cues resulted in progressive deleterious impact on plant growth and development. Priming of stressed plants with low doses of exogenous protective agents accelerates the defense capabilities and thereby increases tolerance to intensified direct biotic stresses. Plethora of previous studies of plants under in-vivo and in-vitro conditions have suggested significant participation of BRs in activation of plant defense mechanisms. Current advancement in the understanding of molecular and signal transduction has revealed interplay between phytohormones and other signaling networks in inducing enhanced tolerance. Accumulating research evidence also indicate involvement of PTI cascade and modulation in sulfur and NO metabolism in regulation and integration of plant defense responses. In conclusion, although notable development has been made over recent years to understand immunity enhancing role of BRs, still further elucidation of molecular contrivance via BRs interaction with other defense cascades will enhance our knowledge of balanced plant defense responses and development of novel approaches for eco-friendly and durable disease tolerance enhancement under varied agronomic settings.

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Chapter 14
Anticancer Potential of Brassinosteroids

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Abstract In recent years, it was shown that brassinosteroids (BS) exert their effects not only on plants but also on animals and man. Eventually, some of these effects allowed considering BS as potential anticarcinogenic agents. The background for searching new chemotherapeutic agents among representatives of this group of phytohormones is their antiproliferative activity shown on a number of cancer cell lines, their ability to inhibit angiogenesis, and low cytotoxicity to normal cells. A higher efficiency was found for some synthetic derivatives of BS. It is believed that BS are involved in the regulation of the cell cycle and induce apoptosis through the pathways independent of androgenic and estrogenic receptors. However, in general, the molecular mechanism of action of BS remains largely unclear. Nowadays, BS and their anticarcinogenic properties are actively studied in many laboratories worldwide. The purpose of this review is to analyze and summarize the data obtained on the topic by our research group. Among various aspects of BS anticancer activity, their cytotoxicity in cancer cells, participation of reactive oxygen species in BS-mediated death of tumor cells, and BS inhibition of procarcinogen activation will be discussed in more detail.

Keywords Brassinosteroids · Cancer Cell Viability · Intracellular ROS Level · Procarcinogen Activation

1 Introduction

Despite significant advances in oncology, the number of annual deaths reaches eight million and tends to increase (Torre et al. 2015). The absolute majority of known antitumor drugs have pronounced side effects, low specificity, and can cause the development of drug resistance (Remesh 2012; Housman et al. 2014). It highlights the need to search for new chemotherapeutic agents, as well as establishing the mechanism of their action.

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For a long time, plants have been used as a source of medicines. More than 50% of known compounds with antitumor activity are of natural origin or synthetic derivatives of natural compounds (Ouyang et al. 2014; Cragg and Newman 2005). Of particular interest in this respect are brassinosteroids (BS). This group of steroidal phytohormones plays an important role in the regulation of growth, development and homeostasis of plants (Khripach et al. 1999; Sakurai et al. 1999; Hayat and Ahmad 2003; Hayat and Ahmad 2011; Pereira-Netto 2012). Comprehensive biological studies of BS revealed also their beneficial effects in relation to non-plant organisms (Zhabinskii et al. 2015). Apart from antiviral, anabolic, adaptogenic and other effects with respect to warm-blooded animals, BS revealed pronounced anticancer potentials (Hoffmannova et al. 2012; Oklestkova et al. 2015). It should be noted that BS can not only directly affect tumor growth (while showing no toxicity against normal cells), but also prevent cancerogenesis by changing the activity of CYP450 enzymes, that take part in activation of procarcinogens and metabolism of drugs.

The first work on the influence of BS on the growth of mammalian cells was a study in which the effects of epibrassinolide (10^{-16}–10^{-9} M) on cultured murine hybridoma cells were studied (Franek et al. 2003). An increase in the potential of the mitochondrial membrane, a decrease in the level of intracellular antibodies, an increase in the cell fraction in the G_0/G_1 phase of the cell cycle, and a decrease in the number of cells in the S phase were noted. The viability of the cells was significantly higher (relative to control) under the action of epibrassinolide at a concentration of 10^{-13} and 10^{-12} M. The ability of BS in a dose dependent manner to inhibit cell growth in cancer cell lines was shown by another Czech group (Swaczynová et al. 2006a, b; Malikova et al. 2008). Experiments with natural BS (epibrassinolide and homobrassinolide) exhibited that BS induced apoptosis in MDA-MB-468, LNCaP cells and arrested MCF-7, MDA-MB-468 and LNCaP cells in G_1 phase of the cell cycle (Malikova et al. 2008). It is important that toxic effects were not observed in untransformed human fibroplasts. Structure-activity relationships studies among a wider range of natural BS and their (22*S*,23*S*)-analogues showed that 6-ketones were more active in comparison with the corresponding 6-oxo-7-oxalactones (Hoffmannova et al. 2012). This was confirmed by experiments with a set of AB-functionalized cholestanes. With regard to functional groups in the cycle A, 3-hydroxy, 3-keto, 2α,3α-dihydroxy and 3α,4α-dihydroxy derivatives proved to be the most active (Malikova et al. 2008; Rarova et al. 2016).

Although being positioned as BS, many studied for anticancer activity steroids are quite far from this class of phytohormones from a chemical point of view (Rarova et al. 2016). These include also 3-substituted 6-oxo-7-oxa- and 7-oxo-6-oxa-B-homo-cholestanes, which showed a distinct cytotoxicity against cancer cells MGC 7901, HeLa and SMMC 7404 (Gan et al. 2012).

A distinguishing feature of the current stage of BS studies is that newly synthesized compounds of this series are tested both on plants and on cancer cell cultures. A number of BS analogues with perfluoroalkylated side chains were prepared by using alkene cross-metathesis and studied for anticancer and brassinolide-type activities (Eignerova et al. 2009). Although these derivatives were quite active in the bean second-internode bioassay, their anticancer activity was insignificant. Similar

results were obtained for BS analogues with a phenyl group in the side chain (Kvasnica et al. 2016; Korinkova et al. 2017). While a high activity (sometimes exceeding that for brassinolide) was observed on plants, only some synthesized in the study compounds showed moderate cytotoxic activity. A number of monohydroxylated BS analogues with a carboxylic group in the side chain were tested for plant growth promoting and anticancer effects (Kvasnica et al. 2014). Only weakly active inducing elongation of the second bean internode was observed, whereas none of the tested BS showed any detectable activity on cancer cell lines.

In contrast to plants, for which the BS signaling pathway is well established (Gruszka 2013; Yang et al. 2011), the mechanism of action (including cytotoxic effects against cancer cells) of these phytohormones on animal organisms is still a challenging problem (Stegerova et al. 2010; Hoffmannova et al. 2012). Many studies revealed that the observed cytotoxicity of BS was caused by induction of apoptosis. A time and concentration dependent cytotoxicity of brassinolide in prostate cancer PC-3 cells was explained by a caspase-induced apoptosis (Wu and Lou 2007). Experiments with epibrassinolide on androgen-responsive LNCaP and irresponsive DU145 cells showed that a caspase-dependent apoptosis was mediated by modulating Bcl-2 family members (Obakan et al. 2014a, b). It was also observed that catabolic enzymes of polyamines in both cell lines were involved in the programmed cell death. Further studies on the same cell lines indicated that the induced by epibrassinolide apoptosis was independent from p53 expression (Obakan et al. 2014a, b). The effect of epibrassinolide on cell viability and colony formation in HCT 116 and HT-29 colon carcinoma cells was explained by the inactivation of PI3K/AKT signaling pathway which promotes a FOXO3a-dependent apoptosis (Coskun et al. 2015). The apoptotic potential of BS in prostate cancer cells (both androgen responsive LNCaP and DU145 cells with nonfunctional androgen receptor) was studied by SILAC (Stable Isotope Labeling by Amino Acids in Cell Culture) analysis (Obakan et al. 2015). It was found that treatment of cells with epibrassinolide resulted in alteration of the expression profile of 160 proteins, the most strongly altered of which was calreticulin. This is a localized in endoplasmic reticulum chaperone that plays an important role in protein folding and buffering Ca^{2+} ions (Obakan-Yerlikaya et al. 2017). The alteration of calreticulin may cause endoplasmic reticulum stress, which, if prolonged, will result in apoptotic cell death.

Hormone-sensitive breast and prostate cancer cells were shown to be more susceptible to BS compared with hormone insensitive ones (Malikova et al. 2008). This may indicate possible modulation of estrogen and androgen mediated responses by BS (Oklestkova et al. 2015).

Another mechanism of how BS can act as anticancer agents is connected with their antiangiogenic properties. Inhibition of angiogenesis is an important factor for anticancer therapy. A group of natural BS was tested for their ability to inhibit in vitro angiogenesis of primary endothelial cells. It was found that homocastasterone and epibrassinolide at 30 μM considerably reduced migration of human umbilical vein endothelial cells (Rarova et al. 2012). Some of the tested BS also decreased the number of tubes. Similar activity was observed for a BS analogue BR4848 (Rarova et al. 2018).

The past years have witnessed a large advance in treating various cancers by combinational drugs (Yin et al. 2018). It is natural therefore that similar studies have been started with BS as one of the components. Combined application of epibrassinolide with etoposide and doxorubicin (1:1 ratios) on drug-resistant small-cell lung carcinoma VPA17 cells showed synergism between BS and these commonly used chemotherapy drugs (Sadava and Kane 2017). A combination of BS with cisplatin was shown to inhibit growth of A549 (lung carcinoma) and HepG2 (hepatocellular carcinoma) cancer cells more effectively than cisplatin alone. Both (22*S*,23*S*)-homocastasterone and (22*S*,23*S*)-epibrassinolide reduced IC_{50} of cisplatin by almost two times (Panibrat et al. 2018a, b). Flow cytometry data on distribution of cell cycle phases showed that a combination of (22*S*,23*S*)-homocastasterone or (22*S*,23*S*)-epibrassinolide with cisplatin reduced the amount of cells in S-phase causing the cell cycle arrest.

2 Cytotoxicity of BS in Cancer Cells

Cholesterol derivatives bearing one or more hydroxyl groups (oxysterols) were shown to possess cytotoxic properties, and some of them were considered as potential cancer chemotherapeutic agents (Schroepfer 2000; de Weille et al. 2013). From a chemical point of view, BS belong to oxysterols, and it is therefore quite natural that similar studies were conducted with this group of phytohormones and their numerous analogues.

A number of 22,23-dihydroxy and epoxy stigmastanes were tested for their effect on human hepatoma Hep G2 and human breast carcinoma MCF-7 cells using MTT assay (Drozdov et al. 2007; Misharin et al. 2008). Among the tested compounds, the greatest cytotoxicity was observed for triols **1** and **2** (Table 14.1). Compound **3** was toxic only to MCF-7 cells. In general, the cytotoxicity of the (22*R*,23*R*)-derivatives exceeded that of (22*S*,23*S*)-stigmastanes. The cytotoxicity of 22,23-oxygenated stigmastanes depended on the number and structure of substituents in the steroid skeleton. The 3β-hydroxy-5-ene derivatives were more toxic than the corresponding Δ^4-3-ketones and Δ^4-3,6-diketones.

The relationships between the stereochemical configuration of C22 and C23 atoms in the 22,23-dihydroxystigmastane derivatives and the cytotoxicity of these compounds was evaluated on human breast carcinoma MCF-7 cells, human ovary carcinoma CaOv cells, and human prostate carcinoma LnCaP cells (Misharin et al. 2010). Cytotoxicity of the studied compounds followed the order LnCaP> MCF-7> CaOv (Table 14.2). It was shown that (22*R*,23*R*)-isomers were more active than their (22*S*,23*S*)-counterparts (**9** and **10**, **11** and **12**, **13** and **14**, **15** and **16**, respectively). The observed difference was attributed to a more rigid side chain of (22*R*,23*R*)-diols in comparison with the corresponding (22*S*,23*S*)-isomers. Compounds **1** and **2** containing the equatorial 3β-hydroxyl group showed the greatest cytotoxicity. The most polar homobrassinolide (**9**) and homocastasterone (**11**) containing the 2α,3α-diol function exhibited the lowest activity. Low-polar compounds

Table 14.1 Effects of 22,23-dihydroxy and epoxy stigmastanes on MCF-7 and Hep G2 cell viability

Compound	IC_{50}, μM	
	Hep G2	MCF-7
(22*R*,23*R*)-stigmast-5-en-3β,22,23-triol (**1**)	10	9
(22*R*,23*R*)-stigmast-5-en-7-on-3β,22,23-triol (**2**)	13	6
(22*R*,23*R*)-stigmast-4-en-3,6-dion-22,23-diol (**3**)		6
(22*R*,23*R*)-22,23-epoxystigmast-5-en-3β,7α-diol (**4**)	16	22
(22*S*,23*S*)-22,23-epoxystigmast-5-en-3β,7α-diol (**5**)	28	
(22*R*,23*R*)-stigmast-4-en-3-on-22,23-diol (**6**)		29
(22*R*,23*R*)-5α,6α-epoxystigmastan-3β,22,23-triol (**7**)		30
(22*R*,23*R*)-stigmastan-3β,5α,6β,22,23-pentaol (**8**)	30	23

Table 14.2 Effects of 22,23-dihydroxy stigmastanes on MCF-7, LnCaP, and CaOv cell viability

Compound	IC_{50}, μM		
	MCF-7	LnCaP	CaOv
Homobrassinolide (**9**)	45	8	>60
(22*S*,23*S*)-homobrassinolide (**10**)	>60		
Homocastasterone (**11**)	60	5	>60
(22*S*,23*S*)-homocastasterone (**12**)	>60		
(22*R*,23*R*)-5α-stigmast-2-en-6-on-22,23-diol (**13**)	3	1.7	6
(22*S*,23*S*)-5α-stigmast-2-en-6-on-22,23-diol (**14**)	>80		
(22*R*,23*R*)-3α,5-cyclo-5α-stigmastan-6-on-22,23-diol (**15**)	5	1.6	5.5
(22*S*,23*S*)-3α,5-cyclo-5α-stigmastan-6-on-22,23-diol (**16**)	>90		
(22*R*,23*R*)-stigmast-4-en-3,6-dion-22,23-diol (**3**)		3.5	21
(22*R*,23*R*)-stigmast-4-en-3-on-22,23-diol (**6**)		3	28
(22*R*,23*R*)-stigmast-5-en-3β,22,23-triol (**1**)		0.7	6
(22*R*,23*R*)-stigmast-5-en-7-on-3β,22,23-triol (**2**)		1.1	5

having no hydroxyl groups in the steroid backbone **13**, **15**, **3** and **6** revealed intermediate toxicity with respect to all cell lines used. Flow cytometry analysis of MCF-7 cells incubated with the most toxic compound **1** showed that cell death was caused by necrosis and not by apoptosis. It was also found that the presence of phospholipids in the medium reduced the cytotoxic effects of (22*R*,23*R*)-dihydroxy-stigmastanes. The authors (Misharin et al. 2010) speculated that triol **1** may have an affinity for phospholipids of bilayers and lipid components of cell membranes. The interaction of certain oxysterols with the cell membrane is known to be important for their cytotoxic activities (Massey 2006).

Studies on homologous 22,23-dihydroxy derivatives of campestane, ergostane and cholestane series showed that a substituent at C24 had little effect on cytotoxic activity. In experiments with MCF-7 cells ergostane and campestane derivatives had the same level of activity as the corresponding stigmastanes (Khripach et al. 2010). Moreover, greater toxicity was also observed for (22*R*,23*R*)-diols **17** and **19** in comparison with their (22*S*,23*S*)-isomers **18** and **20** (Table 14.3). Introduction of a lactone

Table 14.3 Effects of 22,23-dihydroxy ergostane and campestane on MCF-7 cell viability

Compound	IC_{50}, μM
(22*R*,23*R*)-5α-ergost-2-en-6-on-22,23-diol (**17**)	1.6
(22*S*,23*S*)-5α-ergost-2-en-6-on-22,23-diol (**18**)	49
(22*R*,23*R*)-5α-campest-2-en-6-on-22,23-diol (**19**)	1.8
(22*S*,23*S*)-5α-campest-2-en-6-on-22,23-diol (**20**)	Nontoxic

Table 14.4 Effects of 22,23-dihydroxy cholestanes on MCF-7 and LnCaP cell viability

Compound	IC_{50}, μM	
	MCF-7	LnCaP
(22*R*,23*R*)-5α-cholest-2-en-6-on-22,23-diol (**22**)	18	12
(22*R*,23*R*)-2α,3α-epoxy-5α-cholestan-6-on-22,23-diol (**23**)	27	28
(22*R*,23*R*)-5α-cholestan-3,6-dion-22,23-diol (**24**)	40	20
(22*R*,23*R*)-5α-cholestan-6-on-3β,22,23-triol (**25**)	>60	>100

functionality into the cycle B resulted in decreasing the cytotoxic activity against MCF-7 cells (Andreeva et al. 2018).

The effect of substituents in cycle A on cytotoxicity of 22,23-diols was studied on derivatives with the cholestane backbone (Table 14.4; Khripach et al. 2012). These studies confirmed earlier results (Misharin et al. 2010), indicating that polar substituents in cycle A decrease cyclotoxicity. As in the case of the above 22,23-dihydroxy steroids, compound **21** having in the molecule a Δ^2-double bond showed the most potent inhibitory activity. It should be noted that similar Δ^2-steroids were found in plants as biosynthetic precursors of BS (Antonchick et al. 2003).

The estimation of cytotoxicity of diols **21–23** using the MTT test correlated with the results of inhibition of DNA biosynthesis by these compounds in LnCap cells in various concentrations (Khripach et al. 2012). In the presence of **21**, LnCap cells stopped their growth and remained viable for 72 h. A longer incubation at a concentration of 20 μM for 4 days resulted in a partial detachment of cells. Apoptosis (32%) and blockage of the cell cycle in the S and G_2/M phases was observed with exposure of LnCap cells with **21** at a concentration of 20 μM.

3 Participation of Reactive Oxygen Species (ROS) in BS-Mediated Death of Tumor Cells

BS are known to assist plants in overcoming various environmental stresses. The latter trigger an excessive production of reactive oxygen species (ROS) that cause oxidative damage to cellular components and may lead to cell death (Sharma et al. 2012; Czarnocka and Karpinski 2018). Apart from their destructive behavior, ROS serve as signaling molecules being a part of the plant antioxidative defense mechanisms. In this respect, the delicate balance between production and scavenging of

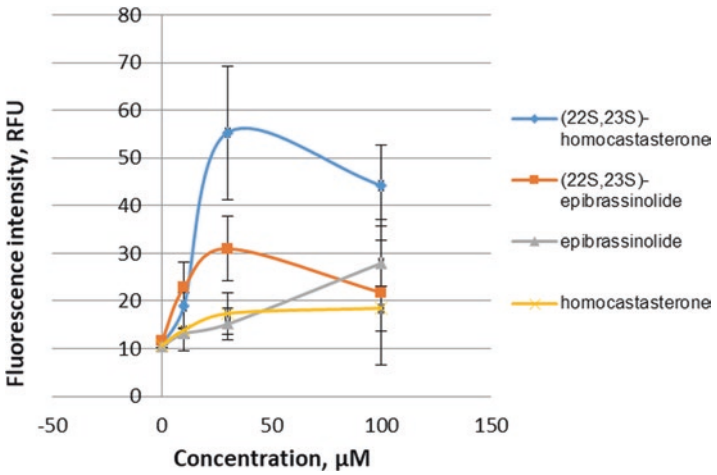


Fig. 14.1 The dependence of the average fluorescence value of DCF on the BS concentration

ROS is essential for normal plant development. In experiments with cucumber, the induction of stress tolerance by BS was shown to involve the generation of ROS (superoxide anion-radicals and hydrogen peroxide) (Xia et al. 2009). Similar results were obtained for wheat (Karpets and Kolupaev 2018). These findings led to the initiation of studies aiming to evaluate the influence of BS on the level of ROS production in cancer cells. The level of ROS in tumors is elevated (Liou and Storz 2010), and its further increase is more harmful for tumorous cells than for healthy ones thus making it possible to preferentially eliminate cancer cells by pharmacological ROS insults (Trachootham et al. 2009).

The BS impact on the level of ROS in cancer cells was studied on the lung adenocarcinome line A549 (Kisselev et al. 2016, 2017). ROS measurements were performed by staining A549 cells with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Inside the cells, DCFH-DA after cleavage of the acetate groups by intracellular esterases and oxidation by ROS is converted into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Chen et al. 2010). Its luminescence parameters reflect the intracellular ROS level (Knerr et al. 2006). Figure 14.1 shows the dependence of the average fluorescence value of DCF on the BS concentration. For all the compounds studied (except of epibrassinolide), the maximum effective concentration was 30 μM, and its further increase either led to a stabilization of the effect (28-homocastasterone) or to its decrease ((22*S*,23*S*)-homocastasterone and (22*S*,23*S*)-epibrassinolide).

The maximum efficiency was found for (22*S*,23*S*)-homocastasterone, for which a nearly sixfold increase in luminescence intensity was observed in comparison with the control at its 30 μM concentration.

Futher experiments were conducted on determining relationship between the ROS generation by BS and cancer cell death. Using two-colored fluorescent cytometry (Et-Br vs. DCF), it was shown that the level of cell death is in direct proportion

Table 14.5 The level of apoptosis in A549 cells after the treatment with BS

Probe	[Brassinosteroid], μM	% apoptosis
Control of A549	1% DMSO	12
Epibrassinolide	100	23
Homocastasterone	100	24
(22*S*,23*S*)-epibrassinolide	100	16
(22*S*,23*S*)-homocastasterone	50	14

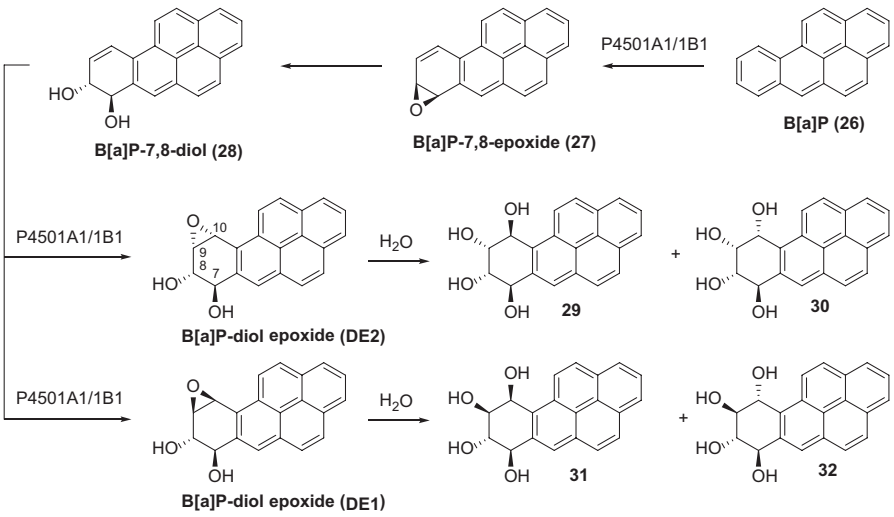
to the concentration of synthetic brassinosteroids (Kisselev et al. 2017). The high level of ROS that were generated as the result of BS influence cause death of the cells. As follows from the study with the addition of (22*S*,23*S*)-homocastasterone, a direct correlation was observed between the intensity of ethidium bromide cell fluorescence and the level of ROS. This is due to the fact that the increase in the level of ROS leads to a gradual disruption of the permeability of the cell membrane for ethidium bromide, which is a sign of necrosis.

In all cases the effect depended on the BS's structure of the side chain and was more pronounced in the case of the SS orientation of the hydroxyl groups at the position C22 and C23 ((22*S*,23*S*)-homocastasterone). This compound also possessed greater antiproliferative activity, which suggests a possible relationship between the induction of ROS and the cytotoxicity of the substances studied.

Necrosis was also proved by determining the level of apoptosis (Table 14.5). Natural BS caused a twofold apoptosis as compared with the control, although there was no changes for synthetic (22*S*,23*S*)-BS.

4 BS as Inhibitors of Procarcinogen Activation

A number of xenobiotics (e.g., polyaromatic hydrocarbons) are known to possess strong procarcinogenic properties as a result of their transformation into highly carcinogenic products by enzymes of cytochrome P450 superfamily. The CYP450 catalytic activity is very sensitive to exogenous influences and can be increased by substrates of these enzymes by a process called substrate induction (Obakan et al. 2014a, b). It can result in an increased generation of carcinogens. Thus, the cytochrome P450 oxidation of the classical xenobiotic benzo[a]pyrene (B[a]P) leads to the formation of a mixture of B[a]P-7,8-diol epoxides DE2 and DE1 (Scheme 14.1), the first of which is carcinogenic, and the other has no adverse effect (Schwarz et al. 2001; Castell et al. 2005). Apart from the activation of carcinogens, highly expressed levels of individual cytochrome isoforms may decrease or increase drug metabolism (Zanger and Schwab 2013). As it was demonstrated on cancer cells MCF-7, the plant-derived substances flavonoids, in particular quercetin, kaempferol, myricitin and apigenin inhibited the activity of CYP1A1 and CYP1B1 xenobiotic metabolising isoforms and the formation of DE2 (Chaudhary and Willett 2006).



Scheme 14.1 Metabolism of B[a]P by CYP1A1/1B1

The influence of BS on the activity of xenobiotic metabolising and drug metabolising isoforms of CYP450 and on the formation of DE2 during the oxidation of B[a]P was studied using monooxygenase enzyme systems of rat liver microsomes (Sysa et al. 2010), tumor hormone-dependent MCF-7 cell line (Sysa et al. 2011) and hormone-independent cell line Hep G2 (Panibrat et al. 2018a, b) as model objects. The highest content of all CYP450 enzymes (including drug-metabolising) was noted in the liver. The CYP1 isoforms taking part at xenibiotic metabolism were also expressed at detectable level in nonhepatic tissues (Pavek and Dvorak 2008). In order to increase the level of isoenzymes of cytochrome P450 (CYP1A1, CYP1A2, CYP1B1) in the microsomal fraction of rat hepatocytes and cancer cells, 20-methylcholanthrene was initially administered to the rats (Sysa et al. 2010), and the cancer cells were exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Tompkins and Wallace 2007). 7-Ethoxycoumarin, 7-ethoxyresorufin and B[a]P) were used as substrates for determining enzyme activity.

The first substrate is used to characterize the drug-metabolizing function of the liver and, first of all, reflects the catalytic activity of such isoenzymes in the human body as CYP3A4, CYP2E1 and CYP2D6 (Fujii-Kuriyama and Mimura 2005). 7-Ethoxyresorufin reflects the activity of all the isoforms of cytochrome P450 induced by xenobiotics (20-methylcholanthrene, TCDD, etc.), namely CYP1A1, CYP1A2, CYP1B1. B[a]P served to assess the detoxifying function of the monooxygenase system by the rate of conversion of B[a]P to its hydroxy derivatives, which after conjugation are removed from the cell (Sysa et al. 2010), and for determining production of DE2 carcinogen (Panibrat et al. 2018a).

Among B-lactones, the change in the type and configuration of the substituent at the C24 position (the ethyl group instead of the methyl group in homobrassinolide in contrast to epibrassinolide) affected only the reaction with 7-ethoxyresorufin.

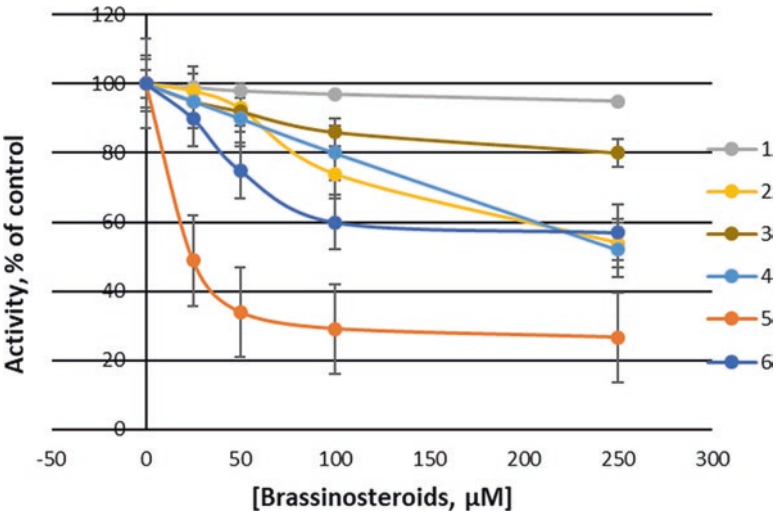


Fig. 14.2 Dependence of the 7-ethoxyresorufin monooxygenase system oxidation rate on the concentration of brassinosteroids. *1* – epibrassinolide, *2* – homobrassinolide, *3* – homocastasterone, *4* – epicastasterone, *5* – (22*S*,23*S*)-homobrassinolide, *6* (22*S*,23*S*)-epibrassinolide

However, the effect was poor and it was not possible to achieve a twofold reduction of the reaction rate (which is necessary for the determination of IC_{50}) even with an increase in the concentration of homobrassinolide in the reaction medium up to 250 μM.

At the same time, a pronounced inhibitory effect was caused by a change in the configuration of 22,23-diol groups of the side chain (*R,R* to *S,S*). The IC_{50} value for (22*S*,23*S*)-homobrassinolide (25 μM) in the reaction with 7-ethoxyresorufin was comparable to that of homocastasterone (13 ± 2.6 μM) and castasterone (16 ± 5.3 μM) (Fig. 14.2). In reaction with 7-ethoxycoumarin, the inhibitory effect of 22*S*,23*S*-hydroxy derivatives was less pronounced (Fig. 14.3). It may be a reflection of the fact that the studied compounds affect only the monooxygenase activity of cytochrome P450 isoenzymes induced by 20-methylcholanthrene and does not or only slightly affect the processes catalyzed by drug-metabolising monooxygenases.

In experiments to assess the effect of BS on the oxidative dealkylation reaction of 7-ethoxyresorufin in the monooxygenase system of the MCF-7 cancer cell line lysate, an inhibitory effect at 50 μM concentration was shown for epibrassinolide and homobrassinolide (Fig. 14.4; Sysa et al. 2011).

The compounds with a 6-oxo-7-oxalactone structure, epibrassinolide and its (22*S*,23*S*)-stereoisomer, proved to be the most effective in this respect (Fig. 14.4; Sysa et al. 2011). Homocastastasterone also reduced the rate of microsomal oxidation, but to a lesser extent. Such changes in the catalytic parameters of the reaction can be attributed to the effect of BS on the induction in the MCF-7 cell line of at least two isoenzymes: CYP1A1 and CYP1B1, which induced by TCDD.

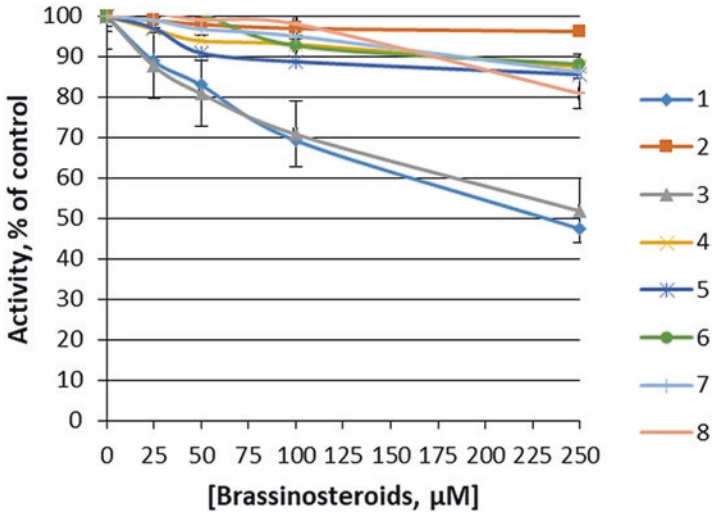


Fig. 14.3 Dependence of the 7-ethoxycoumarin monooxygenase system oxidation rate on the BS concentration. *1* – homobrassinolide, *2* – epibrassinolide, *3* – (22*S*,23*S*)-homobrassinolide, *4* – (22*S*,23*S*)-epibrassinolide, *5* – homocastasterone, *6* – epicastasterone, *7* – ecdysterone, *8* – α-ecdysone

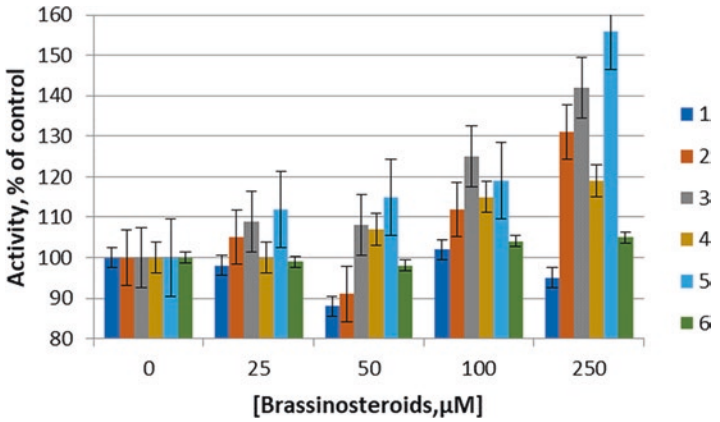


Fig. 14.4 Dependence of monooxygenase system oxidation rate of 7-ethoxyresorufin from brassinosteroid concentration in MCF-7 cell line. *1* – epibrassinolide, *2* – homobrassinolide, *3* – homocastasterone, *4* – epicastasterone, *5* – (22*S*,23*S*)-homobrassinolide, *6* – (22*S*,23*S*)-epibrassinolide

It should be noted that the BS had no significant influence on such an important function of the monooxygenase system as the hydroxylation of B[a]P, which is necessary for its removal from the body (Fig. 14.5).

When the Hep G2 cells were co-incubated with BS and TCDD (Panibrat et al. 2018a), different effects on the formation of the carcinogenic diol epoxide

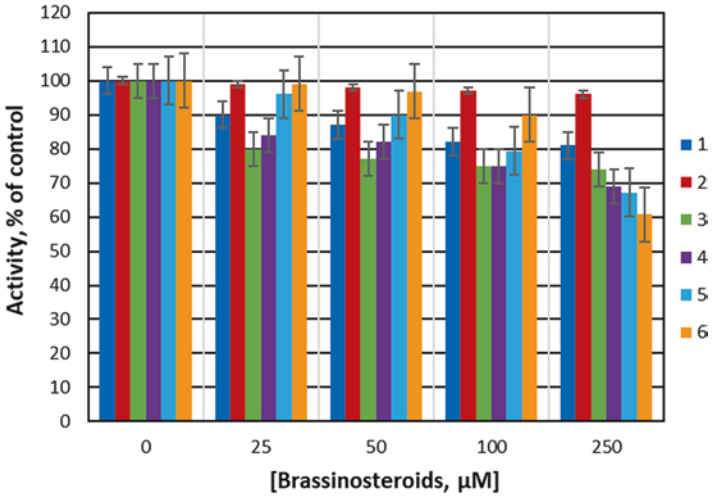


Fig. 14.5 The rate of 3-hydroxybenzo[a]pyren accumulation in the presence of BS. *1* – epibrassinolide, *2* – homobrassinolide, *3* – homocastasterone, *4* – epicastasterone, *5* – (22*S*,23*S*)-homobrassinolide, *6* – (22*S*,23*S*)-epibrassinolide

DE2 were observed (Table 14.6). In the presence of TCDD alone, the activity of cytochromes P450 increased 6.7 times, which was evident from the product/substrate ratio. The amount of diol epoxide DE2 in the induced cells was increased by 2.5 times as compared with the control cells. Under the action of (22*S*,23*S*)-homocastasterone, the ratio of DE2/total reaction product decreased approximately by 2 times and was comparable to that of control cells.

As for the degree of induction of CYP450 activity, the synthetic BS significantly reduced it: (22*S*,23*S*)-epibrassinolide – 9 times, (22*S*,23*S*)-homocastasterone – 30 times, which is 4.5 times less, than the activity of cytochromes P450 in intact cells. Natural epibrassinolide reduced induction by 5 times, while homocastasterone increased it twofold. Inhibition of induction of CYP activity by BS can also indirectly affect drug methabolism.

5 Conclusions

At present, there is sufficient evidence at the cellular and molecular level that BS and their synthetic analogues can act as potential antitumor compounds. Compared with traditional cytostatics, BS have a number of advantages: they are nontoxic for normal cells, have anti-angiogenic activity, and also inhibit the activity of monooxygenase enzymes involved in the activation of carcinogens and the metabolism of drugs. It should be emphasized that very few natural substances are known that can selectively affect tumor cells without affecting the growth of normal cells.

Table 14.6 The effect of BS on the formation of the genotoxic product DE2 as a result of cytochrome P450 catalyzed oxidation of B[a]P-7,8-diol (**28**) in Hep G2 cells

Product	Control	TCDD (10 nM)	TCDD + epibrassinolide (50 μM)	TCDD + homocastasterone (50 μM)	TCDD + (22*S*,23*S*)-epibrassinolide (50 μM)	TCDD + (22*S*,23*S*)-homocastasterone (50 μM)
DE2	22%	61%	47%	63%	52%	22%
Total products	88%	98%	91%	99%	84%	62%
B[a]P-7,8-diol (**28**) (substrate)	12%	2%	9%	1%	16%	38%
DE2/product	0.25	0.62	0.52	0.64	0.62	0.35
Product/substrate	7.33	49	10.11	99	5.25	1.63

Antiproliferative activity of BS was tested on normal cells and on a number of tumor cell lines of different origin. It has been shown that BS are not toxic to normal cells and exhibited in tumor lines IC_{50} in the range of 1–200 μM. Based on in vitro experiments, it was found that there is a dependence of biological activity on the structure of the compounds under study. Thus, in the series of natural BS, compounds with 6-keto function in ring B showed more pronounced antiproliferative properties than compounds with 6-oxo-7-oxalactone function. Also, apparently, the presence of the 2α,3α-vicinal diol group is important. For 22,23-dihydroxystigmastane derivatives, the presence of the equatorial hydroxyl group at the C-3 position is important. The (22*R*,23*R*)-isomers with the conformationally rigid chain are more toxic than the (22*S*,23*S*)-derivatives. The same dependence was observed in the evaluation of the effects of campestane, ergostane and cholestane derivatives.

BS were more effective in suppressing the growth of hormone-dependent tumor lines. It was suggested that the cystostatic activity of BS was partly related to the interaction with steroid receptors. Breast carcinoma studies (line MCF-7) showed that a typical suppression of cell growth in response to the action of antiestrogens is accompanied by a decrease in the number of cells in the S-phase of the cell cycle and a simultaneous increase in the proportion of cells in the G_0/G_1 phase, which is also observed in the presence of BS. The same occurred with the influence of BS on estrogen and androgen-dependent tumor lines. It should be noted that in some cases there was an increase in the number of cells in the G_2/M phase with a decrease in the proportion of cells in other phases of the cell cycle.

It was shown that the type of cell death (apoptosis or necrosis) can also be caused by the structure of the active BS, and the presence of phospholipids in the medium leads to a decrease in the cytotoxicity of the BS. Apoptosis in hormone-dependent tumors was initiated by the violation of the cell cycle, the accumulation of cells in the G_0/G_1 phase, a decrease in their number in the S-phase of the cell cycle. In hormone-independent tumors activation of caspases 3 and 7 occured. Some synthetic (22*S*,23*S*)-analogues of natural BS can cause necrosis through an increase in the intracellular level of ROS in tumor cells, leading to destruction of the cell membrane. This allows to assume that one of the ways of BS influence on cancer cells can be via initiation of the oxidative stress, which leads to the cell death by ROS-dependent mechanisms. Cell necrosis was also observed under the action of trihydroxystigmastane derivatives.

The anti-angiogenic activity of BS was manifested by inhibition of migration of endothelial cells and the formation of vessel tubes. These data indicate the potential use of BS to prevent the formation of metastases.

The influence of BS on the monooxygenase enzyme system of mammalian cells was first revealed and, thus, the possibility of direct influence of BS on enzymatic processes in the mammalian organism, not mediated by steroid receptors, was shown. Brassinosteroids can realize their antitumor potential both by inhibiting the expression of certain cytochrome P450 isoenzymes involved in the activation of procarcinogenic substances, and by direct effect on the enzymatic reaction. It was found that BS primarily inhibit enzyme systems involved in the metabolic activation

of procarcinogenic substances, and the degree of their influence directly depends on the structure of the studied phytosterols. The maximum inhibitory effect was shown by (22*S*,23*S*)-derivatives (22*S*,23*S*)-homoborassinolide, (22*S*,23*S*)-epibrassinolide, (22*S*,23*S*)-homocastasterone.

To date, considerable experience has been accumulated concerning the use of BS as potential antitumor agents with low cytotoxicity against normal cells. The obtained results contribute to a better understanding of the molecular mechanisms of the influence of BS on the biochemical processes in the mammalian organism and can serve as a basis for the directed search and creation of new generations of antitumor drugs.

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Chapter 15
Harnessing the Potential of Brassinosteroids in Abiotic Stress Tolerance in Plants

Navdeep Kaur and Pratap Kumar Pati

Abstract Brassinosteroids (BRs) are the steroidal plant hormones that play a pivotal role in growth and development of plants. They are ubiquitous within the plant kingdom and are well known for their pleotropic effects including growth, rhizogenesis, seed germination, flowering, maturation, senescence and abscission. In the past recent years, brassinosteroids are in the limelight for their potential to confer abiotic stress tolerance in plants. They are known to modulate a plethora of stress responsive pathways that in turn promotes the vigor of the plant under unfavorable conditions. The use of different genetic, biochemical and molecular tools have provided us convincing evidence and valuable insights on the regulation of abiotic stress tolerance using BRs. However, in depth knowledge of the different mechanisms how BRs confer abiotic stress adaptation in plants is still elusive. The present chapter is focused upon understanding the current knowledge of BR mediated abiotic stress tolerance in plants and highlighting the knowledge gaps in the area.

Keywords Abiotic stress · Plant growth regulators · Brassinosteroids · Stress tolerance · Reactive oxygen species · Transcription factors

1 Introduction

The sensitivity of plants towards a range of abiotic stresses is a major threat for enhancing the productivity of different agricultural crops (Kosova et al. 2018; Martinez et al. 2018). These stresses including salinity, drought and temperature together are responsible for almost 50% reduction in the global yield of food crops causing an economic loss of almost $14–19 million (Martinez et al. 2018). Climate change is expected to further escalate the prevalence and potency of these stresses in the coming years (Vaughan et al. 2018). Thus, enhancing the abiotic stress endurance of plants is an immediate worldwide concern for achieving a sustainable food security for the rising world population.

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A number of strategies have been employed for achieving abiotic stress resistance in plants from time to time, including the most classical breeding and the recent one genome editing (Hoang et al. 2016; Miglani 2017); but a single practical solution to solve this problem has not been achieved yet due to the pros and cons of each of these strategies. Conventional breeding and marker assisted selection are the most economical and socially accepted approaches that give fruitful results (Turan et al. 2012). However, they are very time consuming and further their success is hindered by the existing reproductive barrier and narrow genetic variability (Turan et al. 2012, Chantre Nongpiur et al. 2016). Modern approaches such as genetic engineering and genome editing have given promising results for improving the stress endurance in plants. But they also faces severe challenges due ethical concerns, including germplasm cross-contamination, health risks associated with their consumption and lack of public acceptance (Hoang et al. 2016; Miglani 2017). In this scenario, researchers are compelled to suggest an environmental friendly strategy which can give required results in less time.

Plant growth regulators are present in all the plants in minute amounts and regulate a plethora of their morphological, physiological, biochemical and molecular features (Benkova 2016; Ahmad et al. 2018). Their role in agriculture for improving the growth and production of various crops has also been well explored (Rademacher and Jung 2018). But from the past few years, they have attained huge attention due to their remarkable effect on the modulation of abiotic stress responses in plants (Krishna et al. 2017). PGRs act as secondary messengers in response to environmental stress stimuli and ameliorate the effects of these stresses in plants (Benkova 2016). Thus, in the current scenario, the timely and judicious exogenous use of PGRs provides adequate scope for overcoming the hazard of abiotic stress in plants.

2 Brassinosteroids

Brassinosteroids are the steroidal plant hormones which are present ubiquitously in all plants (Ahmad et al. 2018). They were originally discovered independently by two research groups including Nagoya University in the Japan and United States Department of Agriculture (USDA) (Mitchell et al. 1970; Ahmad et al. 2018). Researchers analyzed organic extracts from the pollens of different plant species in an attempt to discover new plant hormones. During these analyses of almost 30 years, the most potent growth extract was identified from *Brassica napus* pollen and was named as “brassins” (Mitchell et al. 1970). The chemical nature of brassins was later identified by collaboration between different laboratories of USDA in 1979. Through extensive experimentation with pollens of *Brassica napus* and using X-ray crystallography, brassins were found to be steroidal lactone which was called as brassolide (Grove et al. 1979). In the almost past three decades (1979–2009), 70 different types of BRs have been reported from the plants which can be further

classified into various groups as C_{27}, C_{28} or C_{29} depending upon the number of carbons they contain (Bajguz and Hayat 2009; Vardhini and Anjum 2015). These diverse types of BRs have been found to occur in almost all the major organs of the plants including seeds, pollen, leaves, fruits, roots, etc. (Bajguz and Hayat 2009).

BRs are perceived at the cell surface via Brassinosteroid Insensitive 1 (BRI1) protein that is a cell surface receptor kinase. BRI1 after induction by BRs interact with BRI1 Associated Receptor Kinase 1 (BAK1). These two interplay with each other and then induce a relay of phosphorylation and dephosphorylation signals. Due to these signaling events, the stimuli perceived at the cell membrane is transduced to the nucleus thus regulating the expression of an array of genes involved in various physiological and biochemical processes (Nakamura et al. 2017, Belkhadir and Jaillais 2015; Sharma et al. 2013c).

Brassinosteroids are well known for their pivotal role in regulation of plant growth and development. They regulate multiple plant processes including photomorphogenesis, stem elongation, xylem differentiation, epinasty, leaf bending, reproductive development, proton pump activation, photosynthesis and protein synthesis, etc. (Clouse and Sasse 1998; Ahmad et al. 2018). Along with these well-documented roles of BRs, their potential for the regulation of an array of abiotic stress responsive pathways in plants has also been well realized.

3 Role of Brassinosteroids in Abiotic Stress Tolerance

In the past decade, researchers have established a direct link between modulation of brassinosteroids levels in plants and abiotic stress adaptation (Sharma et al. 2017). They act as a connecting hub between environmental stimuli and plant stress adaptive pathways for conferring abiotic stress tolerance (Krishna et al. 2017). BRs are well known for their potential to modulate almost all the basic to advanced cellular processes for increasing the vigour of the plants against adverse environmental conditions (Sharma et al. 2017; Fig. 15.1).

3.1 BRs Regulated Physiological and Biochemical Mechanisms

BRs have been reported to trigger the complex multi-component signaling pathways linked to different physiological and biochemical mechanisms involved in abiotic stress adaptation (Sharma et al. 2017) (Table 15.1). These pathways then work in a co-ordinated manner for acclimatization of plants to promote their survival under abiotic stress conditions.

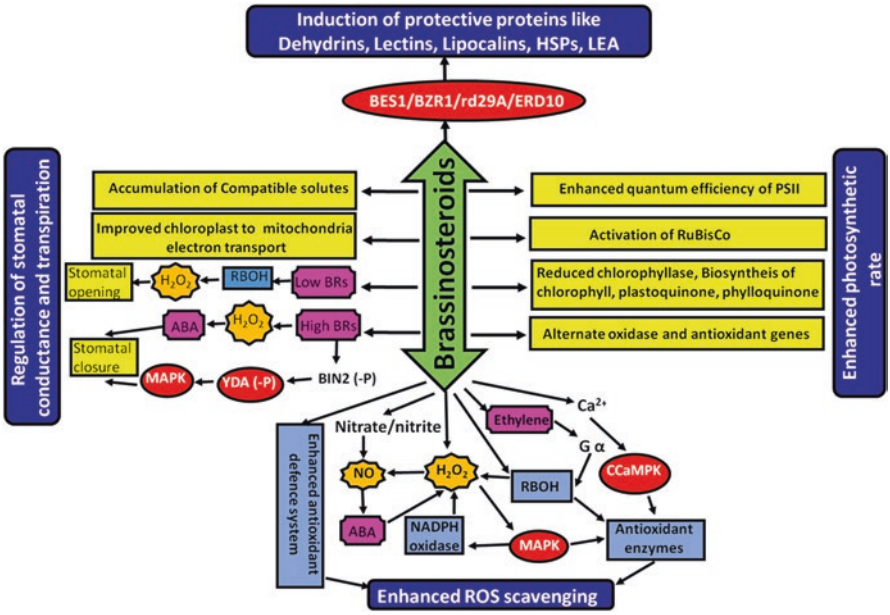

Fig. 15.1 Multiple effects of BRs at various levels to enhance abiotic stress tolerance. Red circles represent transcription factors, orange stars represents ROS/RNS and pink boxes represent phytohormones. Abbreviations are as follows *HSPs* heat shock proteins, *LEA* late embryogenesis proteins, *ABA* (abscisic acid), *RBOH* Respiratory Burst Oxidase Homolog, *MAPK* Mitogen-activated protein kinase, *YDA* YODA, *BIN2* BR-insensitive 2, *RuBisCo* Ribulose-1,5-bisphosphate carboxylase/oxygenase, *NO* nitric oxide, *CCaMPK* calcium/calmodulin-dependent protein kinase

3.1.1 The Restructuring of Cell Wall

Cell wall is the foremost important organelle of plants that serves multiple roles in their life cycle (Houston et al. 2016). It is mainly composed of cellulose, hemicelluloses, pectins, lignins and different structural proteins which are present in varying amounts (Tenhaken 2015; Houston et al. 2016; Rao and Dixon 2017). The dynamics of these cell wall components in response to a spectrum of environmental stimuli often serves as a first line of defence against stress in plants (Sharma et al. 2017). The increasing body of evidence has suggested the influence of BRs in modulation of plant cell wall architecture for ameliorating the effects of abiotic stresses. BRs regulate the expression of *CesA* gene family, *xyloglucan endotransglucosylase/hydrolase (XTHs)* and *expansions* (*EXPs*) genes for restructuring the cell wall in response to stress stimuli (Rao and Dixon 2017; Sharma et al. 2017). *CesA* genes are involved in the biosynthesis of cellulose that plays a critical role in expansion and elongation of the cells in response to multiple stresses (Rao and Dixon 2017). Various genetic and chromatin immunoprecipitation (ChIP) experiments conducted in BR deficient mutants of Arabidopsis have clearly demonstrated the BRs mediated modulation of *CesA* genes. In this study, BR activated transcription factor BES1

Table 15.1 Brassinosteroids regulated abiotic stress responsive genes

S. No	Gene	Mechanism	Function	References
1	*CESA*	Restructuring of cell wall	Biosynthesis of cellulose	Xie et al. (2011) and Rao and Dixon (2017)
2	*Xyloglucan Endotransglucosylase/ Hydrolase (XTHS)*	Restructuring of cell wall	Loosening of plant cell wall	Rao and Dixon (2017)
3	*Expansions (EXPS)*	Restructuring of cell wall	Loosening of plant cell wall	Rao and Dixon (2017)
4	*VND6*	Restructuring of cell wall	Lignification of cell wall	Zhong et al. (2008) Yamaguchi et al. (2010), Zhao and Dixon (2011), Didi et al. (2015), and Li et al. (2016)
5	*VND7*	Restructuring of cell wall	Lignification of cell wall	Zhong et al. (2008), Yamaguchi et al. (2010), Zhao and Dixon (2011) Didi et al. (2015), and Li et al. (2016)
6	*D1*	Protection of photosynthetic apparatus	Repair of photosystem II (PSII)	Siddiqui et al. (2018)
7	*RBCL*	Protection of photosynthetic apparatus	RuBisCO biosynthesis	Perdomo et al. (2017)
8	*RBCS*	Protection of photosynthetic apparatus	RuBisCO biosynthesis	Perdomo et al. (2017)
9	*Chlorophyllase*	Protection of photosynthetic apparatus	Chlorophyll degradation	Hayat et al. (2012) and Sharma et al. (2017)
10	*YODA (YDA)*	Stomatal regulation	Stomata production	Kim et al. (2012)
11	*Speechless (SPCH)*	Stomatal regulation	Stomata production	Gudesblat et al. (2012) and Serna (2013)
12	*Betaine aldehyde dehydrogenase (BADH)*	Osmoregulation	Glycine betaine biosynthesis	Rattan et al. (2014)
13	*RBOH*	Redox homeostasis	ROS generation	Xia et al. (2015)
14	*SOD*	Redox homeostasis	ROS scavanging	Ahmad et al. (2018)
15	*MAPK genes*	Signal transduction	Kinases (phosphorylation)	Divi et al. (2016)
16	*PP2A*	Signal transduction	Phosphatases (dephosphorylation)	Di et al. (2011)

(continued)

Table 15.1 (continued)

S. No	Gene	Mechanism	Function	References
17	*WRKY*	Regulation of gene expression	Transcription factor	Chen and Yin (2017)
18	*DREB*	Regulation of gene expression	Transcription factor	Kagale et al. (2007) and Lata and Prasad (2011)
19	*BZIP*	Regulation of gene expression	Transcription factor	Che et al. (2010)
20	*Heat shock proteins (HSPS)*	Cellular protection	Molecular chaperone	Derevyanchuk et al. (2016)
21	*Late Embryogenesis Proteins (LEA)*	Cellular protection	Protects proteins from aggregation	Kagale et al. (2007) and Duan and Cai (2012)
22	*TUD1 (E3 ubiquitin ligase)*	Signaling protein	Protein ubiquitination	Sharma et al. (2017)

was observed to interact with the CANNTG E-box motif present in the upstream promoter region of most of the *CesA* genes (Xie et al. 2011; Rao and Dixon 2017). The expression levels of BR receptor BRI1 and *Ces*A genes have also been observed to increase together in response to stress in plants (Xie et al. 2011; Rao and Dixon 2017). XTHs and expansions are the genes associated with the loosening of plant cell wall that stimulates the growth of stress affected plant organs (Tenhaken 2015). Exogenous application of BRs significantly enhance their expression in plants (Rao and Dixon 2017).

Lignin is another abundant polymer present in the secondary cell wall of plants. The content of lignin has been earlier reported to increase in response to a range of abiotic stress stimuli in plants (Moura et al. 2010). Exogenous treatment of BRs leads to the enhanced accumulation of lignin through BES1 TF. They regulate the expression of the genes involved in the lignification of cell wall, including *VND6*, *VND7* and *MYB* (Zhong et al. 2008; Yamaguchi et al. 2010; Zhao and Dixon 2011; Didi et al. 2015; Li et al. 2016). Apart from these, BRs increase the activities of different peroxidases that further catalyze the process of lignin polymerization (Tenhaken 2015; Rao and Dixon 2017). Thus, BRs play a significant role in remodeling of plant cell wall in response to various abiotic stresses.

3.1.2 Protection of Photosynthetic Apparatus

Photosynthetic apparatus is one of the most sensitive component of plant metabolic pathways which is seriously affected by abiotic stresses (Gururani et al. 2015). BRs have been implicated to protect this apparatus against the damage caused by different stress conditions thus increasing the vigour of plants (Sharma et al. 2017; Siddiqui et al. 2018). The major effect of environmental stresses on photosynthesis is the repression of photosystem II (PSII), a phenomenon known as photo

inhibition (Gururani et al. 2015). It is mainly caused due to the oxidative stress induced by environmental cues. BRs have been observed to protect the plants against stress induced photo inhibition by regulating the action of D1 protein which is involved in repair of PSII (Siddiqui et al. 2018). They also increase the quantum and photo efficiency of the PSII for mitigating the adverse affects of stress on photosynthetic apparatus (Gururani et al. 2015; Sharma et al. 2017). Further, BRs have been observed to alter the thylakoid structure for protecting the plants against stress induced damage. However, further insights are required to understand the detailed role of BRs in modulation of thylakoid assembly in response to stress (Gururani et al. 2015).

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) is a critical enzyme which catalyzes the major step of photosynthesis. Its activity is seriously hampered by various environmental stresses. Exogenous treatment of BRs has been found to positively regulate the expression of different genes (*rbcl* and *rbcs*) that encode for the functional subunits of RuBisCO. Along with this, BRs also modulate the activity of the rubisco activase (RCA) enzyme that plays a critical role in the activation of RuBisCO (Perdomo et al. 2017).

Chlorophyll degradation is another major effect of environmental stresses in plants (Taibi et al. 2016). The turnover of chlorophyll in plants is mainly regulated by the enzyme chlorophyllase involved in its catabolism (Taibi et al. 2016). BRs have been reported to enhance the net content of chlorophyll in plants under abiotic stress conditions by down-regulating the expression of chlorophyllase (Hayat et al. 2012; Sharma et al. 2017). Apart from this, BRs treatment also up-regulates the transcript levels of critical genes involved in the biosynthesis of chlorophyll in response to stress stimuli (Hayat et al. 2012; Sharma et al. 2017; Siddiqui et al. 2018).

3.1.3 Stomatal Regulation

Stomata are the specially designed epidermal microscopic pores necessary for the gaseous exchange, photosynthesis and transpiration in plants (Haworth et al. 2011; Kim et al. 2012). Plants tend to regulate their stomatal aperture for acclimatization under stress conditions (Shabala 2013; Kaur and Pati 2017). Exogenous treatment of BRs negatively regulates the uptake of K^+ ions in the guard cells thus resulting in the closure of stomatal pores of *Vicia faba* (Daszkowska-Golec and Szarejko 2013). Further, the higher concentrations of BRs leads to closure of stomata in tomato by regulating the dynamics of hydrogen peroxide (H_2O_2) (Xia et al. 2014).

BRs also modulate the stomatal density in crosstalk with mitogen activated protein kinases (MAPK) pathway in response to stress stimuli (Kim et al. 2012). Various experiments conducted in BR deficient mutants elucidate that they negatively influence the stomata generation in plants. The inhibition of BIN-2 function in response to BR treatment in turn deactivates the YODA (YDA, a MAPK kinase). YDA suppresses the initiation of stomata production in plants resulting in lower stomatal conductance (Kim et al. 2012). However, in another report, BRs have

observed to positively regulate the stomata density through the inhibition of phosphorylation of SPEECHLESS (SPCH) which is a basic helix-loop-helix transcription factor involved in stomata development (Gudesblat et al. 2012; Serna et al. 2013). Thus, the exact role of BRs in modulation of stomatal development is still obscure and further insights are necessary to solve this mystery.

3.1.4 Osmoregulation

A number of osmolytes accumulate in the plant cells in response to abiotic stress cues that maintains their turgor pressure and also stabilizes the biomolecules and cellular machinery (Kaur and Pati 2017). These osmolytes mainly includes proline, glycine betaine, mannitol, polyamines and myo-inositol (Sharma et al. 2017). Exogenous treatment of BRs have been observed to stimulate the accrue of proline which results in better survival rates of plants under stress conditions (Sharma et al. 2017). The level of proline in plants is regulated by a fine tune balance between two critical genes viz. *pyrroline-5-carboxylate synth*etase1 (*P5CS1*) and *proline dehydrogenase* (*PDH*) involved in its anabolism and catabolism, respectively (Kaur et al. 2016b, 2017). Although, a direct link of BRs in the regulation of *P5CS1* and *PDH* genes has not been established yet, their possible interaction has been well realized through different BR signaling and biosynthetic mutants (Zeng et al. 2010). The mutant plants with mutation in the genes involved in BRs biosynthesis (*det-2*) and BR signaling (*bin-2*) were found to show the reduced accumulation of proline in response to abiotic stress conditions in plants. But whether this reduction is due to the possible direct interaction between the BR pathway and proline metabolic genes or it is due to the crosstalk between BR with other phtohormones like ABA is still need to be elucidated. Glycine betaine is another important metabolite that protects the chloroplast against osmotic injury (Kurepin et al. 2017).BRs have been found to significantly enhance the content of glycine betaine in plants upon exposure to stress by increasing the activity of the enzyme betaine aldehyde dehydrogenase (BADH) involved in its biosynthesis (Rattan et al. 2014). The content of mannitol also increases in plants under stress conditions in response to BR treatment (Rattan et al. 2014), but the precise mechanism how BRs influence mannitol content in plants still needs to be addressed.

3.1.5 Ion Homeostasis

Plants require an optimum level of a range of metal ions which regulate their growth and development (Dalcorso et al. 2014; Arif et al. 2016). But during the stress conditions specifically salinity and heavy metal stress, the level of these ions increases to toxic levels (Kaur and Pati 2017; Shahzad et al. 2018). Plants regulate the content of these ions during stress conditions through the process of ion homeostasis which removes these excessive ions from their cytosol (Kaur and Pati 2017). In the past few years, the role of BRs in the regulation of ion homeostasis has also been well

established. Exogenous 24-epibrassilide (EBR) treatment reduced the content of sodium (Na^+) and chloride (Cl^-) ions in *Solanum melongena* upon treatment of 90 mM of NaCl (Ding et al. 2012; Liu et al. 2014). Pre-treatment of BRs also prevented the leakage of potassium (K^+) ions by modulating the depolarization-activated K^+ ion channels under the influence of salt stress (Azhar et al. 2017). Further, BRs application leads to the reduced uptake and accumulation of heavy metal ions in plants (Shahzad et al. 2018). However, the detailed signaling cascade involved in BR mediated regulation of ion homeostasis needs to be elucidated.

3.1.6 Redox Homeostasis

The level of reactive oxygen species (ROS) molecules increases rapidly in response to various stresses in plants (Kundu et al. 2018; Saini et al. 2018). Traditionally, this surge in ROS was considered as a harmful effect of stress on plants. But, with the use modern research approaches, they have been well realized to play a dual role in plants (Kaur et al. 2016a). BRs have been reported to modulate the levels of ROS under the stress conditions that in turn regulates different stress adaptive signaling pathways (Jakubowska and Janicka 2017). BRs influence the activity of both ROS generating (NADPH oxidases) as well as ROS scavenging system in plants in response to stress (Jakubowska and Janicka 2017; Ahmad et al. 2018). Among different types of ROS, hydrogen peroxide (H_2O_2) is the critical signaling molecule that modulates the activities of an array of proteins involved in stress adaptation (Sies 2018). H_2O_2 is produced when superoxide dismutase (SOD) acts on the superoxide ions produced by NADPH oxidase (Kaur and Pati 2017). BRs have been reported to positively influence the activity of both NADPH oxidase and SOD enzymes in response to abiotic stress stimuli (Sharma et al. 2013b; Song et al. 2018). The BR signal perceived through the BRI-1 receptor induces the influx of calcium (Ca^{2+}) ions into the cytosol that inturn activates the NADPH oxidase enzyme. Ca^{2+} ions bind to the EF- motif present at the N-terminal of RBOH protein for inducing the production of ROS (Xia et al. 2015). Apart from Ca^{2+} dependent pathway, BRs increases the expression of genes involved in the mitogen activated protein kinase (MAPK) cascade that regulates the different NADPH oxidase genes (Zhu et al. 2013).

BRs also regulate the ROS antioxidant defense machinery for ameliorating the effects of oxidative stress in plants (Ahmad et al. 2018). Exogenous treatment of BRs results in the enhanced activities of different antioxidant enzymes in response to stress stimuli in plants (Sharma et al. 2013a). Further, they have been found to positively induce the accumulation of different non-enzymatic antioxidants in response to abiotic stresses (Sharma et al. 2017; Zhou et al. 2018). Application of BRs results in the increased levels of ascorbate and glutathione upon exposure to heavy metals and pesticide stress (Ahmad et al. 2018; Hou et al. 2018; Zhou et al. 2018).

Nitric oxide (NO) is a unique diffusible signaling molecule that orchestrates a plethora of processes involved in plant growth, development and stress adaptation (Yu et al. 2014). The exogenous application of BRs leads to increased production of

NO in plants that in turn offers abiotic stress resistance by positively regulating the ABA accumulation in plants (Zhang et al. 2010). Further, NO acts in the downstream of H_2O_2 in the BR signaling pathway that modulates antioxidant machinery for abiotic stress adaptation (Sharma et al. 2017).

3.2 BRs Mediated Regulation of Abiotic Stress Adaptation at the Molecular Level

Abiotic stress adaptation is a multi functional process regulated by a wide range of genes. In the past two decades, the potential of BRs in the modulation of these genes involved in the amelioration of the effects of abiotic stress has been well established (Table 15.1). These BR regulated genes can be classified broadly into two types viz. regulatory and functional genes.

3.2.1 BR Responsive Regulatory Genes

Regulatory genes mainly comprise of different kinases, phosphatases and transcription factors (TFs) (Kaur and Pati 2017). Kinases and phosphatases are the most critical regulatory enzymes responsible for phosphorylation and dephosphorylation, respectively of different signaling proteins (Ho 2015). BIN2 kinase and BSU1 phosphatase are the key players of BR mediated signaling in plants (Sharma et al. 2013c). Among other types of kinases, mitogen activated protein kinases (MAPK) cascade is of prime importance as it regulates a multitude of abiotic stress responsive pathways (Raja et al. 2017). Transcriptome analysis of genes induced in response to BR treatment under stress conditions illustrates the synergistic role of BRs in the induction of MAPK pathway genes in plants (Divi et al. 2016). Moreover, BRs regulate stomata development in crosstalk with MAPK genes during abiotic stress adaptation (Kim et al. 2012). PP2A is a critical phosphatase enzymatic protein that plays a dual role in BR signaling. It interacts with TF BZR1 for the induction of BR responsive genes and deactivates the BR pathway by dephosphorylation of BRI1 receptor (Di et al. 2011).TFs are the regulatory proteins that bind to motifs present in the upstream promoter region of different genes and regulate their expression. Major TFs that modulate the expression of different abiotic stress responsive genes include WRKY, GRAS, MYB/MYC, DREB, bZIP, NPR and NAC (Wang et al. 2016). Different genetic and molecular experiments have illustrated that BRs regulate these TFs either directly or indirectly for abiotic stress adaptation (Sharma et al. 2017). WRKY TFs comprise of one of the most critical plant specific super family of TFs that reprogram the transcription of many abiotic stress responsive genes (Banerjee and Roychoudhury 2015). They were initially known to operate either up-stream or down-stream of the different BR mediated signaling pathways (Bakshi and Oelmuller 2014). In a recent report, a group of abiotic stress responsive WRKY

TFs including WRKY46, 54 and 70 have been found to confer drought stress tolerance in conjunction with BR responsive TF BES1 (Chen and Yin 2017). The involvement of GRAS and MYB/MYC TFs in BR signaling pathways has also been well established using different genetic and microarray experiments (Goda et al. 2002; Tong et al. 2009). BR responsive BZR-1 directly modulates the expression of MYB TF that regulates lignin biosynthesis in response to stress stimuli (Rao and Dixon 2017). DREB family comprises of the most crucial TFs that govern the reprogramming of different abiotic stress responsive genes at the transcript level (Erpen et al. 2017). BRs application increases the expression of different DREB TFs under stress conditions that in turn leads to the accumulation of various stress ameliorating protective proteins (Kagale et al. 2007; Lata and Prasad 2011). bZIP are the phylogenetically conserved TFs involved in a plethora of plant processes (Droge-Laser et al. 2018). They have been known to integrate the endoplasmic reticulum (ER) stress signaling and BR signaling pathways in response to stress stimuli (Che et al. 2010). NAC belongs to the largest family of plant TFs (Fang et al. 2015). They were initially hypothesized to regulate abiotic stress adaptation in plants in crosstalk with other PGRs including ABA (Sharma et al. 2017). But in a recent report, NAC TF has been reported to negatively influence the BR biosynthesis in Arabidopsis (Jia et al. 2018). NPR is the TF predominantly involved in biotic stress adaptation, but their involvement in the amelioration of the effects of abiotic stress is also well documented (Sharma et al. 2017). They have been reported to act as a mediator of BR mediated temperature and salinity stress tolerance in crosstalk with salicylic acid (Divi et al. 2010).

3.2.2 BR Responsive Functional Genes

A number of genes serving diverse functions have been found to be differentially regulated by environmental stimuli in plants (Kaur and Pati 2017). BRs modulate the expression of many of these genes, including heat shock proteins (HSPs), lectins, late embryogenesis proteins (LEA), cytoskeleton proteins, dehydrins, those involved in redox homeostasis and PGR metabolism (Sharma et al. 2017). HSPs are the molecular chaperones which play a critical role in the first line of defence against environmental stress in plants (Haslbeck and Vierling 2015; Chen et al. 2018). BRs stimulate the synthesis of HSPs in mitochondria in response to stress in plants (Derevyanchuk et al. 2016). Further, BR regulated TF BES1 has been found to interact directly with HSP90 protein to modulate the levels of BR in Arabidopsis (Shigeta et al. 2015). Lectins are the sugar binding proteins known for their role in regulation of innate immune responses in different organisms. Recently, their role in signaling pathways involved in abiotic stress adaptation is also well realized. BR application boosts the accumulation of wheat germ agglutinin (WGA) in response to salt stress in an ABA mediated pathway in plants (Bajguz and Hayat 2009). They were also found to up-regulate the transcript level expression of three jacalin-related lectins1-3 (JAC-LEC1-3) in response to abiotic stress (Divi et al. 2016). In another investigation, the BR treatment was found to down-regulate the salt stress induced

expression of a mannose binding lectin *SalT* (Sharma 2014). Hence, the exact role of BR in modulation of lectins is still vague. The LEA family of multi functional proteins is well known for its role in abiotic stress tolerance (Mertens et al. 2018). BRs directly regulate the expression of different LEA encoding genes, including *rd29* and *erd10* in response to abiotic stress (Kagale et al. 2007; Duan and Cai 2012). Dehydrins are the major proteins belonging to the LEA family of proteins (Kosova et al. 2014). Pre- treatment with BRs leads to the accumulation of dehydrins in wheat that helps in the amelioration of drought and heavy metal stress (Allagulova et al. 2015; Shakirova et al. 2016). Reorientation of cytosketol structures in response to various environmental cues also plays a crucial role in stress tolerance (Lin et al. 2014). BR deficient mutant plants were found to have altered cell shape due to the possible role of BRs in modulation of the dynamics of cytosketol proteins (Liu et al. 2018). Along with these above mentioned proteins, BRs have been implicated in the regulation of the expression of ferritin (iron storage protein), TUD1 (E3 ubiquitin ligase), lipocalins (signaling protein) and abscisic acid stress ripening (ASR)- like protein (Sharma et al. 2017). Furthermore, the role of BRs in the modulation of genes responsible for micro-RNA mediated regulation of abiotic stress adaptation is emerging (Sharma et al. 2017).

4 Conclusion

The use of plant growth regulators has been found to be highly successful in increasing the vigour of plants under abiotic stress conditions. There has been an overemphasis in the literature on the stress ameliorative effects of BRs. These are ecofriendly chemicals which modulate a plethora of stress related responses for producing high-yielding abiotic stress resistant agriculture crops. Although, their role in the regulation of various cellular to molecular mechanisms is well established, further insights are necessary to unravel these complex intricate mechanisms in much detail.

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Chapter 16
Emerging Trends on Crosstalk of BRS with Other Phytohormones

Puja Ohri, Renu Bhardwaj, Ravinderjit Kaur, Shivam Jasrotia, Ripu Daman Parihar, Anjali Khajuria, and Nandni Sharma

Abstract Brassinosteroids (BRs), a class of steroidal hormones, play diverse roles in plant growth, development, signaling and defense against various biotic and abiotic stresses. It is broad spectrum key regulator in plants that participates in various molecular processes. Exogenous application of BRs vanish various constrains in the path of agricultural development. The present book chapter highlights the interaction and crosstalk of brassinosteroids with other phytohormones such as auxins, gibberellins, jasmonic acid, abscisic acid, salicylic acid, polyamines, ethylene and strigolactones in regulation of various physiological and developmental processes in plants. Various pathways reveal the versatile role of brassinosteroids in various hormonal interactions.

Keywords Brassinosteroids · Phytohormones · Crosstalk · Signaling

1 Introduction

Brassinosteroids (BRs) are endogenous steroidal phytohormones that have polyoxygenated structure and are found to regulate various physiological and metabolic processes at very low concentrations (Youn et al. 2018). It modulates various growth and development related processes such as microspore and seed germination, embryogenesis, regulation of cell division and differentiation, development and growth of thecae and pollen tubes, initiates flowering, regulate leaf senescence,

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vascular-differentiation, reproduction, root development, photomorphogenesis, and also respond to various biotic and abiotic stresses (Sreeramulu et al. 2013; Ahammed et al. 2014; Saini et al. 2015; Sharma et al. 2015, Li et al. 2016, 2017; Ahmad et al. 2018). In addition, BRs acts as important factors in stress modulation and defense in plants (Youn et al. 2018). Mutants deficient or plants insensitive to BRs exhibit a range of growth defects, including dwarf phenotypes (Vukašinović and Russinova 2018), photomorphogenesis in the dark, altered stomatal development and reduced male fertility (Ye et al. 2011; Kim et al. 2012). Because of their immense role for plant development and possible use as a tool for crop yield enhancement, BRs have attracted the attention of researchers in the past two decades. As a result, the BR signaling cascade is conceivably one of the preeminent characterized signaling pathways in plants (Youn et al. 2018). Endogenous regulation of BR is critical for various fundamental functions in plants. Furthermore, BRs act as a master regulator in plant disease resistance and defensive responses to pathogen attack. BRs also enhance tolerance to abiotic stress, including high temperature stress in a range of crop species (Ahammed et al. 2014). BRs maintain the polarization of cell membrane, proton pumping to apoplast and into a vacuole by stimulation of transmembrane ATPases, as well as increasing the efficiency of photosynthesis by increasing the level of CO_2 assimilation through Rubisco activity. Previous studies reveal that stress ameliorative effects of BR are attributed to BR-induced enhancement in secondary metabolism in plants (Ahammed et al. 2013; Çoban and GöktürkBaydar 2016; Li et al. 2016). BRs concentration is found to be higher in pollen grains and immature seeds, whereas low concentration is observed in mature organs. BR mutant plants show various types of deformities, visualised as plant height reduction, dwarfism, dark green leaves, male sterility, delayed flowering, and senescence (Youn et al. 2018). They also stimulate the expression of alfa- and beta-tubulin genes and affect reorientation of cortical microtubules, which influence arrangement of cellulose microfibrils. Leaf senescence proved to be stimulated by this group of hormones as well. Application of low concentration of BRs promotes rooting whereas at higher concentrations, root inhibition was observed. Moreover, BRs regulate the processes of photo- and skotomorphogenesis (etiolation) and are known to have a positive impact on reproductive development and regulation of flowering time. Many reports have shown their significant role in both stress-protection and stress-amelioration (Bari and Jones 2009; Bajguz 2010). Physiological functions of plants and their responses to biotic and abiotic stresses are also elucidated regarding the dramatic recent progress in understanding the BRs-other phytohormones crosstalk.

2 Brassinosteroids-Auxins

Innumerable phases of plant growth and development are regulated by BR-Auxin crosstalk (Hao et al. 2013; Saini et al. 2013; Chaiwanon and Wang 2015). Although this interaction was known for years, but the genetic and physiological evidences for exact mechanism underlying have been discovered only recently (Li et al. 2018a).

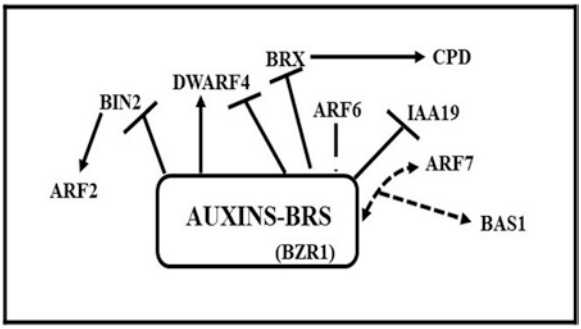

Fig. 16.1 Genes and factors involved in BR-Auxins crosstalk. Arrows shows induced effects, bars indicate negative effects, dashed lines indicate co-regulation while dotted line indicates direct control

These recent investigations have shown that an intact auxin signaling pathway aided by key signaling components such as *BZR1* (BRASSINAZOLE-RESISTANT 1), IAAs (INDOLE-3-ACETIC ACID) and *ARF*s (AUXIN-RESPONSE FACTORS) is necessary for BR responses (Li et al. 2018a, b). In *Arabidopsis*, Oh et al. (2014) reported that *BZR1* binds directly to the promoters of *IAA19* and *ARF7* thereby repressing the expression of *IAA19* while that of *ARF7* was induced (Fig. 16.1). In another investigation, microarray analysis by Youn et al. (2016) showed that in order to modify certain plant growth and developmental events, BR regulate a number of downstream target genes by using *IAA19* and *ARF7*. Additionally, in controlling hypocotyl cell elongation, *BZR1* and *ARF7* besides having a usual protein-DNA interaction, these two showed a physical protein-protein interaction and co-regulates PHYB-4 ACTIVATION-TAGGED SUPPRESSOR 1 (*BAS1*) transcription. Moreover, *BZR1* interacts directly with *ARF6* (another auxin response factor). This interaction induces their mutual activity and regulates a large number of common target genes. Thus, to coordinate plant growth and development, BR and auxin pathways are integrated via its signaling components *BZR1* and *ARF*s via multiple modes. In earlier investigations also, cooperation between *BIN2* (BR INSENSITIVE 2) and *ARF2* was established which showed link between BR and auxin for plant development and improvement (Vert et al. 2008). Additionally, *BRX* (BREVIS RADIX) which is necessary for rate limiting BR biosynthesis, positively controls the *CPD* (CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM) and *DWARF4* genes (Tanaka et al. 2005). Usually, BR represses *BRX* expression but external application of BR can recover *brx* mutant defects. Reversibly, auxins strongly enhance *BRX* gene expression but diminishes in *brx* mutants. This signifies the link between BR biosynthesis and auxin signaling involving expression of *BRX* (Mouchel et al. 2006). In lateral root development, again BRs and auxins shows synergistic roles since BRs plays a role in initiation only and auxins helps in both initiation as well as emergence of lateral root primordia (Casimiro et al. 2001; Bhalerao et al. 2002; Benkova et al. 2003; Bao et al. 2004).

On the other hand, antagonistic role of BR and auxin has also been reported in certain aspects. In *Arabidopsis*, external application of auxin adequately enhances the transcript levels of *DWARF4* gene which induces *BRX* protein to increase BR biosynthesis endogenously. However, auxin can constrain the joining of *BZR1* to

DWARF4 promoter (Chung et al. 2011; Yoshimitsu et al. 2011). But, when the required amount of BR has been synthesized, then BR itself causes feedback inhibition of *DWARF4* (Maharjan et al. 2011). Furthermore, for optimum root growth, transcription factor *BZR1* is required and is constituted mainly by three factors viz. local BR catabolism, synthesis of auxin and signaling of BR. Here, *BZR1* stimulates the genes that are expressed in transition-elongation zone, but suppress genes of the quiescent centre along with stem cells that surround it. But, auxins show reversible effect to BR on spatiotemporal gene expression (Chaiwanon and Wang 2015).

3 Brassinosteroids-Abscisic Acid

It is well acknowledged that ABA and BRs play antagonistic roles in plant growth and development. In plants, ABA inhibits seed germination and regulates seed dormancy during embryo maturation. While, BR boosts seed germination and post-germinative growth processes (Steber and McCourt 2001; Finkelstein et al. 2008; Hu and Yu 2014; Wang et al. 2018). However, physiological, biochemical and genetic studies conducted so far revealed that both BR and ABA jointly control the expression of nearly 100 genes but detailed molecular mechanism of whole cross-talk needs to be explored (Nemhauser et al. 2006; Zhang et al. 2009). Recent investigations reported physical interaction between *BIN2* and *ABI5* (ABSCISIC ACID-INSENSITIVE5; key ABA signaling component) where *BIN2* positively controls ABA responses (Fig. 16.2). However, improper response of ABA was observed when mutant proteins were formed due to mutations on *ABI5* for the *BIN2* phosphorylation sites. Thereby, affirming that *ABI5* is phosphorylated and stabilized by *BIN2*. On the other hand, when BR was applied, ABA mediated response was antagonized by controlling *ABI5* by *BIN2* (Hu and Yu 2014). In another study, *AIB3* transcription was inhibited by the formation of transcriptional repressor complex such as *BES1*, *TPL* (TOPLESS) and *HDA19* (HISTONE DEACETYLASE 19) that aids in histone deacetylation of *ABI3* chromatin (Ryu et al. 2014). Furthermore, the binding of *BZR1* to G-box of *ABI5* promoter, suppresses the expression of *ABI5* thereby increasing the sensitivity of plant to ABA. However, in the mutant

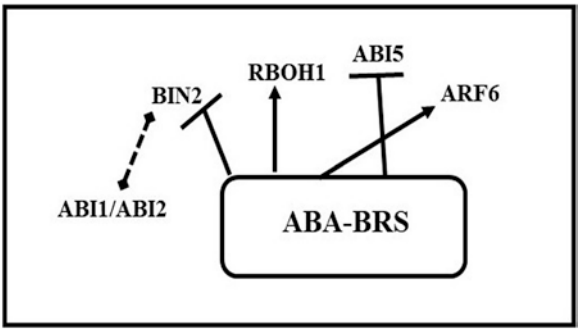

Fig. 16.2 Genes and factors involved in BR-Abscisic acid crosstalk. Arrows shows induced effects, bars indicate negative effects, dashed line indicates direct interaction

*bzr1-1D*the sensitivity was reduced (Yang et al. 2016). Recently, in vitro mimicking of ABA signal transduction and RNA-sequencing analysis demonstrated that in order to control the phosphorylation of BES1, both ABI1 and *ABI2* interacts as well as dephosphorylate *BIN2*. Analysis carried on revealed that ABA through ABA receptors promotes phosphorylation of *BIN2* by suppressing *ABI2*. Moreover, ABA obstructs BR signaling by using primary signaling components of ABA along with its receptors and *ABI2* (Wang et al. 2018).

Synergistic interactions between BR and ABA have been documented in mutant studies (Zhou et al. 2014). It was observed that both BR and ABA activated the generation ofH_2O_2, expression of *RBOH1* (*RESPIRATORY BURST OXIDASE HOMOLOG1*), activity of NADPH oxidase and in conciliating heat and oxidative stress tolerance. In case of ABA-deficient mutant *notabilis* (*not*), BR enhances these responses while in BR synthesis mutant *d^im*, these were strong and lasted for longer time (Zhou et al. 2014).

4 Brassinosteroids-Gibberellins (GA)

In order to coordinate varied physiological processes including seed germination, stem elongation, hypocotyl elongation, expansion of leaf and hypocotyl, maturation of pollens, flowering, plant cell elongation, seedling growth etc., BRs interacts with GA (Ueguchi-Tanaka et al. 2007; Sun et al. 2010; Sun 2011; Li et al. 2012; Tong et al. 2014; Hu et al. 2017; Fig. 16.6). It is one of the best studied crosstalk between different hormones and *BZR1*/*BES1* family plays an important role by interacting both via protein-DNA and protein-protein interactions (Vanstraelen and Benkova 2012; Li and He 2013; Li et al. 2018a, b).

In *Arabidopsis* and rice, during direct interaction *BZR1*/*BES1* binds to the promoters of numerous GA metabolic genes and then controls their expression (Li et al. 2018a, b; Fig. 16.3). In *Arabidopsis*, to control the expression of GA biosynthetic gene *GA20ox1* (*GA 20-oxidase 1*) both *BZR1*/*BES1* joins to its non-E-box motif in a BR induced manner (Unterholzner et al. 2015). Alternately in rice, *BZR1* promotes cell elongation by directly joining to *GA20ox-2*, *GA30ox-2*, *GA2ox-3* promoters to enhance GA biosynthesis and repressing its inactivation (Tong et al. 2014). Direct interaction between BR-GA crosstalk have also been identified in numerous studies where *BZR1*/*BES1* physically interacts with the master negative regulator of GA signaling, the *DELLA* proteins (Bai et al. 2012; Gallego-Bartolome et al. 2012; Li et al. 2012). In another investigation using ChIP (chromatin immune precipitation) study, of the five *DELLA*-encoding genes, four genes viz. *RGA* (REPRESSOR of GAI-3), *GAI* (GIBBERELLIC ACID INSENSITIVE), *RGL1* and *RGL3* were directly targeted by *BZR1* thereby suggesting direct control of *DELLA*-encoding gene expression (Sun et al. 2010).

Investigations pertaining to cell expansion during photomorphogenesis revealed synergistic role of BR and GA simultaneously through the occurrence of BR-activated *BZR1* and GA-inactivated *DELLA* transcription regulators. In the

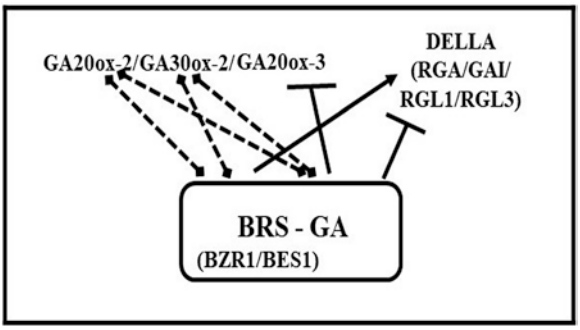

Fig. 16.3 Genes and factors involved in BR-GA crosstalk. Arrows shows induced effects, bars indicate negative effects, dashed lines indicate direct effects

study, it was found that BR signaling is essential for GA promoted cell elongation. On the contrary, GA-deficient dwarf phenotype can be suppressed by BR or active *BZR1* (Gallego-Bartolome et al. 2012). Also, in both in vitro and in vivo studies, direct interaction of *DELLA* with *BZR1* was seen leading to the inhibition in recognizing environmental signals necessary for elongation of cell and etiolation of seedling (Bai et al. 2012; Gallego-Bartolome et al. 2012; Li and He 2013). Similar investigations for strong GA response due to presence of active *BZR1* protein have also been carried on which reported that the expression of *GA20ox* was responsive to exogenous BR, thereby, demonstrating synergistic effects of BRs and GA (Stewart Lilley et al. 2013).

Antagonistic role of BRs and GA have also been reported in rice root immunity during root oomycete, *Pythium graminicola* infection. It was observed that the pathogen used BRs as virulent factors thereby controlling BR machinery in rice to inflict symptoms of disease (Nakashita et al. 2003; Bajguz and Hayat 2009). Furthermore, the above immunosuppressive effect of BRs was explained due to opposite GA crosstalk by increasing the stability of rice *DELLA* protein *OsSLR1* (SLENDER RICE1) which acts as an important regulator of resistance for *P. graminicola* in rice (Li and He 2013). In another study, it was observed that the expression of *OsSLR1* can be enhanced both by pathogen infection as well as by exogenous treatment of BR. Thus, these studies suggested that BRs may constrict the GAs regulated defense responses in rice by interfering in GA signaling (De Vleesschauwer et al. 2012).

5 Brassinosteroids-Jasmonic Acid (JA)

Brassinosteroids are found to promote rice plants' susceptibility to Brown Plant Hopper (BPH) infestation by modulating the Jasmonic acid (JA) pathway (Pan et al. 2018). It was found that BR pathway was inhibited by BPH whereas JA pathway was found activated. qRT-PCR exhibited that decrease in BZR1 (BRASSINAZOLERESISTANT 1) – a BR signaling component and BRI – 1 (BR insensitive 1) -a BR receptor was observed post BPH infection (24 h). Also, for the genes (D2 and D11) related to biosynthesis of BR, similar expressions have been

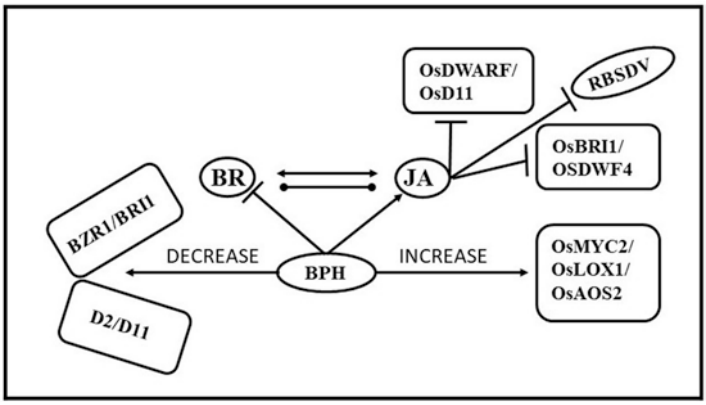

Fig. 16.4 Genes and factors involved in BR-JA crosstalk. Here, arrows show induction/activation, bars show suppression, bar with dots show antagonistic relation while double arrow heads show synergistic relation

recorded (Feng et al. 2016). Like other phytohormones, JA also plays a role in providing defense to the plants against insects (Aljbory and Chen 2018), so investigation of genes related to such defensive ways was also done. It was found that after 24 h of BPH infection, expression of OsMYC2, OsLOX1 and OsAOS2 was found enhanced unexpectedly in rice plants which were also treated with BL. Induction of these JA related genes was observed in BR overproducing plants whereas their suppression was observed in the BR deficient plants post BPH infestation (Pan et al. 2018; Fig. 16.4).

Hormonal crosstalk of BRs with JA also plays an important role in the developmental processes of plant and its stress responses as reported by Ren et al. (2009), Campos et al. (2009), Yang et al. (2011), Kim et al. (2018) and Per et al. (2018). Nahar et al. (2013) also, expressed antagonistic interaction between BR and JA in *O. sativa*. It was revealed that OsDWARF and OsD11 (BR biosynthetic genes) were negatively regulated by JA in the roots of *O. sativa* and on the other hand, JA biosynthesis was also affected negatively where OsAOS2 expression was found down regulated. Therefore, BR biosynthesis was found suppressed by JA in a mutually antagonistic manner. Similarly, DWARF4 expression was also found negatively regulated in CoI1-dependent manner in *Arabidopsis*, where again BR was found to inhibit root inhibition and JA – dependent gene induction (Ren et al. 2009; Kim et al. 2011, 2013; Fig. 16.4).

Another study was conducted by He et al. (2017) regarding JA and BRs interaction where suppression in BR mediated Rice Black Streaked Dwarf Virus (RBSDV) infection was observed by the treatment of JA in rice plants. Application of Brassinazole or Methyl Jasmonate to the infected plants through foliar spray significantly reduced RBSDV infection whereas, it increased when treated with epibrassinolide. This BR mediated susceptibility and JA mediated resistance was demonstrated by using mutants- coi1-13 and Go. Efficient suppression in the expression of BR genes due to methyl jasmonate application was related to OsCO/1 (JA coreceptor) (Fig. 16.4).

Synergistic relationship of JA with BRs was also observed in enhancing the tolerance in plants against abiotic stress. In rice plants under stress, Kitanaga et al. (2006) found improvement in the jasmonic acid level due to BR. Effect of brassinazole was also found on JA level by Peng et al. (2011) where they observed anthocyanins accumulated due to JA hindrance in *Arabidopsis*. When effect of brassinosteroid was low, the transcript level of JA initiated signaling gene and JA biosynthesis quality genes were found down regulated but when focus of BR was high, both the transcript level of JA signaling as well as biosynthesis gene were found up regulated (Peng et al. 2011). Moreover, exogenously applied JA down regulated OSBRI1 and OsDWF4 (BR signaling and biosynthesis genes) thereby exhibiting counter communication in between JA and BR in roots of rice plants (Nahar et al. 2013).

6 Brassinosteroids-Ethylene

Brassinosteroids are found interacting with ethylene antagonistically (Banerjee and Roychoudhary 2018). Ethylene is reported to play a role in gravitropic reorientations in seedlings and in fruit ripening where such gravitropic reorientations were observed during desiccation stress (Vandenbussche et al. 2013) whereas, BRs shows negative regulation of shoot gravitropism. Buer et al. (2006) also reported the BR-ethylene antagonistic relation during root gravitropic responses and found the suppressive effect of ethylene and promotion by BRs. BRs and ethylene were also found in an antagonistic relationship in terms of regulation of AOX (alternative oxidase) activity in *Carica papaya* during fruit ripening (Mazorra et al. 2013). The activity of antioxidative oxidase is in response to the changes in the phytohormone-mediated signals, electron transport chain, metabolites which are associated with respiration (respiratory metabolites) and reactive oxygen species (Vanlerberghe 2013). Moreover, ethylene signaling is also regulated by RAVL1 via activating the EIL1 in rice where RAVL1 is an upstream component of brassinosteroid signaling and biosynthesis (Zhu et al. 2018; Fig. 16.5).

Effect of overproduction of ethylene by using eto1-1 (ethylene over producer 1) on other plant hormones has been also investigated (Li et al. 2018b). Hormonal contents (for various hormones) and transcript level of their associated biosynthetic genes were determine in wild type (WT) plants and 10 days old *Arabidopsis* eto1-1 mutant and then comparative analysis was made between these two. Overproduction of ethylene didn't affect JA level which was found to be due to the unaltered expression of allene oxide synthase (a rate limiting JA biosynthetic gene) (Li et al. 2018a, b).

Interaction of ethylene and BRs was also observed by Zhu et al. (2016) in tomato fruits under salt stress. This interaction was mediated by H_2O_2, as ROS scavenger when applied, underwent significant blocking of ethylene production induced by brassinosteroids. So, due to reduction in ethylene production by using 1-MCP, the reversion in tolerance (BR-induced) to salt stress was observed thereby indicating the downstream action of ethylene to exhibit tolerance against salt stress (Banerjee and Roychoudhary 2018).

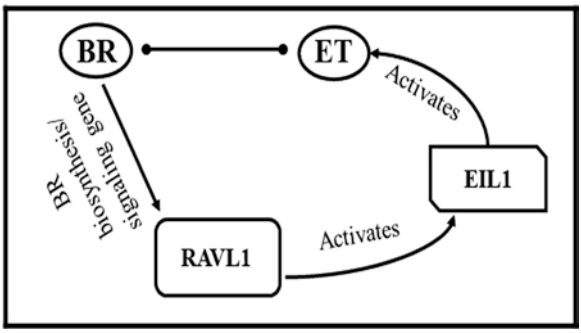

Fig. 16.5 Genes involved in BR-ET crosstalk where arrows show activation while the bar shows antagonistic relation

7 Brassinosteroids-Salicylic Acid (SA)

BR plays a significant role in plant response to both biotic and abiotic stress and at the same time SA shows a remedial effect during abiotic (salinity) stress (Ahmad et al. 2017) (Fig. 16.6). Studies have shown that crosstalk between BR and SA exist via non-expressor of pathogenesis-related genes 1 (NPR1); which regulates SA mediated genes involved in plant defence (Ohri et al. 2015). NPR1 is a redox- sensitive protein which is also an important component of EBR- mediated increase in salt tolerance and thermotolerance. NPR1 bring about this stress tolerance by controlling BZR1 and BIN2; which are important components of BR signaling (Divi et al. 2010). Further it has been found that NPR1 protein is not required for induction of PR-1 (PATHOGENESIS-RELATED1) gene expression mediated by EBR. This shows that BR can show anti-stress activity independently also (Divi et al. 2010). Earlier studies on tobacco plant has shown that BR increases the resistance against Oidium sp. (the fungal pathogen), *Pseudomonas syringae pv. Tabaci* (the bacterial pathogen) and *tobacco mosaic virus* (the viral pathogen) independent of SA (Nakashita et al. 2003). Similar studies on rice plant have shown that BR increases resisitance against *Xanthomonas oryzae* (the bacterial pathogen) and *Magnaporthe grisea* (the fungal pathogen) (Nakashita et al. 2003). Earlier it was thought that the Plant innate immunity was positively regulated by BR. But some studies have shown that *Pythium graminicola* uses BR as virulence factor and exploits BR machinery of rice plant to cause disease, which shows a negative crosstalk between BR and SA (De Vleesschauwer et al. 2012). Moreover studies have shown that suppression of SA defence responses mediated by BR occur downstream of SA biosynthesis and upstream of OsWRKY45 and NPR1 gene (De Vleesschauwer et al. 2012). SA induces the expression of Transcription factor OsWRKY45 which plays an important role in plant stress response (Huangfu et al. 2016). Studies on *Brassica juncea* L. seedlings has revealed that lead (Pb) toxicity is reduced by a collective effect of salicylic acid and 24-epibrassinolide, thereby advocating for modulating various metabolites (Kohli et al. 2018).

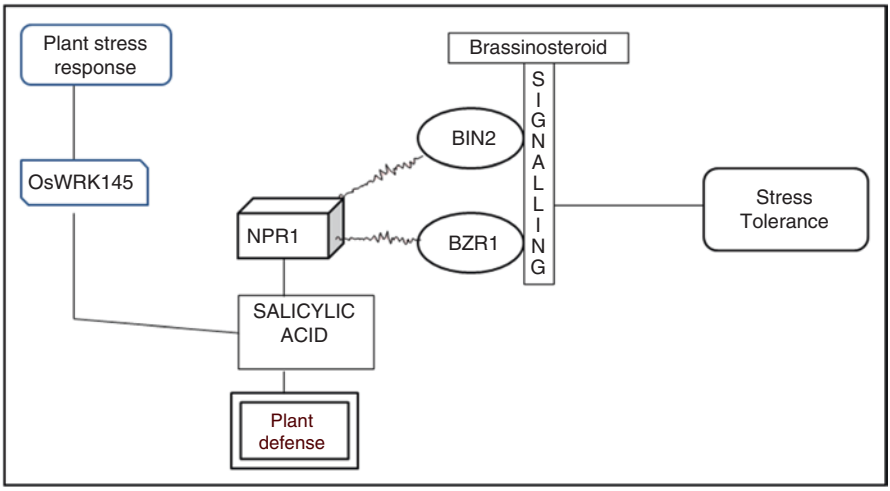

Fig. 16.6 Genes involved in BR-SA Crosstalk. Uneven lines indicates control over genes and straight bar indicates induction of genes

8 Brassinosteroids-Polyamines (PAs)

Brassinosteroids play an important role in stem elongation and polyamines are associated with ageing and diseases (Fig. 16.7). It has been established that Brassinosteroids signaling or biosynthesis pathways are not affected by Polyamines (Anwar et al. 2015). A crosstalk between BR and PA is at its beginning stage. But a co-application of both has shown better results in copper stress tolerance and nodulation. Studies have shown that an exogenous application of EBr and Spd can enhance Cu tolerance in radish. Their collective application reduces the Cu uptake which can be associated with down regulation of genes like RsCOPT2 (6.9-fold) and RsCOPT1 (220-fold) (Choudhary et al. 2012). RsHMA5 is another gene involved in Cu assimilation (Andres-Colas et al. 2006). It has been found that a combined application of EBr and Spd decreased the expression of RsHMA5 by 3.9-folds where as it increased the expression of RsCCH1 genes by 1.8-folds (Choudhary et al. 2012). Studies have also shown that 24-epibrassinolide (EBL) and polyamines (PAs) play an important role in the regulation of nodule formation in plants. In 2016, Lopez-Gomez et al. has reported that in response to EBL treatment to the roots there is an increase in the level of PAs in shoot which collectively suppresses the nodule formation in rhizobium-legumes. Another example of EBL and PAs crosstalk is found in growth of plants under stress. Under salt stress, EBL increases the level of spermine (Spm) which further restores growth (Lopez-Gomez et al. 2016). These studies suggest a great potential of crosstalk between BR and PAs which requires further studies to establish the modulation of the expression of various genes encoding PA enzymes and its effects on other phytohormones. Studies have also revealed that in rice, phytochelatin synthesis may be influenced by polyamines under Cd stress (Pal et al. 2017).

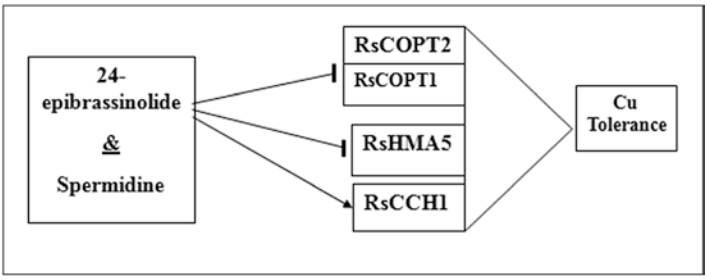

Fig. 16.7 Genes involved in Br-PAs Crosstalk. Here, Bars indicates inhibition of gene expression and arrow indicates stimulation of gene expression

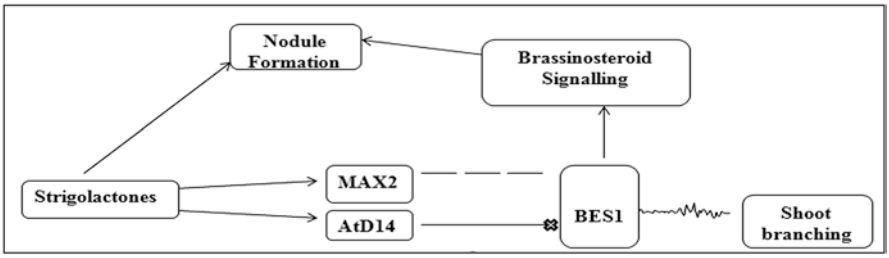

Fig. 16.8 Genes involved in Br-SL Crosstalk. Here, Uneven line indicates control over shoot branching, arrow indicates interaction between genes and cross indicates degradation

9 Brassinosteroids-Strigolactones (SL)

Strigolactone is a terpenoidphytohormone that plays a significant role in suppression of shoot branching (Fig. 16.8). A crosstalk between BR and SLs revolves around a common transcription factor BES1. BES1 (bri1-EMS-suppressor 1) is a positive regulator of BR signaling pathway (Yin et al. 2002). MAX2 is a key component of SL signaling which interacts with BES1 and regulates SL-responsive gene expression. Moreover, AtD14, a putative receptor of SLs degrades the transcription factor BES1. Removal of BES1 from max2-1 mutant results in suppression of branching phenotype. This shows that both BR and SLs regulate BES1 distinctly in order to control some specific developmental processes related to shoot branching (Wang et al. 2013). Formation of nodules in leguminous plants is another example where both BR and SL show positive interaction. Studies have shown that BR has a positive role in nodule formation in pea plant (Ferguson et al. 2005). Similarly, SL has also shown a positive result in development of nodules in pea plants (Soto et al. 2010; Foo and Davies 2011; Liu et al. 2013). It shows a crosstalk between BR and SL in nodule formation which is genetically controlled by AON (autoregulation of nodulation) pathway. But studies on mutant pea plants have shown that BR and SL plays a key role in nodule formation but act independent of AON pathway (Foo et al. 2014).

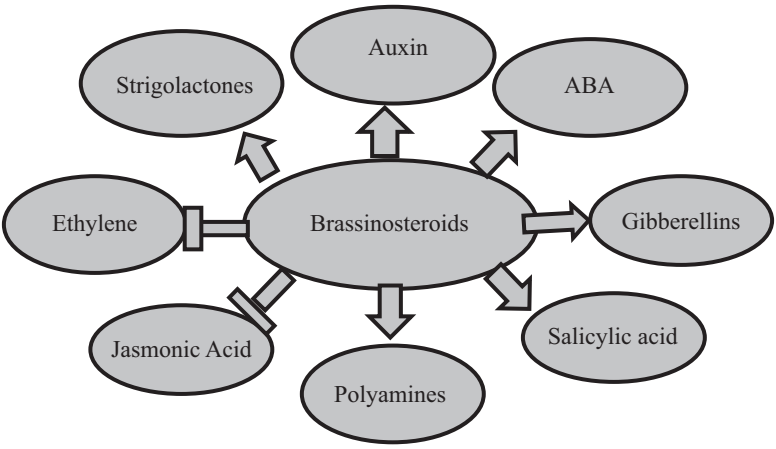

Fig. 16.9 Hormonal crosstalk of Brassinosteroids with other phytohormones. BRs showed synergistic behaviour with auxin, ABA, gibberellins, salicylic acid, polyamines and strigolactones whereas antagonistic behaviour with ethylene and jasmonic acid

10 Conclusion

Brassinosteroid acts as a powerful plant growth regulator due to its involvement in various functions. The wide range of functions is accredited to its manifold targets and complex regulatory mechanisms. Serious and rigorous global efforts are being carried out in understanding the complexity of the hormonal crosstalk of BRs with other phytohormones. The pace of BR research is accelerating rapidly, and with the proliferation of cloned genes and advances in micro-chemical techniques, the range of experimental approaches in understanding BR action continues to expand. Hormonal crosstalk of BRs with other phytohormones showed growth promoting effects as well as inhibitory effects (Fig. 16.9). Although there is vast knowledge of BRs but there are unravelling interactions with these phytohormones will add new dimension to BR research in future.

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