REVIEW

Physiological and molecular mechanisms of brassinosteroid-induced tolerance to high and low temperature in plants

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Abstract

Brassinosteroids (BRs) are plant hormones that were isolated for the first time in the 1970s. This group currently includes more than 70 compounds that differ in their structure and physiological activity. BRs are present in plants in a free form or in the form of conjugates. BRs are known as plant growth regulators, but they also play a role in the plant response to environmental stresses. In the case of plants that are exposed to low/high temperature, exogenous BRs can counteract growth inhibition and reduce biomass losses as well as increase plant survival. BRs show a multidirectional activity in regulating the metabolism of plants exposed to extreme temperatures. The following BRs actions can be distinguished: changes in membrane physicochemical properties, regulation of the expression of selected genes (including stress-responsive genes), as well as indirect effects on metabolism through other hormones or signalling molecules (such as hydrogen peroxide). This review summarizes the current knowledge about the effects of BRs on the physiological and biochemical processes that occur in plants during exposure to low or high temperatures.

Additional key words: plant acclimation, cell membranes, cold, freezing, gene expression, heat, oxidative damage, photosynthesis, proline.

Introduction: discovery, occurrence, biosynthesis and hormonal nature of brassinosteroids

Brassinosteroids (BRs) were isolated for the first time from *Brassica napus* pollen (Grove *et al.* 1979). This group currently includes more than 70 steroids that differ in structure and physiological activity. BRs are present in a free form and as conjugates in higher plants and algae (Bajguz and Tretyn 2003). In higher plants, BRs occur in all organs in a nano- and pico-gram amount per gram of fresh mass. BRs can be divided into three main groups depending on the number of carbon atoms in the molecule: C_{27}, C_{28}, and C_{29}. Sterols are precursors in the synthesis of all BRs, and campesterol and 24-β-methyl-cholesterol are considered to be precursors of C_{28} BRs (Fujioka and Yokota 1997, Takatsuto *et al.* 1999, Park *et al.* 2000, Schaller 2003, Kim *et al.* 2004, 2006), cholesterol of C_{27} BRs (Fujioka and Yokota 1997, Schaller 2003, Kim *et al.* 2004, 2006), and sitosterol of C_{29} BRs (Fujioka and Yokota 1997, Takatsuto *et al.* 1999, Schaller 2003, Kim *et al.* 2006) (Fig. 1). The main and the first BR biosynthetic pathway was discovered in cultured cells of *Catharanthus roseus* and *Arabidopsis*

thaliana for BR type C_{28} brassinolide (Yokota *et al.* 1990, Fujioka and Yokota 1997, Clouse 2015). The intermediates in this pathway are cathasterone, teasterone, typhasterol, and castasterone in the early C6 oxidation pathway and 6-deoxocathasterone, 6-deoxoteasterone, 6-deoxotyphasterol, and 6-deoxocastasterone in the late C6 oxidation pathway (Fujioka and Yokota 1997).

Brassinosteroids are perceived by the cell surface receptor kinase, BRI1. Binding BR to the extracellular domain of BRI1 induces activation and dimerization of kinase with its co-receptor kinase BAK1 (BRI1-associated receptor kinase1) or its homologue SERK1 (somatic embryogenesis receptor kinase 1). Sequential trans-phosphorylation between the kinase domains of BRI1 and BAK1 activates the kinases and BRI1 in turn phosphorylates the members of two groups of plasma membrane-anchored cytoplasmic kinases, BSK1 (brasinosteroid-signaling kinase1) and CDG1 (constitutive differential growth1). CDG1, and possibly also BSK1, phosphorylates the BRI1-supressor1 (BSU1) phosphatase, which then dephosphorylates and inactivates the GSK3-like kinase brassinosteroid insenitive2 (BIN2). BIN2 directly regulates the phosphorylation status and nuclear accumulation of two homologous transcription factors, BZR1 (brasinazole resistant1) and BES1 (bri1-EMS-suppressor1) (Wang *et al.* 2006, Li and Jin 2007, Wang *et al.* 2014).

Although it was already known in the 1970s that the exogenous BRs stimulates the elongation of stems and cell division in plants (Mitchell *et al.* 1970, Grove *et al.* 1979), these compounds were recognised as a new class of plant hormones only in the 1990s when several genes responsible for the biosynthesis and perception of BRs were discovered. The mutations that are associated with the loss of function of these genes usually lead to growth disturbances (including short hypocotyl, dwarfing of seedlings and mature plants) or to dark green leaves (Yang *et al.* 2011). The ability of these compounds to
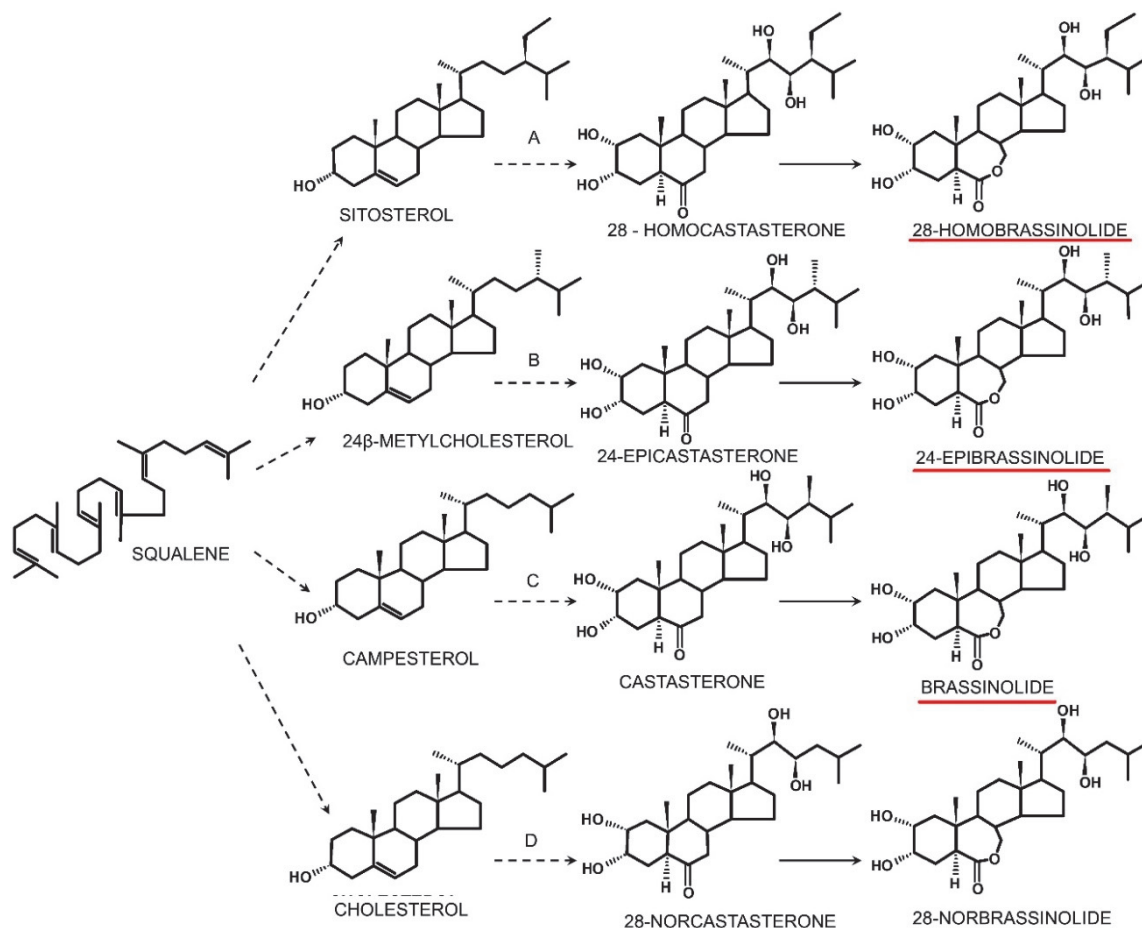
Fig. 1. Simplified model of biosynthesis of selected BRs in plants: *A* - type C_{29} (Takatsuto *et al.* 1999, Schaller 2003, Kim *et al.* 2006), *B* and *C* - type C_{28} (*B* - Winter *et al.* 1999, Park *et al.* 2000, *C* - Takatsuto *et al.* 1999, Park *et al.* 2000, Schaller 2003, Kim *et al.* 2004, 2006), *D* - type C_{27} (Schaller 2003, Kim *et al.* 2004, 2006). BRs tested in experiments with high and low temperature are underlined.

counteract the negative effects of environmental stresses in plants should, however, be considered as a particularly interesting characteristic of BRs due to the possibility of their practical application in agriculture/horticulture (Krishna 2003, Song *et al.* 2016, Shu *et al.* 2017). During their life cycle, plants are usually exposed to different biotic and abiotic stresses, which may result in a decrease in the yield. Among the abiotic stresses, too low or too high temperatures are particularly serious problems in agriculture and horticulture. Species, such as cucumbers, tomatoes, or maize are very sensitive to cold, while frost, especially when occurs under insufficient snow cover on fields, can cause significant yield losses of winter crops, *e.g.*, oilseed rape or cereals. High temperature, in turn, is particularly dangerous to plants when combined with drought. Table 1 Suppl. summarises the data for the temperature ranges, species, and brassinosteroids that were used in order to ameliorate the plant damage.

The structure and properties of cell membranes

Low temperature significantly increases membrane lipid peroxidation due to the increased production of free radicals. Membrane lipid peroxidation is usually estimated based on a spectrophotometric determination of the malondialdehyde (MDA) content (the final oxidation product of polyunsaturated fatty acids) by a reaction with thiobarbituric acid (TBA), which generates a coloured TBA-MDA complex (Hodges *et al.* 1999). An increase in membrane lipid peroxidation was observed in tomato seedlings exposed to day/night (d/n) temperatures of 12/6 °C for 12 d (Cui *et al.* 2016), as well as in tomato fruits that were stored at 1 °C for 3 weeks (Aghdam *et al.* 2012). Spraying of seedlings or fruits with 24-epi-brassinolide prior to cold considerably decreased the MDA content compared to seedlings or fruits without BR application. A similar phenomenon was found for the fruits of pepper, eggplant seedlings, and cucumber plants (Hu *et al.* 2010, Wang *et al.* 2012, Jiang *et al.* 2013, Wu *et al.* 2014a). Moreover, cell suspensions of *Chorispora bungeana* that were cultured with 24-epibrassinolide (0.05 mg dm^{-3} or 0.1 μM) showed a marked decrease (by 20 to 40 %) of MDA content compared to the culture that was grown at 0 - 4 °C (for 3 - 5 d) but without 24-epi-brassinolide (Liu *et al.* 2009, 2011). The participation of BRs in membrane protection against peroxidation in cold was confirmed by studies on BR mutants. Qu *et al.* (2011) applied 0 °C (24 h) to *A. thaliana* plants and found that the *bri1-116 (-/-)* mutant (BR insensitive null allele of the BR receptor BRI1) reached significantly higher MDA content than the Col-0 wild type. Finally, in tomatoes with a partial deficiency of BRs that were exposed to cold, membrane lipid peroxidation was increased while the overexpression of the BR biosynthetic gene *Dwarf* or the application of 24-epibrassinolide reduced the cold-induced oxidative damage (Xia *et al*. 2017).

In addition to MDA accumulation, ion leakage measurement can be used, which indicates cell membrane permeability. Increased ion leakage is usually interpreted as membrane damage. In the primary leaves of oilseed rape kept at 2 °C, 24-epibrassinolide (0.05 and 1 μM) decreased ion leakage (by about 80 %) compared to the control (Janeczko *et al.* 2007). The authors noted temperature-, tissue-, and concentration-dependent effects of BRs on this parameter. Mango fruits that are stored at 5 °C for 28 d have electrolyte leakage about 40 %, but BR treatment (10 μM) prior to storage reduced this value to 20 % (Li *et al.* 2012). In tomato fruits, stored at 1 °C for three weeks, electrolyte leakage was approximately 85 %, whereas this value was decreased to 70 % in fruits that were treated with 3 μM brassinolide (Aghdam *et al.* 2012). A cell suspension of *C. bungeana* cultured at 0 and 4 °C (3 d) with 24-epibrassinolide (0.1 μM) had approximately a 20 % lower ion leakage compared to the stressed cultures without hormone (Liu *et al.* 2011). Qu *et al.* (2011) applied 0 °C for 24 h to *A. thaliana* plants and found that the *bri1-116 (-/-)* mutant reached approximately 50 % higher ion leakage than the Col-0 wild type. Eremina *et al.* (2016) measured electrolyte leakage in cold-acclimated and frozen leaves of *A. thaliana* wild type and two mutants: BR-hyper-signalling line (*BRI1oe*) and BR-signalling defective mutants (*bri1-301*). After freezing at -10 °C (6 h), *BRI1oe* had a lower (by about 20 %) whereas *bri1-301* had a higher (by about 20 %) electrolyte leakage in comparison to the wild type.

Typically, during the process of acclimation to low temperature, membrane structural changes aim at the increasing proportion of unsaturated fatty acids, which improves the fluidity of the membranes. Brassinosteroids may affect the fatty acid composition of cell membranes. An increase in content of unsaturated fatty acids, linoleic acid C18:2 and linolenic acid C18:3, in mango fruits that were treated with brassinolide and stored at 5 °C was higher than in the control. Simultaneously, the content of palmitic acid C16:0, palmitoleic acid C16:1, stearic acid C18:0, and oleate acid C18:1 was slightly reduced by BR (Li *et al.* 2012). In monogalactosyldiacylglycerols (MGDG), a fraction that was isolated from a winter oilseed rape green callus acclimated at 2 °C, 24-epibrassi-nolide decreased the molar percentage of saturated fatty acids and increased the percentage of unsaturated fatty acids (18:1, 18:2, and 18:3) compared to a callus grown in the cold without BR (Janeczko *et al.* 2009). The MGDG fraction (next to the fraction of digalacto-syldiacylglycerols) is considered to be abundant especially in the thylakoid membranes (Johnson and Williams 1989).

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Li *et al.* (2012), analysed the plasma membranes of mango fruits that were stored at 5 °C for 28 d by measuring the phase transition temperature using differential scanning calorimetry (DSC) and polar lipid fluidity by electron paramagnetic resonance (EPR). The phase transition temperature in the fruits that are not treated with brassinolide is constant and about 6.5 °C during the storage period, while phase transition temperature in brassinolide-treated plants is markedly decreased. Polar lipid fluidity is determined on the basis of the EPR spectra, which enable the order parameter (S) and rotational correlation time (τ_c; inversely proportional to membrane fluidity) to be calculated. A gradual increase in the value of both parameters is observed with storage time in brassinolide-untreated plants, and there is a decrease in S and τ_c in plants to which this BR was applied. This indicates increased membrane fluidity in the presence of brassinolide. These results correspond to last studies, which were done using a Langmuir bath by Filek *et al.* (2017). When 24-epibrassinolide and 24-epicastasterone are introduced into lipid monolayers, their physicochemical properties changed. Lipids for monolayer formation were isolated from the leaves of wheat growing at 5 and 20 °C and 24-epibrassinolide incorporation into lipid monolayer resulted in the formation of more flexible surface structures (Filek *et al.* 2017). However, 24-epicastasterone induced the opposite effect. These results show importance of the chemical structure of BRs for their interaction with membranes.

High temperature also increases lipid peroxidation. In melon seedlings, a d/n temperature of 42/32 °C for 2 d increased the MDA content compared to the control (Zhang *et al.* 2014). Pretreatment with 24-epibrassinolide (1 mg dm^{-3}) reduced MDA accumulation by 35 %. A similar effect was observed in rice plants grown at high d/n temperatures of 40/30 °C for 7 d (Thussagunpanit *et al.* 2015b). In eggplants at d/n temperatures of 43/38 °C (8 d), 0.1 μM 24-epibrassinolide reduced the MDA content by 16 % compared to plants that were not treated (Wu *et al.* 2014b). Mazorra *et al.* (2011) studied

the effect of 45 ºC (4 - 8 h), on tomato plants (wild type and brassinosteroid mutants (mutant with altered BRs sensitivity *curl3^{-abs}*, extremely dwarf mutant d^x and dwarf-overexpressing line 35SD). An increase in MDA content was found after 7 d of recovery from heat stress in the Wt, d^x, 35SD and *curl3^{-abs}* plants but 24-epibrassinolide (1 μM) reduced the MDA content.

Concluding remarks: Cell membranes may act as "thermal sensors", which is the primary cause of many other metabolic changes within a cell including the expression of certain genes (Horvath *et al.* 2012). From this point of view, the ability of BRs to induce structural/functional changes in the cell membrane is interesting. Based on presented knowledge, various possible directions of BR action on a membrane can be noted. The first is the influence of BRs on the fatty acid composition towards an increased proportion of unsaturated fatty acids. This suggests the involvement of BRs in the biosynthesis of fatty acids or fatty acid transport and incorporation into cell membranes. Secondly, BRs (similarly as some other sterols) may enter cell membrane directly and modify its properties through, for example, increasing the distance between fatty acid chains, which also may improve the functioning of the membrane under unfavourable temperature conditions. Another mechanism of BR action could be its indirect effect on the inactivation of the excess of reactive oxygen species (ROS) that are generated under stress conditions (see next chapter). ROS-induced damage of constituent membrane elements is manifested, as is mentioned above, by the accumulation of the products of lipid peroxidation. BR-stimulated deactivation of free radicals probably indirectly protects cell membranes and thus lowers lipid peroxidation. A separate issue is the fact that important functional proteins such as water transporting channels and proton pumps are present in the cell membranes. Knowledge of the effect of BR on membrane proteins is rudimentary (Morillon *et al.* 2001, Yang *et al.* 2003) and especially requires studies for low or high temperature conditions.

Mobilization of cellular antioxidant system

Low temperature: A temperature that is too low for optimal growth is accompanied by increased free radical formation (H_2O_2, $O_2^{\bullet-}$, and $^{\bullet}OH$) in cells, which is associated with the activation of the antioxidant system. The antioxidant system consists of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and non-enzymatic antioxidants, including ascorbic acid (AsA) or tocopherols (Liu *et al.* 2009, 2011, Hu *et al.* 2010, Jiang *et al.* 2013, Cui *et al.* 2016). In eggplant seedlings, d/n temperatures of 10/5 for 8 d increased SOD, POD, ascorbate peroxidase (APX), and CAT activities (Wu

et al. 2014a). The application of 0.1 μM 24-epibrassinolide prior to cold, additionally stimulated the activity of these enzymes, by 31, 120, 33, and 64 %, respectively. Treatment of pepper fruits with 15 μM 24-epibrassinolide caused higher CAT and glutathione reductase (GR) activities during storage at 3 ºC for 18 d (Wang *et al.* 2012). Further, the application of 24-epibrassinolide to two cold-exposed tomato cultivars (tolerant Zhongza9 and sensitive Zhongshu4) increased SOD, POD, and CAT activities by about 50 % (Cui *et al.* 2016). In cold-exposed cucumber plants, 28-homo-brassinolide and 24-epibrassinolide stimulated SOD activity (Hu *et al.*

2010, Farriduddin *et al.* 2011) and Jiang *et al.* (2013) emphasised the significant role of irradiance for post-cold plant regeneration and brassinosteroid action. Cucumber plants were exposed to cold (10/7 °C, 6 d), and then they were regenerated 4 d under high (600 µmol m^{-2} s^{-1}) and low (100 µmol m^{-2} s^{-1}) irradiance. Plants that were treated with 0.1 µM 24-epibrassinolide (spraying on day 5 of cold) and recovered at a high irradiance demonstrated a marked increase in the activities of SOD, APX, monode-hydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and GR as well as a reduced glutathione/oxidised glutathione (GSH/GSSG) ratio and ascorbate/dehydroascorbate (AsA/DHA) ratio. The plants that recovered under low irradiance had only a slightly higher SOD activity. Finally, the most recent studies of Xia *et al.* (2017) shows that in tomato, BR increases the accumulation of the transcripts of respiratory burst oxidase homolog1 (*RBOH1*) and glutaredoxin (*GRX*) genes, and that BR-induced cold tolerance is accompanied by a higher ratio of reduced/oxidised 2-cysteine peroxiredoxin (2-Cys Prx) and the activation of antioxidant enzymes. The authors suggested that in tomato, BRs enhance cold tolerance through a signalling cascade that involves RBOH1, GRXs, and 2-Cys Prx.

The effect of BRs on antioxidant enzyme activities has also been confirmed in cell suspensions. The application of 24-epibrassinolide (0.1 µM) to *C. bungeana* cell cultures grown at 0 and 4 °C for 3 or 5 d resulted in additional stimulation of the APX, CAT, GR, POD and SOD activities and increase in AsA and GSH content compared to the cell cultures without the added hormone (Liu *et al.* 2009, 2011).

High temperature: The stress caused by a high temperature also activates the antioxidant system. Wu *et al.* (2014b) studied eggplants in the five-leaf stage subjected to high d/n temperatures of 43/38 °C for 8 d and they found a significant increase in the activities of SOD, POD, CAT, and APX by 35, 37, 26, and 14 %, respectively, as well as the AsA and GSH content (by 28 and 25 %, respectively) compared to plants that were not exposed to high temperature. The plants that were previously treated with 0.1 µM 24-epibrassinolide showed increased SOD, POD, CAT, and APX activities by 30, 103, 68, and 22 %, respectively, compared to the plants that were exposed to a high temperature without BR treatment. A marked increase in the AsA and GSH content (48 and 27 %, respectively) was also observed in BR-treated plants compared to stressed, but not BR-treated plants. High temperatures (42/32 °C, 2 d) stimulated the activity of SOD, POD, CAT, and APX in melon (Zhang *et al.* 2014). In this case, 24-epibrassi-nolide (1 mg dm^{-3}) pre-treatment further enhanced SOD, POD, CAT and APX activities. 24-Epibrassinolide also alleviated the high temperature-induced oxidative stress by increasing the enzymatic and non-enzymatic antioxidant and glyoxalase systems in seedlings of *Ficus concinna* (Jin *et al.* 2015). On the other hand, no effect of 24-epibrassinolide (1 µM) on the activity of antioxidative enzymes was observed in heat-exposed tomatoes (Mazorra *et al.* 2011), which suggests that the effect of BRs may be species or cultivar dependent.
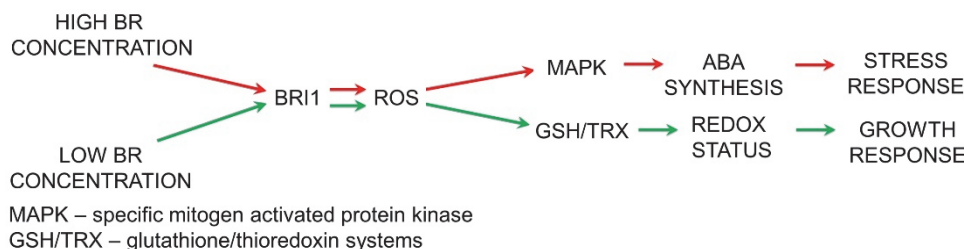

MAPK – specific mitogen activated protein kinase
GSH/TRX – glutathione/thioredoxin systems

Fig. 2. BR concentration-dependent model of growth and the stress response of plants - role of ROS (modified from Xia *et al.* (2015).

Concluding remarks: According to the best of our knowledge, there is lack of data showing the genetic background of the impact of BR on the antioxidant system under high or low temperature. The results described by Xia *et al.* (2009a) for cucumbers grown under optimal conditions suggest that one of the possible reasons why the activity of antioxidant enzymes increases in the presence of BRs may be higher expressions of the genes coding these enzymes. Xia *et al.* (2009a) also noticed that cucumber plants treated with 24-epibrassinolide (0.1 µM) have higher relative abundance of *CAT*, *POD*, and *MDAR* transcripts than plants that were not treated with BRs. According to many researchers, reactive oxygen species (ROS), in addition to the damaging action on cellular components and structures, also play a signalling role (Kreslavski *et al.* 2012). The concept of BR action on the antioxidant cell system proposed by Xia *et al.* (2009a, 2015) is interesting in this context (Fig. 2). BR receptor activation (as a result of ligand-receptor binding) leads to the production of ROS, *e.g.* H_2O_2, however, spatial and temporal changes in the ROS content depend on the BR content in the tissues. Low BR content can cause a temporary increase in ROS, which stimulates the cell antioxidant system. This leads to a shift in the cell redox balance towards reducing processes and acts as a signal, *e.g.* stimulating photosynthesis and developmental processes. A high BR content in the tissues results in long-term ROS

accumulation, which in turn induces a phosphorylation cascade of mitogen-activated protein kinase. In this case, ROS and kinase stimulate the biosynthesis of abscisic acid, which is the main hormone associated with stress tolerance. In this context, the increased accumulation of BRs as a result of low or high temperature is worth noting (Dockter *et al.* 2014, Pociecha *et al.* 2016). Dockter *et al.* (2014) found that the content of casta-sterone during the growth of barley is 7.43 pmol g^{-1}(f.m.) at 14 °C, but it increased to 10.31 pmol g^{-1}(f.m.) in plants grown at 26 °C. In turn, Pociecha *et al.* (2016) showed

that the castasterone content in rye grown at 18 °C is 4 - 5 pmol g^{-1}(f.m.), however, as a result of six weeks of cold, this content increases to about 14 - 16 pmol g^{-1}(f.m.). This phenomenon can result in the activation of mechanism where ABA is involved (proposed by Xia *et al.* 2015; Fig. 2). On the other hand, BR supply may also slightly increase ROS production even before the onset of stress, which results in a higher antioxidant system activity. The increased activity of antioxidant system in non-stressed plants that were treated with BRs was also reported by Jiang *et al.* (2013).

Photosynthetic pigments

Low temperature: One of the most important symptoms of stress that is caused by a low temperature is a decrease in the content of photosynthetic pigments. Soaking pepper fruits in brassinolide solutions (5, 10, and 15 μM) increases the chlorophyll content by 23, 17, and 24 %, respectively, on the 18th day of storage at 3 °C, compared to plants that were not treated with BR (Wang *et al.* 2012). The senescence of cut leaves of oilseed rape is accelerated at 2 °C, but leaf infiltration with 24-epibrassinolide (0.001 - 1 μM) significantly reduces this phenomenon (Janeczko *et al.* 2007). In 30-d-old cucumber plants, a 40 % reduction in the chlorophyll content after 18 h at 3 - 5 °C was limited to only 16 % after pretreatment with 28-homobrassinolide (10^{-8} M) (Farriduddin *et al.* 2011). In eggplant seedlings, the content of chlorophyll *a*, *b,* and (*a+b*) decreased by 32, 57, and 39 %, respectively, during 8 d at 10/5 °C and 24-epibrassinolide (0.05 - 0.2 μM) alleviated this detrimental effects of cold (Wu *et al.* 2014a). He *et al.* (1991) observed that brassinolide improves the greening of etiolated leaves in maize exposed to cold.

High temperature: Stress caused by a high temperature is also accompanied with a decrease in chlorophyll content. In eggplant seedlings exposed to 43/38 °C for 8 d, pretreatment with 0.1 μM 24-epibrassinolide increased the content of chlorophyll *a, b,* and (*a+b*) by 17, 27, and 20 %, respectively, compared to heat-exposed plants that were not BR-treated (Wu *et al.* 2014b). The decrease in chlorophyll content was milder in rice supplemented with 24-epibrassinolide prior to exposure to 40/30 °C for 7 d relative to the control (Thussagunpanit *et al.* 2015a). In another study, this rice cultivar treated with 24-epibrassi-nolide showed an increase in the content of chlorophyll *a, b,* and (*a+b*) and carotenoids by 86, 74, 83, and 28 %,

respectively, on the 7th day of a high temperature exposure, compared to plants that were not treated with BR (Thussagunpanit *et al.* 2015 b). In the plants that are treated with 24-epibrassinolide, the content of individual pigments achieved after 7-d recovery the level of the plants that are not exposed to heat. In maize growing in a net house with maximum temperature of 17.6 - 24.5 ºC and minimum temperature of 2.8 - 7.4 ºC (21 d), decrease in the chlorophyll content is milder when the seedlings are supplemented with 1 μM 24-epibrassinolide (Singh *et al.* 2012). 24-Epibrassinolide also ameliorates photo-synthetic pigment losses in melon seedlings exposed to 42/32 ºC for 2 d in the stage of 3 - 4 leaves (Zhang *et al.* 2014).

Concluding remarks: Considering the fact that chlorophyll degradation occurs in plants under stress (Sakuraba *et al.* 2014), it can be assumed that BRs restrict this process. Presumably, BRs affect the synthesis or activity of the enzymes that participate in the processes of chlorophyll breakdown such as Chl *b* reductase (Hörtensteiner and Kräutler 2011). BRs may also participate in regulating chlorophyll biosynthesis. BR-deficient barley mutants at the optimal temperature accumulated a few percent less of chlorophylls compared to wild type (Janeczko *et al.* 2016). Simultaneously, the mutants also had lower content of cytokinins, which are stimulators of chloroplast development and chlorophyll biosynthesis (Parthier 1979). This may suggest an indirect effect of BRs on chlorophyll biosynthesis through cytokinin action. The engagement of BR signalling in chlorophyll accumulation is also suggested by Yoshizawa *et al.* (2014). All of these hypotheses require verification in plants that are exposed to low/high temperatures.

Gas exchange, photosystem activities, and CO₂ assimilation

Low temperature: Gas exchange parameters are very susceptible to environmental changes. In cucumber

plants, 28-homobrassinolide (10^{-8} M) and 24-epibrassi-nolide (0.1 mg dm^{-3}) reduce the cold-induced decreases in

net photosynthetic rate (P_N), stomatal conductance (g_s), internal carbon dioxide concentration (c_i), and transpiration rate (E) (Hu *et al.* 2010, Farriduddin *et al.* 2011). The effect is confirmed by the 24-epibrassinolide treated eggplants exposed to 10/5 °C for 8 d (Wu *et al.* 2014a).

Low temperature usually decreases the efficiency of the photosystems (PS). Fluorescence methods permit a detailed characterization of any disturbances in the energy flow especially within PS II. Cucumber plants in the 3-leaf stage that were sprayed with 24-epibrassinolide (0.1 mg dm^{-3}) prior to exposure to 12/8 °C for 3 d reached higher values of the effective quantum yield of PS II photochemistry (Φ_{PSII}) and photochemical quenching coefficient (q_P) and lower nonphotochemical quenching (NPQ) than plants that were not sprayed (Hu *et al.* 2010). The beneficial effect of this hormone on the efficiency of PS II was also observed during the post-stress regeneration (Hu *et al.* 2010). After the treatment of 28-homobrassinolide (10^{-8} M), 30-d-old cucumber plants reached higher values of PS II maximum quantum yield (variable to maximum fluorescence ratio, F_v/F_m) under cold (3 - 5 °C, 18 h) compared to plants without the BR (Farriduddin *et al.* 2011). Further, the F_v/F_m ratio in cucumber was restored to the level before cold by applying 24-epibrassinolide on the 5th day of cold (at 10/7 °C for 6 d), but only under high irradiance (600 μmol m^{-2} s^{-1}) (Jiang *et al.* 2013). No effect of BR on PS I efficiency was observed in this case. In seedlings of eggplant exposed to 10/5 °C for 8 d, the values of F_v/F_m, potential photochemical efficiency (F_v/F_0), Φ_{PSII}, excitation energy capture efficiency of open centres (F_v'/F_m'), and qP decreased by 9, 32, 17, 9, and 14 %, respectively, and 24-epibrassinolide (0.05 - 0.20 μM) significantly counteracted this phenomenon (Wu *et al.* 2014a). Finally, in cold-treated tomato plants, with a partial deficiency in BRs, F_v/F_m is decreased (Xia *et al.* 2017). Overexpression of the BR biosynthetic gene *Dwarf* or the application of 24-epibrassinolide increased the F_v/F_m under cold (Xia *et al.* 2017).

Under cold, BRs stimulated activity of enzymes responsible for various stages of carbon dioxide binding. The activity of carbonic anhydrase (CA), which participates in the transport of inorganic carbon (Henry 1996), decreased by 24 % in cucumber plants under cold but did not change in plants that were sprayed with 28-homobrassinolide (10^{-8} M) prior to cold (Farriduddin *et al.* 2011). Research conducted by Jiang *et al.* (2013) on cucumber plants exposed to 10/7 °C for 6 d showed that BR play a role in regulating CO_2 binding by ribulose-*bis*-phosphate carboxylase/oxygenase (Rubisco). Plants that were sprayed with 24-epibrassinolide prior to cold treatment and recovered under irradiance of 600 μmol m^{-2} s^{-1} reached a significantly higher Rubisco content, total Rubisco activity, and Rubisco activase (RCA) activity compared to the stressed plants that were not sprayed. This phenomenon did not occur during recovery under irradiance of 100 μmol m^{-2} s^{-1}. An increase in Rubisco

activity after 24-epibrassinolide application was also confirmed by Pociecha *et al.* (2016) in a study on winter rye plants exposed to 3 and 6 weeks of cold. On the other hand, in perennial ryegrass, when 24-epibrassinolide (0.25 mg dm^{-3}) was applied before plant exposure to 4 ºC for 6 weeks, the Rubisco activity was reduced by about 30 % but only in sensitive cv. Amarant and not in the resistant cv. Flinston (Pociecha *et al.* 2017). This suggests that the effect of BRs on Rubisco activity in cold may be cultivar dependent.

High temperature: In rice, 24-epibrassinolide limited heat-induced (40/30 °C, 7 d) disturbances of gas exchange. A significant increase was recorded in P_N, g_s, and E by 325, 240, and 235 %, respectively and a decrease in internal CO_2 concentration (c_i) by 97 % in plants treated with 24-epibrassinolide compared to non-treated and heat-exposed plants (Thussagunpanit *et al.* 2015a,b). Positive effect of 24-epibrassinolide (0.005 - 0.20 μM) on the gas exchange in eggplants subjected to 43/38 °C (8 d) was also observed by Wu *et al.* (2014b). In melon plants exposed to 42/32 °C (2 d), spraying with 24-epibrassi-nolide (1 and 1.5 mg dm^{-3}) maintained P_N and g_s on a level close to those at 30/20 °C (Zhang *et al.* 2014). High temperature tolerant and sensitive cultivars of melon grown at 42/32 °C for 2 d also had an improved P_N and higher values of F_v/F_m, F_v'/F_m', Φ_{PSII}, and qP and lower values of F_0 and NPQ after 24-epibrassinolide treatment (Zhang *et al.* 2013, 2014). The decrease in PS II effectivness caused by temperature 43/38 °C (8 d) in eggplant seedlings was compensated by 24-epibrassi-nolide (0.05 - 0.2 μM) (Wu *et al.* 2014b). In rice, 24-epibrassinolide also effectively alleviated the negative effects of 40/30 °C (7 d) on PS II (Thussagunpanit *et al.* 2015 a,b). The minimum fluorescence (F_0) was lower in plants that were sprayed with 24-epibrassinolide before stress, and parameters such as F_m, F_v/F_m, F_v'/F_m' had higher values than those noted in the stressed plants without BR application. The electron transport rate (ETR) in plants treated with 24-epibrassinolide was also significantly improved (Thussagunpanit *et al.* 2015a). An analysis of chlorophyll *a* fast fluorescence kinetics in spring barley exposed to 42 °C (3 h) indicated an abnormal energy flow in PS II that was manifested in a decrease in energy absorption (ABS/CSm), energy flux for trapping (TRo/CSm), and energy flux for electron transport (ETo/CSm) (Janeczko *et al.* 2011). The protective effect of 24-epibrassinolide (0.25 mg dm^{-3}, leaf infiltration before heat) was observed in the first leaf. ABS/CSm, TRo/CSm, and ETo/CSm values were higher by approximately 23, 49, and 69 %, respectively, compared to the values noted in the stressed plants that were not treated with BR. Xia *et al.* (2009a) noticed that after cold (8° C), cucumber plants that were treated with 24-epibrassinolide had higher values of ETR than untreated plants. Application of BR biosynthesis inhibitor brassinazole (Brz) lowered ETR values below the control

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values while treatment of plants with combination of Brz + 24-epi-brassinolide restored the values to the control level.

Concluding remarks: BRs regulate gas exchange in plants exposed to low/high temperatures. Improved P_N is often connected with stimulation of the activity of the enzymatic system. Rubisco activity is lower in BR-deficient mutants of barley (under optimal conditions) which may confirm regulatory role of BRs in functioning of the enzyme (Janeczko *et al.* 2016). The BR-stimulated accumulation of the transcripts of genes encoding Rubisco or RCA (Xia *et al.* 2009a,b) was also observed. Xia *et al.* (2009b) showed that BR promotes but Brz inhibits expression of genes encoding the Calvin cycle enzymes required for Rubisco regeneration. All of the described molecular mechanisms of BR action should be confirmed for plants that are cultured at low/high temperature. The simplest reason for the decreased efficiency of PS II during plant exposure to high/low temperatures seems to be a reduced content of

photosynthetic pigments (described above). Further, the correct structure and functioning of the thylakoid membranes, where electron carriers are located, is essential to maintain high PS efficiency. The stabilising effect of BRs on the structure and properties of the membranes at low/high temperatures is mentioned in another chapter. Although microscopic analyses show that the application of 24-epibrassinolide maintains the typical shape of the chloroplasts and promotes the formation of grana in the stressed plants, more detailed studies of the effect of BR on photosynthetic light reactions in plants growing at low/high temperature should be done, especially using BR-biosynthesis or signalling mutants. The positive impact of BRs on the thylakoid membranes and chloroplast ultrastructure was confirmed in salt-stressed cucumber plants (Yuan *et al.* 2012). Finally, BRs may also have an indirect influence on CO_2 assimilation by improving plant water management, even if values of E and g_s are usually higher in the BR-treated plants grown under both low or high temperatures.
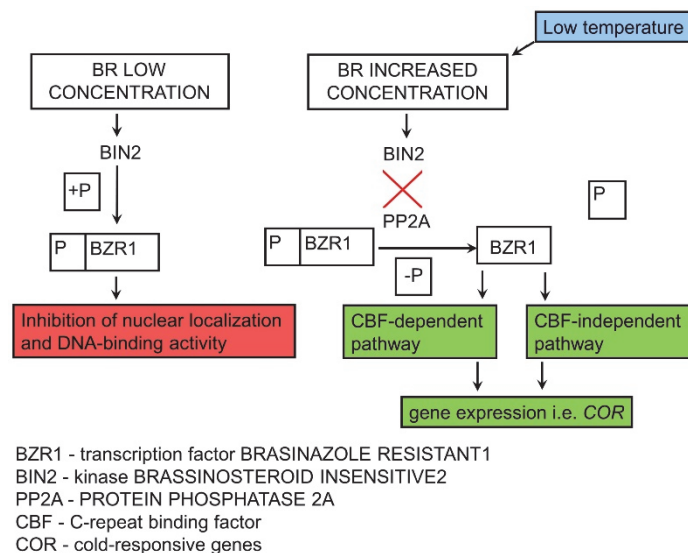

Fig. 3. A model of plant response to low temperature and low [4 - 5 pmol g^{-1} (f.m.)] or increased [14 - 16 pmol g-1 (f.m.)] BR concentration (modified from Wang *et al.* 2014 and Li *et al.* 2017). Brassinosteroid concentrations are exemplary given for rye based on Pociecha *et al.* (2016); P - phosphate. +P - phosphorylation, -P - dephosphorylation, P - phosphate attached to the protein or not attached to protein. The dephosphorylation step of BZR1 is blocked by BIN2 in the case of low BR concentration (Wang *et al.* 2014). A higher content of BRs allows the inactivation of BIN2 and the dephosphorylation of BZR1, which in turn can directly activate BR-responsive genes or act indirectly in a CBF-dependent way (Wang *et al.* 2014, Li *et al.* 2017). For more detail see chapter "Biosynthesis of proteins and accumulation of proline and glycinebetaine".

Sugar accumulation

Low temperature. An increase in sucrose content under cold is especially important for winter plants where it is a part of the hardening before frost. In perennial ryegrass, after pretreatment with 24-epibrassinolide (0.25 mg dm^{-3}) and cold acclimation at 4 °C for 6 weeks, Pociecha *et al.* (2017) observed a significant decrease of glucose and

fructose content (in cv. Amarant by about 38 and 41 % and in cv. Flinston by about 28 and 63 %, respectively) compared to untreated plants. It is important that this effect was accompanied by an increase in content of sucrose (54 to 77 %) and also of protective sugars nystose and 1-kestose in both cultivars. The picture was not so

clear in winter rye plants that were grown at 4 °C for six weeks and pretreated with 24-epibrassinolide (Pociecha *et al.* 2016). The content of glucose and fructose decreased (by 33 and 29 %, respectively) and the content of sucrose and nystose increased only in the moderately winter resistant cv. Stach, but in the winter resistant cv. Dańkowskie Złote, a decrease in the glucose (44 %) and fructose (36 %) content was not accompanied by an increase of the sucrose and nystose content (Pociecha *et al.* 2016). Cold induced the accumulation of sugars also in seedlings of cold sensitive maize inbred line LM-17. Application of 24-epibrassinolide (1 μM) further increased the glucose, sucrose, and starch content by about 15, 28 and 45 %, respectively, compared to cold stressed plants without BR treatment (Singh *et al.* 2012).

High temperature. Changes of sugar accumulation under high temperature are not as crucial as in the case of a cold acclimation. A marked decrease in total soluble sugar content was found in rice plants subjected to 40/30 °C (7 d) compared to the control (Thussagunpanit *et al.* 2015b) but an increase in total soluble sugar content (by 23 %) was recorded in plants that were pretreated with 24-epi-brassinolide compared to plants that were subjected to heat without being sprayed with BR. In the same cultivar grown in the field during the hot season, 24-epibrassinolide increased the total soluble sugar content in the straw (by 107 %). The hormone also increased reducing sugar content in the straw (by 72 %) and the starch content in the straw (by 27 %), husk (by 24 %), and seeds (by 35 %) (Thussagunpanit *et al.* 2015a). Another rice cultivar exposed to a temperature of 35/32 °C for 12 h also demonstrated a decrease in the content of soluble sugars, which was further reduced by a

mixture of plant growth regulators containing BRs (Fahad *et al.* 2016). Compared to rice, eggplant seedlings subjected to 43/38 °C (8 d) have higher soluble sugar content compared to unstressed plants and the application of 24-epi-brassinolide (0.1 μM) enhanced this effect (Wu *et al.* 2014).

Concluding remarks. In the case of heat treatment, the sugar accumulation in plants may fluctuate possibly due to a dependence on the general performance of photosynthesis. However, the increased sucrose concentration under cold lowers the freezing point of the cell solution, and thus it contributes to a higher resistance to frost. The stimulation of sucrose production along with an accumulation of protective sugars (like nystose) by BRs suggests that these hormones play a role in the process of acquiring frost resistance. A BR-stimulated increase in CO_2 assimilation (described in previous chapter), may be the first step in this process, but BRs may also affect the activity of the enzymes that directly participate in sugar biosynthesis. The involvement of BR in the regulation of sucrose synthesis from glucose and fructose was studied for plants grown under optimal conditions, for example by Yu *et al.* (2004) and Janeczko *et al.* (2016). Yu *et al.* (2004) observed an increase in the activity of the enzymes that are involved in sucrose biosynthesis in cucumber after exogenous BR application. According to Janeczko *et al.* (2016), barley mutants with a reduced BR production accumulated more glucose and fructose and less sucrose than the wild type, which may also confirm dysfunctions in sucrose biosynthesis as a result of a BR deficiency. The mechanisms of the action BR on sugar biosynthesis require further confirmation in plants grown at low/high temperatures.

Biosynthesis of proteins and accumulation of proline and glycinebetaine

Low temperature. The acclimation of plants to low temperature requires an adjustment of a significant part of protein metabolism. In maize seedlings grown in a net house (max. temp. 17.6 - 24.5 ºC, min. temp. 2.8 - 4 ºC; 21 d), the content of total proteins was 28 % lower than in the control in a greenhouse (25/18 °C) but 24-epi-brassinolide (1 μM) ameliorated this decrease (Singh *et al.* 2012).

Among the particular proteins with an accumulation stimulated by BRs under cold is the ATP-synthase β subunit. Cold tolerant and sensitive cultivars of tomato sprayed with 24-epibrassinolide before cold (12/6° C, 12 d) accumulated more of the ATP-synthase β subunit, thereby prevented a cold-induced decrease of its content (Cui *et al.* 2016).

In mango fruits stored at 5 °C (28 d), brassinolide (10 μM) increased the accumulation of the gene transcripts encoding the membrane-associated proteins: temperature induced lipocalin (*TIL*), type II SK2

dehydrin (*TSD*), abscisic stress ripening-like protein (*ASR*), and the remorin family proteins (*REM*) (Li *et al.* 2012). These proteins are associated with the stress response and may also perform important functions in membrane formation and repair. Cold-responsive (COR) proteins belong to hydrophilic polypeptides, which help to stabilise membranes, and thus may protect against damage induced by the cold (Tomashow 1998). According to Eremina *et al.* (2016), BRs enhance plant resistance against freezing stress *via* the regulation of the expression of *COR* genes. BR-controlled basic helix-loop-helix transcription factor (CES), can contribute to the constitutive expression of the *C-repeat/dehydration-responsive element binding factors (CBFs)* transcriptional regulators that control the *COR* gene expression. *CBF1, CBF3*, and that *COR15A* transcript accumulation is increased in the wild type *A. thaliana* as a result of the application of 24-epibrassi-nolide (250 nM). *BRI1oe* (BR-hypersignalling line) mutants have higher

transcriptions of *CBF1* and *CBF3* as well as *COR15A, COR15B, COR47,* and *COR78* compared to the wild type. In turn, the BR-signalling defective mutants *bri1-301* and *bri1-1* had a significantly lower accumulation of these transcripts compared to the wild type plants. The effect of BRs on COR proteins was also confirmed by the studies of Kagale *et al.* (2007) and Divi *et al.* (2016), which proved that *COR* genes were up-regulated by BR. According to Li *et al.* (2017), BRs act *via* BZR1 (transcription factor - brassinazole resistant 1) in a CBF-dependent manner in cold but also in a CBF-independent manner (Fig. 3). The dephosphorylation step of BZR1, which is blocked by BIN2 in the case of low BR content, is crucial (Wang *et al.* 2014). A higher content of BRs permits the inactivation of BIN2 and the dephospho-rylation of BZR1, which in turn can directly activate BR-responsive genes or act indirectly in a CBF-dependent way (Wang *et al.* 2014, Li *et al.* 2017).

Proline is a specific amino acid that acts as an antioxidant, osmolyte, signalling molecule, and metal chelator (Hayat *et al.* 2012). In eggplants, exposed to 10/5 °C (8 d), a drastic increase in the proline content (by 793 %) was observed compared to plants that were not subjected to cold (Wu *et al.* 2014a). Pretreatment with 24-epibrassinolide (0.1 μM) additionally enhanced this effect by 87 % (Wu *et al.* 2014a). Fruits of tomato that were treated with brassinolide (6 μM) and stored at 1 °C (3 weeks) also had a 65 % higher proline content compared to low-temperature-exposed fruits without BR application (Aghdam *et al.* 2012). Glycine betaine also acts as osmolyte (Ashraf and Foolad 2007). Seedlings of maize treated with 24-epibrassinolide (1 μM) and exposed to cold show an increase of the glycine betaine content by about 85 % compared to chilled plants that were not supplemented with this hormone (Singh *et al.* 2012).

High temperature. In plants at high temperature, protein content may decrease (melon, 42/32 °C, 2 d) or increase (eggplant 43/38 °C, 8 d) (Zhang *et al.* 2014, Wu *et al.* 2014b). The application of 24-epibrassinolide (0.05 - 1.5 mg dm^{-3}) increased protein content by an average of 35 % in both species. A decrease in the protein content at 35/32 °C in rice was ameliorated by a mixture of plant growth regulators with BRs (Fahad *et al.* 2016). Brassinolide (10 nM) increased also the protein content in cell cultures of *Chlorella vulgaris* grown at 30 - 40 °C (Bajguz 2009).

Under high temperature, heat shock proteins (HSP) play a chaperone function and prevent damage to many heat-sensitive cellular proteins. In seedlings of oilseed rape subjected to 45 °C, *hsp101*, *hsp90*, and *hsp70* genes were expressed, and 24-epibrassinolide promoted their transcripts accumulation, especially of *hsp101* and *hsp90* (Dhaubhadel *et al.* 1999, 2002). The 24-epibrassinolide also stimulated the accumulation of small HSPs (sHSPs) after heat treatment. A significant accumulation of *HSP* transcripts (particularly in plants that were treated with

24-epibrassinolide) was found during heat but after heat their accumulation decreased and in BR-treated plants even to a lower level than in control plants (Dhaubhadel *et al.* 2002). It is worth noting that Dockter *et al.* (2014) reported an increased amount of endogenous BRs in plants grown at high temperature. Finally, Samakovli *et al.* (2014) proposed HSP as important players in BRs signalling. Briefly, if a BR is not bound to a receptor, HSP90 in combination with BIN2 in the nucleus prevents the dephosphorylation of BES1 and BZR1 and the expression of BR-responsive genes. If BR is bound to receptor, the HSP-BIN2 complex is transferred to the cytoplasm and the dephosphorylation of BES1 and BZR1 permits the expression of BR-regulated genes.

Eggplant seedlings subjected to 43/38 °C for 8 d as well as melon seedlings subjected to 42/32 °C for 2 d, were characterised by an increase in the proline content compared to the control (Wu *et al.* 2014b, Zhang *et al.* 2014). The application of 24-epibrassinolide (0.1 μM) to the eggplant and 24-epibrassinolide (0.05 - 1.5 mg dm^{-3}) to the melon enhanced this increase. Rice exposed to 35/32 °C and treated with a hormonal mixture containing BRs also had an increased proline content (Fahad *et al.* 2016).

Concluding remarks. Microarray and proteomic analyses of BR-treated plants and BR-deficient mutants proved that many genes are BR-regulated (Yang and Komatsu 2004, Deng *et al.* 2007). A large part of this knowledge has been obtained from studies of plants grown under optimum temperature. More detailed studies should now be devoted to the aspect of low/high temperature treatments, which would be helpful in explaining the mechanisms of BR action under these stresses. Currently, it appears that BRs are engaged in controlling the *COR* expression at low temperature and *HSP* expression at high temperature. They also promote the accumulation of the ATP-synthase β subunit and the transcripts of the *TIL, TSD, ASR,* and *REM*, which are important in the function, formation, and repair of membranes.

Many studies have indicated a positive relationship between plant stress tolerance and the accumulation of proline and glycine betaine (Ashraf and Foolad 2007). The BR-stimulated accumulation of both osmolytes may then support plant acclimation to low or high temperature. The study of Janeczko *et al.* (2016) on barley BR-deficient mutants demonstrated that these plants - under optimum growth conditions and under drought - indeed accumulated less proline than the wild type, which suggests the importance of BR in regulating the proline content. According to Farriduddin *et al.* (2009), BRs activate the proline biosynthesis enzymes.

A search for other proteins that may directly or indirectly contribute to a brassinosteroid-mediated increase in the tolerance to low/high temperature is an open question.

Other effects of BRs application

Low temperature: The acclimation of plants to a low temperature is connected with the significant changes in hormonal homeostasis, especially in the content of the so-called stress hormones. *C. bungeana* cell cultures that were subjected to 0 and 4 °C (3 d) accumulated abscisic acid [ABA];17 and 22 ng g^{-1}(f.m.)], respectively. Cells cultured with 24-epibrassinolide (0.1 μM) accumulated 33 and 27 ng(ABA) g^{-1}(f.m.) at 0 and 4 °C, respectively (Liu *et al.* 2011).

Application of 24-epibrassinolide (0.25 mg dm^{-3}) to perennial ryegrass combined with cold (4 ºC, 6 weeks) resulted in an increased content of the ethylene precursor (ACC) compared to the plants without BR treatment (Pociecha *et al.* 2017). The jasmonic acid content increased only in cv. Flinston and no effect of 24-epi-brassinolide on salicylic acid content was noted.

In addition to the well-known effect of BRs on Rubisco or antioxidant enzymes activity, the activity of other important enzymes has also been shown. In cucumber plants under cold (5/3 °C, 18 h), nitrate reductase activity was reduced by 27 % compared to unstressed plants; however, 28-homobrassinolide (10^{-8} M) neutralised this effect (Farriduddin *et al.* 2011). Pectin methylesterases (PMEs) play a significant role in plant cold/frost tolerance as they catalyze the dimethyl-esterification of cell wall polygalacturonans (Micheli 2001, Qu *et al.* 2011). The activity of PMEs was markedly increased in the wild type *A. thaliana* grown at 0 °C (24 h) and less increase was observed in the *bri1-116* homozygous mutant, whereas in the *bzr1-D* mutant, the PME activity was similar as in the wild type (Qu *et al.* 2011). Ammonia-lyase (PAL) is a key enzyme in the metabolism of phenols, which help to protect plants against stress (Lafuente *et al.* 2003). Tomato fruits that were stored at 1 °C (3 weeks) had increased PAL activity; brassinolide further enhanced the effect and simultaneously increased the total phenol content (Aghdam *et al.* 2012). An increase in soluble phenolics was also noted in
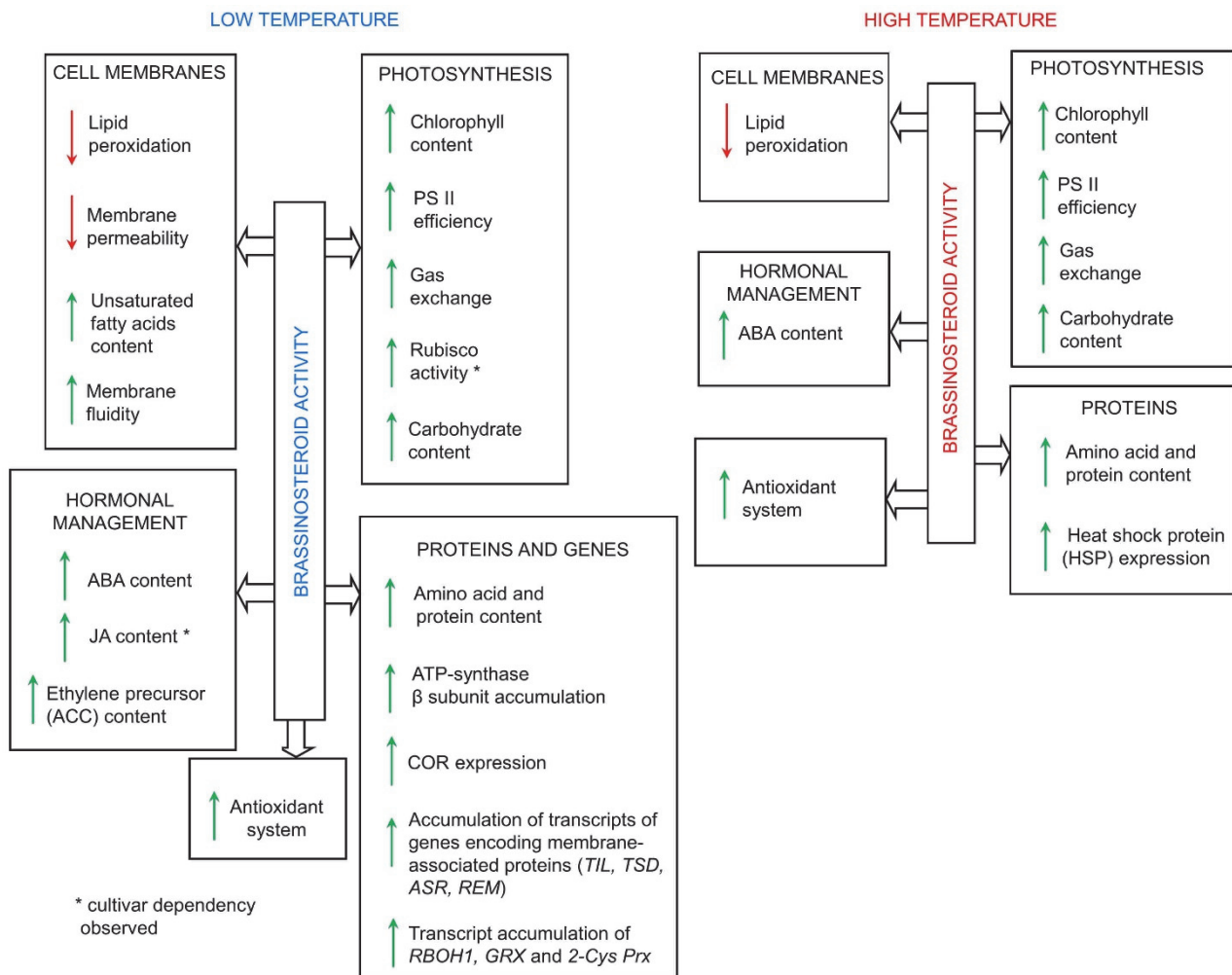

Fig. 4. Multidirectional activity of BRs in the regulation of metabolism of plants exposed to low and high temperatures.

the perennial ryegrass cv. Flinston (but not in cv. Amarant) grown at 4 ºC for 6 weeks and treated with 24-epibrassinolide (0.25 mg dm^{-3}) (Pociecha *et al.* 2017). To the best of our knowledge, there is no data about the effect of BRs on the activation of the above-mentioned enzymes in high temperature.

High temperature: Plant hormonal balance is also changed by a high temperature. *Chlorella vulgaris* cells at temperatures of 30, 35, and 40 °C (3 h) accumulated higher amounts of ABA, which increased along with the increase in temperature to 1.65, 2.2, and 2.6 ng g^{-1}(f.m.), respectively). Addition of 10 nM brassinolide stimulated ABA accumulation at 40 °C to 4.32 ng(ABA) g^{-1}(f.m.) (Bajguz 2009).

Concluding remarks: Plant hormones remain in the connection network and control their own biosynthesis and transport (Weiss and Ori 2007). BRs also contribute to the functioning of this network (Zhang *et al.* 2009), but little is specifically known about their role in terms of temperature stress. As is shown here, BRs increase the accumulation of ABA at low or high temperature. Barley mutants in the *HvDWARF* gene encoding C6-oxidase, which is involved in BR biosynthesis, accumulated a significantly lower amount of ABA compared to the wild type plants (Janeczko *et al.* 2016), which may confirm an interplay between these hormones. During cold hardening of ryegrass, BR also stimulated the accumulation of other stress hormones, jasmonic acid and ethylene precursor ACC. The role of BRs in the hormonal network of plants that are exposed to high temperature and especially to low temperature (significant in hardening to frost) definitely requires further studies.

The detailed mechanisms that are responsible for BR impact on the activity of the aforementioned enzymes remain unknown. We can only theoretically state that BRs act by up-regulating the genes coding those enzymes. It is also possible that there is an indirect effect of BRs *via* the stimulation of the production of chaperones (HSP) that, among others, may help to protect the cell enzymatic system against a loss of activity under temperature stress.

Improvement of plant resistance to low and high temperatures

Low temperature: The viability of cells of *Chorispora bungeana,* measured by 2,3,5-triphenyltetrazolium chloride (TTC) reduction test, decreased during growth at 0 and 4 °C for 3 - 5 d, but supplementation with 24-epibrassinolide (0.05 and 0.1 mg dm^{-3}) counteracted this effect (Liu *et al.* 2009, 2011). Brassinosteroids also reduce the cold damage to plants such as cucumber or maize, which are characterized by a relatively low cold tolerance. About 30 % reduction in the shoot and root length, leaf area, and fresh and dry masses of cucumber exposed to 5/3 °C for 18 h were largely prevented by 10^{-8} M 28-homobrassinolide (Farriduddin *et al.* 2011). In the cucumber cv. Jinchun No. 4, 24-epibrassinolide (0.1 μM) when applied on the 5th day of cold (10/7 °C) counteracted the reductions in the growth and dry mass but the effect was more pronounced in plants treated under high irradiance (Jiang *et al.* 2013). Singh *et al.* (2012) found that seedlings of maize exposed to cold in net house, showed a reduction in plant height (35 %) and fresh mass (24 %) compared to the those in a greenhouse. The pre-treatment with 1 μM 24-epibrassinolide increased plant height and fresh and dry masses by about 15, 36, and 2 %, respectively, compared to the plants without BR pre-treatment. Similar effects were also observed by He *et al.* (1991) for brassinolide pre-treated maize.

The application of BRs provides a chance to minimise fruit damage during cold storage. A marked reduction of chilling injury index (CI) was observed after immersion of tomato cv. Newton fruits in 3 and 6 μM brassinolide solution compared to fruits that were not BR-treated and stored at 1 °C for 3 weeks (Aghdam *et al.* 2012). The similar effect of brassinolide (10 μM) on CI was observed for mango fruits stored at 5 °C for 28 d (Li *et al.* 2012). The BR application limited the sunken lesions of the pericarp and pulp darkening accompanying tissue necrosis. Pepper fruits also responded to storage at 3 °C for 18 d by the occurrence of damage such as surface pitting and calyx discoloration (Wang *et al.* 2012). A gradual increase in the CI index was observed with the duration of fruit storage and the greatest damage was recorded on the last (18th) day. Pre-treatment of the fruits with a 15 μM brassinolide solution alleviated these effects by reducing the CI index by about 69 % compared to the fruits that were stored in the cold without brassinolide treatment.

Relatively new research was dedicated to the possibility of limiting frost damage by BR. Winter rye plants sprayed with 24-epibrassinolide (0.25 mg dm^{-3}) before a 6-week acclimation at 4 °C showed less frost damage and a higher survival rate (Pociecha *et al.* 2016). Winter wheat seedlings sprayed with 24-epibrassinolide (0.25 or 0.05 mg dm^{-3}), cold-acclimated and then exposed to -12°C had a higher survival rate than those that were not BR-treated (Janeczko 2016). Perennial ryegrass acclimated at 4 ºC for 6 weeks and treated with 24-epi-brassinolide (0.25 mg dm^{-3}) had an increased frost tolerance (by 35 %) compared to plants without the BR treatment (Pociecha *et al.* 2017). The importance of BRs in the process of acclimation and frost tolerance was confirmed by a study on *A. thaliana* mutants with impaired BR-signalling (Eremina *et al.* 2016). Compared

to the wild type, BR-hypersignalling mutants *35S:BRI1-GFP (BRI 1oe)* that were exposed to -10 °C (6 h) demonstrated a higher frost tolerance. In turn, *bri1-301* and *bri1-1* mutants with disturbances in BR-signalling exhibited a drastically reduced frost tolerance. The rate of survival of the wild type plants was about 50 %, while for *BRI 1oe*, it was 70 %, and for *bri1-301* only a few percent. Plants of the *bri1-1* mutant did not survive the frost.

High temperature: Bromegrass cell suspension cultures treated with 10 μM 24-epibrassinolide had significantly higher cell viability at high temperatures (40 - 45 ºC, 45 - 90 min) than the untreated cultures (Wilen *et al.* 1995). In one of the first studies that showed the alleviating effects of BRs on whole plants at high temperature, Dhaubhadel *et al.* (1999) assessed the effect of 45 °C (4 h) on oilseed rape and tomato. This temperature was lethal for most of the plants, but 24-epibrassinolide (1 μM) increased plant survival. In the studies of Mazorra *et al.* (2011), all of the tomato cv. Ailsa Craig seedlings and approximately 80 % of cv. Money-maker seedlings survived at 45 °C for 4 h but not for 8 h. 24-Epibrassinolide (1 μM) improved the survival of plants stressed for 5, 6, and 7 h. A temperature of 45 °C (7 h) was then tested on tomato the brassinosteroid mutants: *curl3-abs*, *dx*, and 35SD. Approximately 90 % of the *curl3-abs* seedlings survived but only 18 - 30 % of *dx* and 35SD seedlings survived. The survival rate of the *dx* mutants that were treated with 24-epibrassinolide (1 μM) was approximately 2.6-fold higher compared to the *dx* plants that were not treated. 24-Epibrassinolide also improved the survival of the *curl3-abs* seedlings. In eggplant (cv. Huqie 9), a temperature of 43/38 °C (8 d) reduced plant height, stem diameter, and shoot and root fresh masses by 28, 35, 61, and 58 %, respectively, compared to the control (Wu *et al.* 2014b). 24-Epibrassi-nolide (0.1 μM) increased the values by 26, 43, 55 and 43 %, respectively, compared to the control (high temperature, no BR spraying). A high temperature (42/32 °C, 2 d) decreased fresh masses of

shoots and roots in melon plants (Zhang *et al.* 2013). 24-Epibrassinolide (1 mg dm^{-3}) treated plants had a higher mass by about 14 - 27 %. 24-Epibrassinolide also decreased the heat injury index as measured by observations of leaf yellowing, curling, and senescence compared to the stressed plants that did not undergo hormone spraying. In another work of these authors on the same species, a higher BR concentration (1.5 mg dm^{-3}) was also effective (Zhang *et al.* 2014). In rice, a temperature of 40/30 ºC (7 d) reduced the shoot and root fresh masses and leaf area, but this effect was limited by 24-epibrassinolide (10^{-8} M) (Thussagunpanit *et al.* 2015b). In the same cultivar grown in the field during the hot season (February to May 2012, Thailand), 24-epi-brassinolide increased the number of filled seeds per panicle by 16 % and increased the seed mass by 35 % (Thussangunpanit *et al.* 2015a).

A high temperature during plant development may negatively affect pollen viability. Tomato pollen that were inoculated on a medium with 1 μM 24-epibrassi-nolide and exposed to 35 ºC for 4 h showed a higher germination, enhanced tube growth, and lower pollen bursting compared to the pollen without BR treatment (Singh and Shono 2003). In rice at a temperature of 35/32 °C, a reduction in pollen fertility, anther dehiscence, number of germinated pollens on the stigma and total number of pollen occurred (Fahad *et al.* 2016). Plants administered with a mixture of growth regulators containing BRs had higher values of the aforementioned parameters in comparison to the stressed plants that had not been sprayed.

Concluding remarks: To conclude, plant resistance to high/low temperatures is an important issue in agriculture and horticulture as well as in fruit and vegetable storage. Many methods are used to improve plant resistance, including applications of protective compounds (Senaratna *et al.* 1988). The results summarised in this chapter show that BRs have the potential to reduce many of the negative effects of low/high temperatures, which encourages the future practical application of BRs.

Conclusions and future perspectives

The negative effects of low or high temperature are reduced in plants to which BRs are applied before or during stress. The following ways of BR action are possible: alterations in the membrane physicochemical properties, regulation of the expression of some genes (*HSP, COR*), and regulation of metabolism through other hormones or signalling molecules (ABA or hydrogen peroxide) (Figs. 4 and 5). Most of the literature on this subject is related to the effect of exogenous BRs on plants that are subjected to low/high temperatures. In the future, more emphasis should be placed on studying the impact of low/high temperatures on BR-biosynthesis/signalling

mutants or on plants that are treated with BR inhibitors. This would strengthen the knowledge about the role of these hormones in plant responses to low/high temperatures. Nevertheless, results from studies with the exogenous application of BRs on plants that are exposed to low/high temperatures are a good starting point for agricultural/horticultural practice. Agrochemicals with BRs may help to protect crops against the negative impact of low/high temperatures (*i.e.*, by improving frost resistance). It is worth noting that BRs act in very small concentrations and are natural biodegradable substances. Simultaneously, the use of classical breeding methods or

genetic engineering methods to modulate the BR content in plants or to change the signal transduction pathways that are derived from the BR receptors are promising for the creation of new more stress resistant plants.

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