1 Biological Sciences, Plant Biology

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Brassinosteroids participate in the control of basal and acquired freezing tolerance of plants

Marina Eremina[a], Simon J. Unterholzner[a], Ajith I. Rathnayake[a], Marcos Castellanos[b], Mamoona Khan[a,1], Karl G. Kugler[c], Sean T. May[b], Klaus F. X. Mayer[c,d], Wilfried Rozhon[a] and Brigitte Poppenberger [a,2]

[a]Biotechnology of Horticultural Crops, TUM School for Life Sciences Weihenstephan, Technische Universität München, D-85354 Freising, Germany; [b]Nottingham Arabidopsis Stock Centre, University of Nottingham, School of Biosciences, Loughborough LE12 5RD, UK; [c]Plant Genome and Systems Biology, Helmholtz Center Munich, D-85764 Neuherberg, Germany; [d]TUM School for Life Sciences Weihenstephan, Technische Universität München, D-85354 Freising, Germany; [1]Present address: Institute of Science and Technology Austria, Am Campus 1, A-3400, Klosterneuburg, Austria

[2]**Address correspondence to:**

Brigitte Poppenberger, Biotechnology of Horticultural Crops, TUM School for Life Sciences Weihenstephan, Liesel-Beckmann-Str. 1, D-85354 Freising, Germany; Phone: +49 8161 712401; Fax: +49 8161 712501; E-mail: brigitte.poppenberger@wzw.tum.de

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Brassinosteroids (BRs) are growth-promoting plant hormones that play a role in abiotic stress responses but molecular modes that enable this activity remain largely unknown. Here we show that BRs participate in the regulation of freezing tolerance. BR signalling-defective mutants of *Arabidopsis thaliana* (arabidopsis) were hypersensitive to freezing before and after cold acclimation. The constitutive activation of BR signalling, in contrast, enhanced freezing resistance. Evidence is provided that the BR-controlled basic helix-loop-helix transcription factor CESTA (CES) can contribute to the constitutive expression of the *CRT/DRE BINDING FACTOR* (*CBF)* transcriptional regulators that control cold responsive (*COR*) gene expression. In addition, CBF-independent classes of BR-regulated *COR* genes are identified that are regulated in a BR- and CES-dependent manner during cold acclimation. A model is presented in which BRs govern different cold responsive transcriptional cascades through the posttranslational modification of CES and redundantly acting factors. This contributes to the basal resistance against freezing stress, but also to the further improvement of this resistance through cold acclimation.

SIGNIFICANCE STATEMENT

Cold stress is an influential environmental factor that affects plant distribution and can strongly limit crop productivity. Plants have evolved sophisticated signalling cascades that enable them to withstand chilling or even freezing temperatures. These cascades alter the biochemical composition of cells for protection from damage caused by low temperature stress. In addition, cold stress has a profound impact on plant morphologies, causing growth repression and reduced yields. In this work we reveal that the brassinosteroids, a class of steroid hormones that is known for its role in growth control, also confers freezing tolerance in plants and describe regulatory circuits that contribute to this activity. Implications for the breeding of cold resistant plants are discussed.

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2   Seasonal and diurnal temperature changes are influential environmental factors that affect plant

3   distribution and can strongly limit crop productivity. Whereas chilling-sensitive plants from

4   tropical or subtropical regions suffer damage already even above freezing temperatures, plants

5   from temperate geographical zones, such as *Arabidopsis thaliana* (arabidopsis) commonly

6   display a certain level of constitutive (intrinsic or basal) freezing tolerance. This basal tolerance

7   can be further enhanced by exposure to low but non-freezing temperatures in a process termed

8   cold acclimation that leads to transcriptome reprogramming (1), and induces biochemical,

9   physiological and morphological changes, including growth repression, which allows plants to

10   increase their freezing resistance (2).

11   A group of genes induced by cold and other types of abiotic stress such as drought or

12   osmotic stress is the *COLD-RESPONSIVE* (*COR*) genes. The promoters of certain *COR* genes

13   contain a *cis*-acting element responsible for drought and low-temperature responsiveness, the

14   C-REPEAT/DEHYDRATION-RESPONSIVE ELEMENT (CRT/DRE; (3)), which is bound by the

15   CRT/DRE BINDING FACTOR (CBF/DREB) family of APETALA 2 (AP2) domain transcription

16   factors (4). CBF activity is controlled by upstream components of which the best characterized is

17   INDUCER OF CBF EXPRESSION 1 (ICE1), a basic helix-loop-helix (bHLH) transcription factor

18   (5), which, in response to cold, is modified by SUMOylation and promotes *CBF3* expression (6).

19   Like most physiological traits in plants chilling and freezing tolerance is controlled by

20   phytohormones (7), and evidence suggests that the brassinosteroids (BRs) are involved. When

21   externally applied, BRs increased chilling tolerance (8, 9). However, the molecular modes of this

22   control remain unclear (10). BRs are steroid hormones that regulate vegetative and reproductive

23   development by promoting cell division and cell elongation (11). A plasma membrane-localised

24   receptor complex containing the receptor-like kinase BRASSINOSTEROID INSENSITIVE 1

25   (BRI1) perceives the hormones and initiates signalling to control the activity of BR-regulated

1　transcription factors (TFs; (11)). The best-studied members of BR-controlled TFs are BES1-

2　EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE INSENSITIVE 1 (BZR1) (12, 13). They are

3　substrates of BR-repressed ARABIDOPSIS GSK3/SHAGGY-LIKE KINASES (ASKs) including

4　BRASSINOSTEROID INSENSITIVE 2 (BIN2), which are negatively regulated by BRs and

5　repress BES1/BZR1 activity through phosphorylation (11).

6　　　　In addition, bHLH TFs such as CESTA/HALF FILLED (CES/HAF) are also targets of

7　BIN2 (14). CES is the closest homologue of BRASSINOSTEROID ENHANCED EXPRESSION 1

8　(BEE1) and BEE3 (15) and is required for elongation growth in both vegetative (14) and

9　reproductive tissues (16). BRs control CES protein activity, abundance and subnuclear

10　localisation via BIN2-mediated phosphorylation and phosphorylation-repressed SUMOylation

11　(17).

12　　　　Here we show that BRs promote the freezing resistance of plants and elucidate

13　molecular modes that contribute to this activity. BR-deficient mutants were hypersensitive to

14　freezing stress whereas an activation of BR signalling increased freezing tolerance both before

15　and after cold acclimation. We provide evidence that the BR-regulated bHLH transcription factor

16　CES can directly bind to *CBF* promoters and contribute to the regulation of constitutive *CBF*

17　expression, in particular of *CBF1* and *CBF3*. In addition, non-CBF regulon types of *COR* genes

18　are identified that are regulated by the BR-CES/BEE pathway during cold acclimation. A model

19　for the role of BRs in basal and acquired freezing tolerance is presented and discussed.

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21　**Results**

22　**BR Signalling Contributes to Basal Freezing Tolerance of Plants.** Previously, it was shown

23　that BR treatment improves the chilling tolerance of arabidopsis plants (8-10). In addition, we

24　found that application of the BR epi-brassinolide (epiBL) improved arabidopsis survival following

25　sub-zero temperature exposure (*SI Appendix*, Fig. S1*A*). To investigate if BRs are involved in

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basic freezing tolerance BR-signalling mutant plants were treated at either −6 °C or −8 °C for 4 h, and survival was assessed as the ability to form new leaves after 2 weeks of recovery at 21 °C. Wild type Columbia-0 (Col-0) showed survival rates of approximately 45 % following −6 °C exposure and of approximately 15 % following −8 °C exposure (Figs. 1*A* and *B*). These rates were increased in *BRI1oe* (with approximately 70 % and 30 %, respectively) and strongly reduced in *bri1-1* and *bri1-301,* with <10 % survival (Figs. 1*A* and *B*). The phenotype was associated with a decrease in electrolyte leakage, an indicator of damage to cellular membranes, in *BRI1oe* and with an increase in *bri1-301* (Fig. 1*C*).

To investigate at which step in the BR signalling pathway the regulatory effects may occur, the survival rates of two independent lines overexpressing ASKθ, a BIN2 homologue (18), were assessed following −6 °C treatment. In *ASKθoe* plants BR signalling is constitutively repressed, which results in BR-deficient phenotypes even severer than those found in *bin2-1* (18). Importantly, both lines were hypersensitive to freezing (Fig. 1*D*), providing evidence that BR function in constitutive freezing tolerance is conferred downstream of ASKs.

In cold and freezing stress responses *COR* gene regulation occurs (1). To investigate if BRs may participate, we employed a bioinformatic analysis and determined on a genome-wide scale whether *COR* genes may be constitutively repressed in a BR-deficient situation. This showed that approximately 6 % of all cold-induced genes were repressed in the BR-deficient mutant *det2-1* (*de-etiolated 2;* 18). Among them CBF-induced genes were significantly enriched (Fig. 1*E*; *SI Appendix*, Table S1). To investigate whether BRs are required for the basal expression of *CBFs* and down-stream *COR* genes, transcript levels were measured by quantitative real-time PCR (qPCR) in the BR hyper-signalling line *35S:BRI1-GFP (BRI1oe;* 19), the two BR signalling-defective mutants *bri1-1* (20) and *bri1-301* (21) and the BR biosynthetic mutant *cpd* (*constitutive photomorphogenic dwarf;* (22), which has severer phenotypes than *det2-1* (11). The results showed that BR signalling deficiency repressed *CBF3* transcription in seedlings (*SI Appendix*, Fig. S1*B*), and *CBF1* and *CBF2* transcription in adult plants (Fig. 1*F*).

1	Moreover, the downstream targets *COR15A*, *COR15B*, *COR47*, *COR78* and *KIN1* (*KINASE 1*)

2	were all repressed in BR signalling-deficient mutant backgrounds. In particular *COR15A* and

3	*COR15B* levels were reduced (Fig. 1*F*; *SI Appendix*, Fig. S1*B*). In *BRI1oe* plants,

4	complementary changes occurred, but they were subtler (Fig. 1*F*; *SI Appendix*, Fig. S1*B*).

5	Growth on media containing BR or the BR biosynthesis inhibitor brassinazole (Brz) (23)

6	produced corresponding changes in *CBF* expression (*SI Appendix*, Fig. S2*A* and *B*), confirming

7	previous studies, which had shown BR induction of *CBF1* and *COR* gene expression following

8	BL treatment (8, 24). At early time points after BR treatment *CBFs* were not significantly induced

9	in the conditions we tested (*SI Appendix*, Fig. S2*C*).

10	**BR Signalling Contributes to Cold Acclimation.** Given the evidence that BR signalling

11	promotes freezing tolerance of plants under non-acclimated conditions, we investigated whether

12	BRs also affect freezing tolerance following cold acclimation. Freezing tolerance assays of cold-

13	acclimated BR signalling-deficient mutants showed that the survival rates of *bri1-1* and *bri1-301*

14	were strongly reduced, whereas *BRI1oe* survival was increased in comparison with that of the

15	wild type (Figs. 2*A* and *B*). In agreement, electrolyte leakage of acclimated plants was increased

16	in *bri1-301* and decreased in *BRI1oe* (Fig. 2*C*).

17	Since BRs can impact on basal *CBF* expression levels it was of interest to analyse if BRs

18	may also contribute to the induction of *CBF* transcription in response to cold stress. Therefore,

19	*bri1-1* and *bri1-301* were treated at 4 °C, and mRNA levels of *CBFs, COR15A* and *COR15B*

20	were quantified in a time-course manner by qPCR. The result showed that in response to cold

21	stress the transient induction of *CBF1* expression was slightly reduced in BR-signalling defective

22	mutants, whereas *CBF2* and *CBF3* induction were unaffected (*SI Appendix*, Fig. S3).

23	Consistently, the levels of *COR15A* and *COR15B* were only slightly decreased, with the most

24	prominent effects 24 h after treatment (*SI Appendix*, Fig. S3).

CES and Homologues Promote Basal Freezing Tolerance. To identify BR-controlled TFs that

promote freezing tolerance downstream of ASKs a candidate gene approach was employed.

The dominant *bes1-D* (12), *bzr1-1D* (13) and *ces-D* (14) mutants were tested for their responses

to freezing stress. Sub-zero temperature treatments of non-acclimated adult plants showed that,

whereas the *bzr1-1D* mutation did not confer an effect, the *bes1-D* mutation slightly suppressed

freezing tolerance in this experimental setting (*SI Appendix*, Fig. S4*A*-*C*). On the contrary, the

ces-D mutation clearly increased survival rates, conferring a high level of resistance to −6 °C

treatment (Fig. 3*A* and *B*) and reducing electrolyte leakage in the treated plants (Fig. 3*C*).

In the Col-0 background CES acts redundantly with the BEEs in floral organ development

(16) and it therefore seemed likely that the BEEs would also compensate for a loss of CES

function in freezing tolerance. Accordingly, to evaluate the effects of CES loss of function on

freezing tolerance, a *ces-2 bee1 bee2 bee3* quadruple mutant (*qM*) was generated by

introducing a newly identified CES knock-out mutant allele, *ces-2* (whose characterisation is

shown in Fig. S5), into the *bee1-3* mutant background (15). In addition, the *haf/ces-3 bee1 bee3*

triple mutant (Fig. S5*A* and *B*; (16)) was included in the analysis (termed *tM* from here forward).

The results of freezing tolerance assays showed that the *tM* and *qM* were hypersensitive to

freezing and showed increased electrolyte leakage (Fig. 3*A*-*C*).

To address the molecular basis of the *ces* mutants freezing tolerance phenotypes, a

bioinformatic analysis of available *ces-D* microarray data (14) was carried out. It was determined

to which extent the dominant *ces-D* mutation can induce the expression of *COR* genes on a

genome-wide scale. This revealed that 7.5 % of *ces-D*-induced genes were also cold-inducible

and that the CBF regulon, as defined by Park *et al*., (2015) (25), was significantly enriched

among them (Fig. 3*D*; *SI Appendix*, Table S2). qPCR confirmed that *CBFs* were significantly

induced in *ces-D*. However, the degree of regulation varied between developmental stages with

only *CBF1* induced in seedlings (*SI Appendix*, Fig. S6), but all *CBFs* up-regulated in adult plants

(Fig. 3*E*). This induction was correlated with an increased expression of all down-stream *COR*

genes tested. In the CES *tM* and *qM CBF1* and *CBF3* were reduced by approximately 5-fold in adult plants (Fig. 3*E*), whereas in seedlings a significant reduction on a whole-plant scale was detectable only for *CBF3* (*SI Appendix*, Fig. S6). This was correlated with a reduction of downstream *COR* genes, in particular of *COR15A*, in both developmental settings (Fig. 3*E*; *SI Appendix*, Fig. S6). Moreover, in *tM* and *qM* plants BR treatment was ineffective in inducing *CBF1*, *CBF3* and *COR15A* expression (Fig. 3*F*). Therefore, in summary there is evidence that the CES/BEE bHLH subfamily participates in basal freezing tolerance and the BR-induction of *CBF* expression.

CES and Homologues Participate in Cold Acclimation. To test whether CES and the BEEs may also affect freezing tolerance following cold adaptation *ces* mutant plants were cold acclimated before exposure to sub zero temperatures. Also in these assays, *ces-D* was more resistant to freezing stress than wild type, whereas the *tM* and *qM* lines were clearly hypersensitive (Figs. 4*A* and *B*). Electrolyte leakage assays confirmed that *ces-D* was less affected, whereas *tM* and *qM* plants were more affected by the treatment (Fig. 4*C*).

When in the *ces* mutant lines the expression of CBFs was assessed following cold treatment, it was found that in *ces-D* the induction of *CBF1* was more pronounced than in the wild type (*SI Appendix*, Fig. S7). This was correlated with a more pronounced increase in the mRNA levels of *COR15A* and *COR15B* (Fig. 4*A*). In *qM* plants no significant differences to wild type were detectable.

CES Directly Binds to *CBF* Promoters *in planta*. In view of the evidence that CES has the ability to promote *CBF* expression, it was investigated whether the *CBFs* are direct CES targets. The promoters were searched for CES binding sites and all 3 promoters were found to contain G-box motifs. To investigate whether CES can bind to these regulatory elements, chromatin immuno-precipitation (ChIP) assays were performed with *35S:CES-YFP*-expressing plants before and after cold treatment. The result showed that CES was significantly enriched on the G-

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box containing promoter regions of all *CBFs*, both in untreated conditions and following cold treatment (Figs. 5*A* and *B*). I*n vitro* DNA binding studies with recombinant protein confirmed that CES directly bound to the G-box motifs in the *CBF* promoters (Fig. 5*C*). LUCIFERASE (LUC) reporter assays in arabidopsis protoplasts from the *ces-3/haf bee1 bee3* tM with a fragment of the *CBF1* promoter containing the G-box and one in which the G-box was mutated showed that the G-box was necessary for CES transcriptional activity *in vivo* (Fig. 5*D*).

CES activity is altered by SUMOylation, which is induced in response to an activation of BR signalling (17). Since protein SUMOylation plays a central role in cold stress responses (6), we addressed if the SUMOylation state impacts on CES activity in *COR15A* expression and freezing tolerance. *COR15A* expression levels were determined in plants expressing CES wild-type (35S:CES^{wt}-YFP, line 32; (14) or CES mutant versions, with either impaired (35S:CES^{K72R}-YFP, line 411) or enhanced SUMOylation (35S:CES$^{S75A+S77A}$-YFP, line 310; (17)). The result showed that, as compared to CES^{wt} expressing plants, plants expressing non-SUMOylated CES^{K72R} had decreased, whereas plants expressing constitutively SUMOylated CES$^{S75A+S77A}$ had increased *COR15A* levels (Fig. 5*E*). Also, plants expressing CES^{K72R} suffered more, and plants expressing CES$^{S75A+S77A}$ suffered slightly less damage by freezing than the CES^{wt} expressing control (Fig. 5*F-H*). Therefore, there is evidence that SUMOylation promotes CES activity in freezing tolerance.

BRs and CES Control Common Non CBF-Regulon Genes in Response to Cold. Although there was strong evidence that CES can regulate *CBF* expression, the subtle changes in *CBF* and down-stream *COR* gene expression in BR and CES loss-of-function mutants, in particular during cold acclimation, as compared to their clear freezing hypersensitivity, indicated that, in addition to the CBF regulon also other types of cold-responsive genes are regulated by BRs and CES. To identify these genes we assessed global changes in gene expression in 3-week-old *bri1-301*, *ces-D*, the *qM* and wild type in response to cold stress using the new Affymetrix Arabidopsis Gene 1.1 ST Array.

Comparison of cold-treated (4 °C) and untreated (21 °C) wild-type plants showed that 1,720 genes were significantly induced (>1.5-fold change; FDR <0.05) and 2,183 were significantly repressed in response to cold stress (<-1.5-fold change; FDR <0.05) (*Dataset S1*, Table S3). Among the cold-induced genes a highly significant share (hypergeometric test p-value: $5*10^{-76}$; Fig. 6*A*) was previously identified as CBF-induced genes (25). Also, a significant share of genes repressed (Fig. 6*B*), were previously identified as CBF-repressed (25), although less significantly (p-value: $5*10^{-8}$). A relatively large number of CBF regulon genes, as defined by Park et al. (25) escaped detection in cold-treated wild type plants (Fig. 6*A* and *B*), which may be due to differences in ecotypes, experimental settings and array types used. The Arabidopsis Gene 1.1 ST Array differs from the ATH1, which had been used to define the CBF regulon (25), since it employs a random primer for reverse transcription of RNA and a different design of probe sets, resulting in differences in signal strength between the two array types (26). This is important to consider, in particular when the mutant data is interpreted.

Comparison of wild type and *ces-D* at 21 °C revealed that of 737 *ces-D* induced genes (*Dataset S1*, Table S4), 24 were also CBF-induced ($p=1*10^{-13}$). A further 105 cold-induced genes identified are not members of the CBF regulon ($p=5*10^{-26}$) (Fig. 6*C*). Among those a significant enrichment of annotations associated with membrane was found (*SI Appendix*, Fig. S8*A*). Interestingly, among the 638 *ces-D* repressed genes a highly significant share of 261 cold-repressed genes was present ($p=1*10^{-120}$); of those 7 were CBF-repressed genes, which again is a significant share ($p=5*10^{-8}$; Fig. 6*D*). GO analysis of the 256 CBF-independently up-down-regulated genes revealed a number of terms enriched, many associated with lipid and fatty acid biosynthesis or metabolism (*SI Appendix*, Fig. S8*B*; *Dataset S1*, Tables S5 and S6).

With the microarray analysis relatively few genes were found to be miss-regulated in *bri1-301* and the *qM* at 21 °C (15 up and 41 down in *bri1-301*; 3 up and 19 down in the *qM*; Figure S9). Among the genes induced in *bri1-301* were the BR biosynthesis genes *BR6Ox2* and *ROT3* (*Dataset S1*, Table S4), which are feedback-induced in BR-deficient mutants (23, 27). No

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significant share between genes induced in *bri1-301* or the *qM* and CBF- or cold-induced genes was found (*SI Appendix*, Fig. S9*A-C*). Comparison of genes repressed in *bri1-301* and the *qM* with CBF- and cold-induced genes revealed that only a few CBF-induced genes were also constitutively repressed in these mutants. Although this share was not significant, the microarray analysis did identify *COR15A* as repressed in both *bri1-301* and the *qM* (*Dataset S1*, Table S4). Given that the clear reduction in *CBF1* expression in *bri1-301* and the *qM* and the extent of increase in *ces-D* at 21 °C (Figs. 1*B* and 3*B*), escaped detection with the arrays, it is evident that the arrays were less sensitive than the qPCR analysis we applied. In support, *COR15A*, which, according to qPCRs, was approx. 80-fold increased in *ces-D*, was determined as being increased only approx. 8-fold with the arrays. This reduced sensitivity will have masked changes in gene expression that prevail in the knock-out lines. However, the high stringency also bears benefits, since identified changes can be seen with stronger confidence.

In *bri1-301*, in response to 4 °C, 656 genes failed to be induced and 834 genes failed to be repressed as compared to wild type (Fig. 6*E* and *F*; *Dataset S1*, Table S7 and S8), indicating that they rely on BRI1 function for cold regulation. Among those, CBF regulon members were represented (12 and 2 resp.), but also many additional types of *COR* genes, which are shown in Table S8 and S9 (*Dataset S1*). In the *qM* 369 genes failed to be induced and 576 genes failed to be repressed in response to 4 °C as compared to wild type. Almost all of these genes were not CBF regulon genes (Figs. 6*G* and *H*; *Dataset S1*, Tables S10 and S11) providing evidence that, during cold adaptation, CES largely impacts CBF-independent routes of *COR* gene regulation. Interestingly, in both *bri1-301* and the *qM*, several CBF-induced genes were more strongly up-regulated by cold than in wild type (*Dataset S1*, Tables S8 and S10), which may result from the lower basal levels of CBF regulon expression that prevail in these mutants (Figs. 1*B* and 3*B*).

Importantly, there was a strikingly large overlap of 313 *COR* genes ($p < 9*10^{-98}$) that failed to be induced in both *bri1-301* and the *qM* mutant (Fig. 6*I*; Table S12). GO enrichment analysis revealed terms related to aromatic compound and phenylpropanoid biosynthesis and

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1   metabolism, but also terms associated with defence and immune responses (*SI Appendix*,

2   Figure S10*A*; *Dataset S1*, Table S13). Moreover, there was a highly significant over-lap of 455

3   *COR* genes (p<1*10^{-122}) that failed to be repressed in both *bri1-301* and the *qM* (Fig. 6*J*; *Dataset*

4   *S1*, Table S12). Very interestingly, in addition to GO terms associated with lipids and fatty acids

5   as in *ces-D*, genes involved in cell cycle regulation, cell skeleton and microtubule activity were

6   highly over-represented (*SI Appendix*, Fig. S10*B*; *Dataset S1*, Table S14). Also, a number of

7   cyclins were present (*Dataset S1*, Table S13).

8   In *ces-D*, the expression of a multitude of genes was altered in response to 4 °C (*Dataset*

9   *S1*, Table S3). 1,153 genes showed enhanced expression, while 1,223 had reduced expression

10   as compared to wild type. Notably, the expression of 12 CBF-regulated genes was stronger in

11   *ces-D* than in wild type (Fig. 6*K*), confirming that *ces-D* has a larger capacity to activate the CBF

12   regulon during cold acclimation. Also, 7 CBF-repressed genes were more strongly repressed in

13   *ces-D* than in wild type (Fig. 6*L*). In addition, 269 non CBF-regulon genes were more strongly

14   induced and 298 more strongly repressed in *ces-D*. GO analysis again revealed terms

15   associated with stress responses and lipids biosynthesis respectively, but also ribosome

16   biogenesis and rRNA metabolism (*SI Appendix*, Figs. S8*C* and *D*; *Dataset S1*, Table S5 and

17   S6). Thus, in addition to a clear impact on the CBF-regulon, *ces-D* also affects additional types

18   of *COR* genes. Particularly fatty acid and lipid biosynthesis, metabolism and localisation appear

19   to be targets.

20

21   **Discussion**

22   BRs are steroid hormones with versatile roles throughout plant development (11). In the early

23   days of BR research, it became apparent that in addition to promoting growth, BR application

24   increases plant resistance against different types of abiotic stress, including chilling stress (9). In

25   this study we provide evidence that BRs control freezing tolerance and that this ability is

1   conferred by an effect of BRs on the expression of different classes of *COR* genes, including the

2   CBF regulon.

3   BR-deficient arabidopsis mutants were impaired in the basal expression of *CBFs* and

4   downstream targets, whereas an activation of BR signalling through *BRI1* over-expression

5   increased the basal levels of *CBF*s, in particular of *CBF1* and *CBF3*. This result supports earlier

6   findings of increased basal *CBF* expression in *BRI1oe* plants (8-10) and increased *COR15A*

7   levels in plants over-expressing the BR biosynthesis gene *DWF4* (*DWARF4;* (24)). In contrast, a

8   semi-quantitative analysis had indicated that in the BRI1 allele *bri1-9 CBF* expression is

9   increased (9). Our study has now quantitatively assessed *CBF* and down-stream *COR* gene

10   expression in several BR mutants and provides evidence that BR signalling promotes basal

11   expression of *CBFs*. The fact that BRs did not induce *CBF* expression on a whole-plant level at

12   early time points after BR treatment, but only when plants were exposed to BR for longer periods

13   of time, supports the notion that the BR status affects basal *CBF* expression, but does not

14   significantly impact *CBF* expression in early responses to BR or cold.

15   There is evidence that the activity of BRs in basal *CBF* transcription is mediated by the

16   BR-regulated bHLH TF CES and its homologues BEE1 and BEE3. In the dominant *ces-D*

17   mutant basal expression levels of *CBFs* and down-stream *COR* gene were strongly increased.

18   In *ces bee* knock-out plants basal *CBF1* and *CBF3* levels were decreased and *CBF1* and *CBF3*

19   induction by BRs was compromised. Moreover, in response to cold, the *ces-D* mutant activated

20   the CBF regulon to a larger extend than wild type, which could be due to the elevated basal *CBF*

21   expression levels that exist in this mutant.

22   Although the constitutive changes in *CBF* and *COR* gene expression revealed in BR and

23   CES loss-of-function plants, in particular when compared to their clear freezing hypersensitivity,

24   may be considered mild, there are several things to be considered. First, BR responses at the

25   transcriptional level are small, usually only 2-3 fold (28, 29). Second, tissue specific differences

in *CBF* expression, that may be instrumental for basal freezing tolerance, could have been disguised when whole plants were used for qPCR and microarray analyses. Third, in loss-of-function mutants functional redundancy can mask regulatory effects and it is clear that additional factors and pathways can also regulate *CBF* expression. Therefore, the relatively mild changes in basal *CBF* expression levels in the loss-of-function plants do not exclude a role of the BR-CES/BEE-*CBF1/3-COR* pathways in basal freezing tolerance. However, since we here show that additional pathways participate it will be important to determine the relative contribution of the CBF regulon to BR-CES/BEE conferred basal freezing tolerance.

In addition to their role in basal freezing tolerance, BRs and CES are also of importance for freezing stress resistance that requires cold adaptation, since BR and CES/BEE mutants have clear defects in acquired freezing tolerance. However, in this process BRs appear to act largely in a CBF-independent manner, since in BR and CES/BEE knock-out mutants the CBF regulon is activated to similar extends like in wild type. A whole-transcriptome analysis identified non CBF-regulon types of *COR* genes that depend on BRI1 and CES for activation or repression in response to cold. Importantly, there is a highly significant overlap of genes miss-regulated in both *bri1-301* and *qM* plants following cold exposure, providing evidence that the role of BRs in cold adaptation is conferred to a significant degree by CES and the BEEs. Within the *COR* genes that failed to be repressed in BR and CES knock-out mutants the annotations cell cycle regulation, cell skeleton and microtubule activity were overrepresented. This indicates that without a functional BR-CES/BEE module plants are unable to decrease cell division and metabolism during cold adaptation, which could, at least in part, explain the freezing hypersensitivity of the mutants. Also, annotations for fatty acid and lipid synthesis, metabolism and transport were over-represented. Since it is well established that in response to cold stress changes in the structure and composition of membranes occur, which are dedicated to reduce damage caused by freezing (2, 7), it is possible that a role of CES/BEEs in the synthesis of fatty acid and lipids, which are essential membrane building blocks, contributes to the freezing

hypersensitivity of the mutants. In the future it will be important to investigate which factors are directly regulated by CES/BEE during cold acclimation and to determine their relative contribution to BR-CES/BEE-conferred cold acclimation-dependent freezing tolerance.

Unlike the BEEs, whose expression is BR-induced (15), CES is not BR-regulated at the transcriptional level but is subject to BR-induced post-translational modification. The current postulation is that in response to BR CES phosphorylation by BIN2 is inhibited, CES accumulates in an unphosphorylated state, promoting CES SUMOylation and nuclear compartmentalisation (17). This may restrict CES activity on one type of promoters, for example those of BR biosynthesis genes (14), which are repressed when BR levels become high (23, 27), but may facilitate activity on other types such as *CBFs*. In support of this hypothesis we have first evidence that SUMOylation promotes CES activity in *COR15A* expression and freezing tolerance suggesting a model in which BRs induce CES SUMOylation to alter COR gene expression.

In cold responses, protein SUMOylation plays a key role. The overall SUMOylation of proteins drastically increases in response to cold stress (6), and the SUMOylation of ICE1 enhances its activity in *CBF3* transcription. The manner by which ICE1 SUMOylation is induced is currently unknown, although a phosphodeficient mutant of ICE1 is more readily SUMOylated (30), and also in CES, de-phosphorylation promotes SUMOylation (17). It is thus possible that in response to cold, BR signalling is activated to alter the phosphorylation state of TFs, inducing SUMOylation and activating them in *COR* gene expression. This, in a secondary response, would reduce BR biosynthesis, given that when BR signalling is activated, BR biosynthetic gene expression is repressed (23, 27). In support of this model (illustrated in Fig. 7), the BR biosynthesis gene *CPD*, a direct CES target (14), is down-regulated by cold both in arabidopsis (31) and in mungbean (32), and also in our hands, *CPD,* as well as also *DFW4* and *BR6ox2 (BRASSINOSTEROID-6-oxidase 2),* mRNA abundance were reduced in response to cold (*SI Appendix*, Figure S11). However, importantly, in BR-deficient mutants, unlike in other mutants

with growth defects (33, 34), growth repression is not correlated with increased stress tolerance. This lack of correlation supports the notion that the function of BRs in freezing tolerance is not principally to cause morphological changes that promote plant survival as a secondary consequence, but that BRs directly participate in signalling events required for freezing tolerance.

Although tolerance to different types of abiotic stress can be enhanced by *CBF* overexpression (35), this is not an agronomically feasible strategy, given that overexpressing *CBFs* impairs plant growth by inhibiting GA metabolism and signalling (33). In contrast, the overexpression of *CES* induces *CBF* transcription and enhances freezing tolerance, but does not also produce dwarf plants (14), uncoupling freezing tolerance from growth repression. Different, but not mutually exclusive mechanisms may account for this. First, *CBF* mRNA levels in *ces-D* may be sufficient to increase *COR* expression and freezing tolerance, but not to repress growth. Second, the growth-repressive activity of CBFs may be released by the positive regulatory role of *ces-D* in BR biosynthesis (14), and/or third, *ces-D* may directly interfere with the effects of CBFs on GA biosynthesis and/or signalling.

In conclusion, our study reveals that BRs, in addition to their growth-promoting capacities, enhance plant resistance against freezing stress. We present evidence that this function is conferred by the activity of the CES/BEE proteins in controlling *COR* gene expression. Given that *BRI1-* and *CES* over-expression increases freezing tolerance, but does not also repress growth, this approach may be promising for enhancing crop resistance against freezing, and possibly also other types of abiotic stress. Such a strategy would be of high relevance for plant production in agriculture and horticulture.

Material and Methods

1   **Plant Material.** The T-DNA insertional mutant *ces-2* was obtained from NASC (SALK_124840),

2   and the site of insertion was mapped by PCR (primers CES KO fwd and CES KO rev in

3   combination with LBb1; all primers used in this study are listen in Table S14) to the location

4   1380 downstream of the ATG (*SI Appendix*, Fig. S5*A*). qPCR analysis showed that CES

5   expression was reduced in *ces-2* (*SI Appendix*, Fig. S5*C*).

6   *ces-2* was crossed with *bee1 bee2 bee3* (15), and the F2 offspring were genotyped using

7   the primer pairs CES KO fwd/CES KO rev, BEE1 KO fwd/BEE1 KO rev, BEE2 KO fwd/BEE2 KO

8   rev and BEE3 KO fwd/BEE3 KO rev. These primers, for the wild type, and plants heterozygous

9   for the corresponding gene, yielded amplicons of 484 bp, 1077 bp, 458 bp and 709 bp,

10   respectively, whereas they did not yield amplicons for homozygous plants.

11   For phenotypic analysis of silique filling, 8-week-old, soil-grown *ces* mutant plants were

12   used. Siliques were harvested from a single branch of at least 5 plants of the indicated

13   genotypes. The siliques were incubated in a clearing solution (20 g of chloral hydrate, 4.6 ml of

14   water, 2 ml of glycerol, 87%) for 24 h at room temperature and were assessed by optical bright

15   field microscopy.

16   **Plant Freezing Assay.** Plant freezing assays were performed as described previously (6, 36)

17   with modifications. Plants were grown in soil in Bright Boy growth chambers (CLF Plant

18   Climatics GmbH, Wertingen, Germany) at 21 °C under long-day (LD) conditions (16 h light at 80

19   µmol m^{-2} s^{-1}/ 8 h dark) for 3 weeks before the treatments were performed. For treatments without

20   cold acclimation plants were incubated in a controlled temperature chamber (Panasonic MIR-

21   154, Panasonic Biomedical, Osaka, Japan) for 30 min at 4 °C, then for 1 h at 0 °C, before the

22   temperature was decreased at 2 °C per hour. The final desired subzero temperature was

23   maintained for the indicated period of time before the temperature was again increased at the

24   same rate to 4 °C. The plants were then kept at 4 °C for 1 day before they were returned to 21

1 °C. Survival was scored 2 weeks later, with only those plants able to develop new leaves were

2 counted as survivors.

3   For cold acclimation experiments 3-week-old plants were acclimated for 3 days at 4 °C in

4 the light. Freezing treatment was then performed in the same manner as for non-acclimated

5 plants with the final freezing temperature of −10 °C maintained for 6 h.

6 **Electrolyte Leakage Assays.** Electrolyte leakage from fully expanded rosette leaves of 3-week-

7 old plants was measured as described previously (37) with modifications. Plants were grown in

8 soil at 21°C in LD conditions, and the fifth and sixth leaf pairs were used. The leaves were

9 placed in tubes containing 100 µl of deionised water. An ice chip was added to facilitate

10 nucleation, and the tubes were kept at −2 °C for 2 h followed by a temperature decrease at 2 °C

11 per hour. Samples were removed at the indicated temperature points and were immediately

12 placed on ice for gradual thawing overnight. On the next day, 6 ml of deionised water was added

13 to each tube, and the samples were incubated for 5 h at 21°C with gentle shaking, after which

14 the conductivity of the solution was determined with a conductivity metre (GMH 3430, Greisinger

15 Electronic, Regenstauf, Germany). The tubes were then incubated at 95 °C for 3 h, and the

16 conductivity of the solution was measured again. Electrolyte leakage was quantified as a

17 percentage of the conductivity after treatment relative to total conductivity.

18 **qPCRs.** For qPCRs plants were grown vertically on agar plates or in soil depending on the

19 experiment. Total mRNA was extracted with a Plant RNA Kit (Omega Bio-Tek, Norcross, GA,

20 USA) and treated with DNaseI to digest traces of DNA. First strand cDNA was synthesized from

21 1 µg of RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham,

22 USA) according to the manufacturers' instruction. qPCRs were performed with the SensiFAST

23 SYBR Lo-ROX Kit (Bioline, London, UK) using the Mastercycler Realplex (Eppendorf, Hamburg,

24 Germany). *Ubiquitin-conjugating enzyme 21* (*UBC21;* (38)) was used for the normalisation of the

25 results. qPCRs were typically performed with at least 2 independent biological samples, each

measured in at least 3 technical repeats. For qPCR analysis of cold-treated plants, seedlings were grown vertically on agar plates. Twelve-day-old seedlings were treated at 4 °C in the light, and plant material was collected in a time-course manner. Statistical significance of differences between two samples was assessed using the Student's t-test. For quantitative analyses among multiple samples, statistical significance was assessed using analysis of variance (ANOVA) followed by the Tukey's post-hoc test.

ChIPs. For ChIPs 3-week-old, soil-grown 35S:CES-YFP plants (line 32; (14)) and wild type were treated for 3 hrs with 4 °C or left at 21°C., ~0.6 g of plant material was harvested for each biological replicate. ChIP was performed in 3 biological repeats according to (39).

Protoplast Transformation and Luciferase Transactivation Assays. For transactivation assays, a *35S:CES* construct was used (14). For reporter plasmid generation, a *CBF1* promoter fragment containing the G-box was PCR-amplified with the primer pair CBF1-luc-FW/RV. The primer pair CBF1-luc-FW1/RV was used to mutate the G-box sequence, yielding the mCBF1 promoter fragment. The resulting PCR fragments were cloned into the pGreenII-0800-LUC vector (40).

Protoplasts were isolated from *haf bee1 bee3* plants (16) and transiently transformed using a PEG-mediated transformation protocol. Luciferase assays were performed using a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) and a Lumat LB9501 luminometer (Berthold, Bad Wildbach, Germany) as described previously (17).

Whole Transcriptome Analysis. Whole transcriptome analysis was performed from 3-week-old, soil-grown plants, treated with 4°C in the light for 3 days. Controls were kept at 21°C for 3 days. RNA was prepared from aerial tissues of plants, sample concentration and purity was determined by spectrophotometry and RNA integrity was confirmed using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). Whole transcriptome analysis was conducted by hybridizing total RNA of four independent biological

replicates for each line and treatment (thirty-two biological samples in whole) to Affymetrix Arabidopsis Gene 1.1 ST Array Strips (Affymetrix, Santa Clara, CA, USA).

Gene expression data were analyzed using Partek Genomics Suite 6.6 software (Partek Incorporated, St. Louis, USA). The raw CEL files were normalized using the RMA background correction with quantile normalization, log base 2 transformation and mean probe-set summarization with adjustment for GC content. One 4°C-treated wild-type samples was identified as an outlier using Partek and was thus excluded from further analysis.

Bioinformatic Analyses. The *det2-1* mutant comparison against the wild type was performed using the AtGenExpress data set 'Brassinolide time course in wild-type and *det2-1* mutant seedlings' (TAIR ME00335). The CEL data were normalised using the RMA algorithm as provided by the justRMA method in the Bioconductor Affy package (41). For each time point (30 min, 1 h and 3 h) significantly regulated genes (FDR-adjusted P-value <0.05) were called with the *limma* package (42). The union of these sets was considered to represent the *det2-1*-associated genes. *COR* and CBF regulon-related genes were assigned as described previously (25). The significance of the overlap of *COR* genes and genes related to the CBF regulon was determined with a chi-square test of independence.

For bioinformatics analysis of the microarray data differentially expressed genes (DEG) were identified by a two-way ANOVA, and p-values were adjusted using the FDR (false-discovery rate) method to correct for multiple comparisons. DEG were considered significant if p-value was ≤0.05 at a fold-change (FC) of ≥2 with an FDR <0.05. The raw data was included in the Gene Expression Omnibus (GEO) database. Venn diagrams were constructed using the Venn diagram generator (bioinformatics.lu/venn.php). Significance of overlaps was calculated with the hypergeometric test. Analysis for enrichment of GO terms was performed with agriGO (bioinfo.cau.edu.cn/agriGO/analysis.php).

EMSAs. HEX-labelled probes for EMSAs were prepared by PCR using the primer pairs CBF1-ChIP-FW/HEX-CBF1-ChIP-RV, HEX-CBF2-ChIP-FW/CBF2-ChIP-RV and CBF3-ChIP-FW/HEX-CBF3-ChIP-RV. The amplicons were purified using an E.Z.N.A. Cycle Pure kit (Omega Bio-Tek). The probes (30 ng per reaction) were incubated with purified CES-GST protein and subsequently separated on 6% PAGE gels as described previously (14). The bands were detected using a Molecular Imager FX Pro (Biorad, Hercules, USA) equipped with a 532 nm laser for excitation and a 555 nm long pass emission filter.

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Author contributions

Designed research: M.E., S.J.U., M.C., K.G.K., S.T.M., K.F.X.M., W.R., B.P; performed research: M.E., S.J.U., A.I.R., M.C., M.K., K.G.K., W.R.; analyzed data: M.E., S.J.U., M.C., K.G.K., W.R., B.P.; wrote paper: B.P.

Figure legends

Fig. 1. BR signalling promotes basal freezing tolerance. (*A,B*) Freezing tolerance of non-acclimated *BRI1oe*, *bri1-301* and *bri1-1* plants as compared to wild type. Plants were grown in LD growth conditions at 21 °C. After 3 weeks they were treated at −6 °C or −8 °C for 4 h. Survival was assessed after 2 weeks of recovery at 21 °C. (*A*) shows representative plants of each line and (*B*) shows the quantified results. Error bars show the SDs of 3 biological replicates. (*C*) Electrolyte leakage in leaves of non-acclimated plants of the wild type, *BRI1oe* and *bri1-301* plants grown under the same conditions as described in a and treated with the indicated temperatures. Error bars show the SD of 3 biological replicates. (*D*) Freezing tolerance of non-acclimated *ASKθoe-27* and *ASKθoe-10* plants as compared to wild type. Plants were grown in the same conditions as in *A* and treated at −6 °C for 4 h. Quantified results (left) and representative plants (right) are shown. (*E*) Venn diagram showing the overlap of cold-induced genes (as defined by Park et al. (25); in purple) with genes repressed in *det2-1* (determined from dataset TAIR ME00335; in lavender). The significance of representation of the CBF-regulon of *COR* genes (as defined by Park et al. (25); in yellow) in the overlay was calculated with a chi-square test giving the P-value shown. (*F*) Transcript levels of *CBFs* and *COR* genes were determined in 3-week-old plants of wild type, *35S:BRI1-GFP* (*BRI1oe*), *bri1-301* and *bri1-1* by qPCRs. Error bars show the SDs of 3 biological replicates. The letters indicate significant differences between genotypes (P < 0.05; ANOVA).

Fig. 2. BR signalling contributes to cold acclimation. (*A,B*) Freezing tolerance of *BRI1oe*, *bri1-301* and *bri1-1* plants as compared to wild type after cold acclimation. Three-week-old plants grown in LDs at 21 °C were acclimated for 3 days at 4 °C and then treated at −10 °C for 6 h. Survival was scored after 2 weeks of recovery at 21 °C. Pictures of representative plants (*A*) and the quantified results (*B*) are shown. Error bars show the SDs of 3 biological replicates. (*C*)

Electrolyte leakage in acclimated plants of the wild type, *BRI1oe* and *bri1-301.* Plants were grown and acclimated as described in B, and ion leakage was measured in detached leaves following exposure to the indicated sub-zero temperatures. Error bars show the SDs of 3 biological replicates.

Fig. 3. CES and homologues confer basal freezing tolerance. (*A,B*) Freezing tolerance of non-acclimated *ces-D, tM* and *qM* lines as compared to that of the wild type. Plants were grown in soil in LD growth conditions at 21 °C for 3 weeks and were treated at −6 °C for 4 h. Survival was assessed following 2 weeks of recovery at 21 °C. Shown are representative plants of each line (*A*) and a quantification of the results (*B*). Error bars show the SD of 3 biological replicates. (*C*) Electrolyte leakage in leaves of non-acclimated plants of the wild type, *ces-D, tM* and *qM* plants grown under the same conditions as described in C and treated at the indicated temperatures. Error bars show SDs of at least 2 biological replicates. (*D*) Venn diagram showing the overlap of cold-induced genes (as defined by Park et al. (25); in purple) with genes induced in *ces-D* (as defined by Poppenberger et al. (14); in pink). The significance of representation of the CBF-regulon of cold-induced genes (as defined by Park et al. (25); in yellow) in the overlay was calculated with a chi-square test giving the P-value shown. (*E*) mRNA levels of *CBFs* and *COR* genes in 3-week-old, non-acclimated soil-grown *ces-D, tM* and *qM* plants determined by qPCRs. Error bars show SDs of at least 2 biological replicates. The letters indicate significant differences between genotypes ($P < 0.05$; ANOVA). (*F*) Transcript levels of *CBF1, CBF3* and *COR15A* were assayed in 10-day-old wild-type seedlings grown on ½ MS media supplemented with 250 nM epi-BL. Error bars show SDs of at least 2 biological replicates. Asterisks indicate significant differences (**P* < 0.05, ***P* < 0.01; Student's t-test).

Fig. 4. CES and homologues promote acquired freezing tolerance. (*A,B*) Freezing tolerance of *ces-D, tM* and *qM* plants. Three-week-old plants grown in LD growth conditions at 21 °C were acclimated for 3 days at 4 °C and then treated at −10 °C for 6 h. Survival was scored after 2 weeks of recovery at 21 °C. Images of representative plants (*A*) and quantified results (*B*) are

25

shown. Error bars show SDs of 3 biological replicates. (*C*) Electrolyte leakage in detached leaves of acclimated plants of the wild type, *ces-D, tM* and *qM.* Plants were grown and acclimated as described in b before electrolyte leakage assays were performed. Error bars show SDs of 3 biological replicates.

Fig. 5. CES directly binds to the promoters of *CBFs* and activates their expression. (*A*,*B*) ChIP of CES-YFP followed by qPCR of DNA fragments containing the G-box motifs in the *CBF1* (G-box: −106 from the transcriptional start)*, CBF2* (G-box: −111 from the transcriptional start) and *CBF3* (G-box: −2,151 from the transcriptional start) promoter. Leaves of 3-week-old *CES-YFPoe* plants and wild type grown in soil in LDs, either untreated (*A*) or treated at 4 °C for 3 h (*B*), were used. The *5sr RNA* gene was used for normalisation. *UBQ5* was measured as a control. Values are fold enrichment of CES-bound DNA containing the G-box motif in immuno-precipitated samples relative to the total input DNA. Error bars show the SD of 3 biological replicates. Asterisks indicate significant differences (*P < 0.05, **P < 0.01; Student's t-test). (*C*) CES binds to G-box motifs in the promoters of *CBF1*, *CBF2* and *CBF3 in vitro*. Fluorescent-labelled probes representing parts of the *CBF1*, *CBF2* or *CBF3* promoter that contain a G-box were incubated with CES-GST. Competitor (C) or mutated competitor oligos with the G-box mutated (C*) were added in 10x and 100x molar excess to analyse the specificity of binding. (*D*) Luciferase transactivation assays in arabidopsis protoplasts from the *ces-3/haf bee1 bee3* tM. The LUC reporter constructs (wtCBF1p: wild-type *CBF1* promoter, mCBF1p: *CBF1* promoter with mutated G-box) were transiently expressed in protoplasts either alone (control) or with CES as an effector. Error bars show SDs of 3 biological replicates. Asterisks indicate significant differences (*P < 0.05, **P < 0.01; Student's t-test). (*E*) Transcript level of *COR15A* in plants over-expressing either wild-type CES (CES wt), a mutant impaired in SUMOylation (CES K72R) or a mutant constitutively SUMOylated (CES S75A+S77A: CES AA). Error bars show SDs of 3 biological replicates. The letters indicate significant differences between genotypes (*P* < 0.05; ANOVA). (*F*,*G*) Freezing tolerance of *CESwt-*, CES *K72R-* and *CES AA*-expressing plants.

Three-week-old plants grown in LDs at 21 °C were acclimated for 3 days at 4 °C and then treated at −10 °C for 6 h. Survival was scored after 2 weeks of recovery at 21 °C. Pictures of representative plants (*F*) and the quantified results (*G*) are shown. Error bars show SDs of 3 biological replicates. (*H*) Electrolyte leakage in leaves of non-acclimated plants of -type and *CESwt*-, CES *K72R*- and *CES AA*-expressing plants grown in the same conditions as in b and treated at the indicated temperatures. Error bars show SDs of 3 biological replicates.

Fig. 6. Whole transcriptome changes in BR and CES mutants in response to cold. (*A,B*) Overlap of genes induced (*A*) or repressed (*B*) in wild type in response to 4 °C, wild type grown at 21°C, and CBF-induced or repressed genes. p-values were calculated with the hypergeometric test. (*C,D*) Overlap of genes induced (*C*) or repessed (*D*) in *ces-D* as compared to wild type at 21 °C, cold-induced genes of wild type, and CBF-induced genes. (*E,F*) Overlap of genes induced (*E*) or repressed (*F*) in *bri1-301* in response to 4°C treatment as compared to *qM* grown at 21°C and CBF- and cold-induced or repressed genes. (*G,H*) Overlap of genes induced (*G*) or repressed (*H*) in *qM* in response to 4°C treatment as compared to *qM* grown at 21°C and CBF- and cold-induced or repressed genes. (*I*) Overlap of cold-induced genes that failed to be induced in *bri1-301* and *qM* plants treated with 4°C. (*J*) Overlap of cold-repressed genes that failed to be repressed in *bri1-301* and *qM* plants treated with 4°C for 3 d. (*K*) Overlap of genes up-regulated in *ces-D* in response to 4°C treatment as compared to wild type, CBF-induced genes and cold-induced genes. (*L*) Same as (*K*) but for down-regulated genes.

Fig. 7. Working model for the contribution of BRs to freezing tolerance. Cold stress stimulates BR signalling to induce de-phosphorylation and SUMOylation of CES and activate it in both CBF-dependent and CBF-independent modes of *COR* gene regulation. As a consequence of an activation of BR signalling BR biosynthesis is feedback repressed through positive and negative regulation of activators (including CES) and repressors (including BZR1) of BR biosynthetic gene transcription.