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# Studies of factors involved in cold stress responses of plants

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*Contents*

## Abstract

Seasonal and diurnal temperature changes are some of the most influential environmental factors that affect plant productivity and distribution. Plants from temperate geographical zones display a certain level of constitutive freezing tolerance, which can be significantly elevated after exposure to low, but non-freezing temperatures. This adaptive response, termed cold acclimation, leads to large transcriptome re-programming. The expression of multiple cold-regulated genes is altered, which results in the accumulation of cryoprotective proteins and metabolites that allow plants to adapt to freezing stress. Understanding the molecular mechanisms that plants have evolved to tolerate cold stress may provide new strategies for breeding plants with improved environmental stress tolerance.

This study focused on the characterisation of factors involved in cold stress responses of plants in *Arabidopsis thaliana* (arabidopsis) and shows that the plant hormones brassinosteroids (BR) regulate freezing tolerance. BR-deficient mutants were hypersensitive to freezing before and after cold acclimation, which was correlated with repressed transcription of the *CBF* transcription factors and the CBF regulon of cold-responsive genes. In agreement, enhanced BR signalling increased *CBF* expression and promoted freezing tolerance. It is demonstrated that BR application elevates steady-state transcript levels of *CBFs* and CBF target genes, whereas a reduction in BR levels has opposite effects. Further it is shown that the BR-regulated basic helix-loop-helix transcription factor CES and its homologues BEEs promote expression of *CBFs* and freezing tolerance and the molecular mechanism enabling this activity is revealed.

In a second project this work characterised a bi-functional locus *LOS2/ENO2* that encodes an enolase and a transcription factor AtMBP1 which is involved in cold stress responses of plants. It is shown that *los2/eno2* knock-out lines exhibit severe developmental defects including reduced growth and defective vascular development. These phenotypes correlate with strongly compromised over-all enolase activity, reduced lignin and sinapoyl malate contents and altered fatty acid and soluble sugar composition. It is further demonstrated that developmental defects of the *los2/eno2* lines were partially restored by expression of LOS2/ENO2 protein that lacks the transcription factor activity, providing evidence that the growth defects were caused by ENO2 deficiency. In addition, we reveal that *LOS2/ENO2* promoter activity is feed-back regulated by AtMBP1.

In summary, this work provides evidence for a function of brassinosteroids in CBF-controlled cold responses of plants and contributes to the characterisation of LOS2/ENO2, a bi-functional locus that is thought to act in CBF-independent cold-responses in arabidopsis.

## Zusammenfassung

Temperaturschwankungen, sowohl tageszeitpunkts als auch saisonal bedingt, zählen zu den einflussreichsten Umweltfaktoren, welche sich auf Produktivität und Verbreitung von Pflanzenarten auswirken. Arten, die in gemäßigten Breiten heimisch sind, zeigen ein gewisses Maß an konstitutiver Frosttoleranz, die nach Einwirkung niedriger Temperaturen, signifikant erhöht werden kann. Diese Anpassungsreaktion wird Kälteakklimatisierung genannt und bewirkt beträchtliche Veränderungen des Transkriptoms. Die Expression von kälteregulierten Genen ändert sich, was zu einer Akkumulierung von Proteinen und Metaboliten führt die eine Kältestressanpassung erlauben.

Diese Arbeit beschäftigte sich mit der Charakterisierung von Faktoren, welche die Kältestressantwort von *Arabidopsis thaliana* (Ackerschmalwand) regulieren und zeigt, dass die Hormonklasse der Brassinosteroide (BR) darin eine Rolle spielt. BR-defiziente Mutanten reagierten sowohl mit als auch ohne Kälteakklimatisierung hypersensibel auf Frost, was mit einer unterdrückten Transkription der *CBF* Transkriptionsfaktoren und des CBF Regulons kälteregulierter Gene korrelierte. Im Gegensatz dazu führte eine verstärkte BR Signaltransduktion zu einer erhöhten Expression von *CBFs* und erhöhte die Frosttoleranz. Es konnte gezeigt werden, dass der BR-regulierte basic helix-loop-helix Transkriptionsfaktor CES und dessen Homologe BEEs sowohl die Expression der *CBFs* als auch die Frosttoleranz fördern. Zugrundeliegenden molekularen Mechanismen, welche diese Vorgänge ermöglichen, wurden analysiert.

In einem zweiten Projekt wurde der bifunktionelle Lokus *LOS2/ENO2* charakterisiert, der neben der Enolase ENO2 auch den Transkriptionsfaktor AtMBP1 kodiert welcher in die Kältestresssignaltransduktion von Pflanzen involviert ist. *los2/eno2* knock-out Linien zeigten schwerwiegende Entwicklungsdefekte, welche mit stark beeinträchtigter Enolaseaktivität, reduziertem Gehalt an Lignin und einer veränderten Zusammensetzung von Fettsäuren und löslichen Zuckern einhergingen. Die Entwicklungsdefekte der *los2/eno2* Mutanten konnten durch Expression eines LOS2/ENO2 Proteins, mit reduzierter Transkriptionsfaktoraktivität teilweise wiederhergestellt werden, was Evidenz dafür liefert, dass sie durch ENO2 Defekte bedingt sind. Weiterhin wurde gezeigt, dass *LOS2/ENO2* Promotor Aktivität in einen Rückkopplungsmechanismus durch AtMBP1 reguliert wird.

Zusammenfassend klärt diese Arbeit die Funktion der Pflanzenhormone BRs in CBF-abhängiger Kältestresstoleranz auf und charakterisiert den bifunktionalen Lokus LOS2/ENO2, welcher CBF-unabhängig in der Kältesignaltransduktion agiert.

## Abbreviations

| | |
|---|---|
| 2-PGA | 2-phosphoglycerate |
| ABA | Abscisic acid |
| ACN | Acetonitrile |
| bHLH | Basic helix-loop-helix |
| BR | Brassinosteoids |
| ChIP | Chromatin immunoprecipitation |
| CK | Cytokinin |
| COR | Cold-regulated |
| CRT/DRE | C-repeat/Dehydration-responsive element |
| CTAB | Cetyl trimethylammonium bromide |
| DTT | Dithiothreitol |
| EBSs | EIN3 binding sites |
| EDTA | Ethylenediaminetetraacetic acid |
| epiBL | EpiBrassinolide |
| ET | Ethylene |
| HPLC | High-performance liquid chromatography |
| JA | Jasmonic acid |
| LUC | Luciferase |
| MeJA | Methyl jasmonate |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| NASC | Nottingham Arabidopsis Stock Center |
| NES | Nuclear export signal |
| PEG | Polyethylene glycol |
| PEP | Phosphoenolpyruvate |
| qPCR | Quantitative polymerase chain reaction |
| SA | Salicylic acid |
| TCA cycle | Tricarboxylic acid cycle |
| TF | Transcription factor |
| YFP | Yellow fluorescent protein |

# I. Introduction

## 1. Cold stress tolerance of plants

Seasonal and diurnal temperature change is one of the most influential environmental factors affecting plant productivity and distribution. Plants from temperate geographical zones, such as arabidopsis, usually display a certain level of constitutive (intrinsic, basal) freezing tolerance, which can be significantly elevated after expose to low, but non-freezing temperatures. This process, termed cold acclimation, leads to transcriptome reprogramming (Fowler and Thomashow, 2002) and consequently to biochemical and physiological changes which allow plants to adapt to freezing temperature stress (Cook et al., 2004).

### 1.1 Cold-regulated gene expression

Whole transcriptome analyses of different plant species showed that upon transfer of plants to cold dynamic changes in gene expression occur (An et al., 2012; Chaudhary et al., 2015; Chen et al., 2014; Barah et al., 2013; Fowler and Thomashow, 2002; Jung et al., 2014; Leyva-Pérez et al., 2015; Li et al., 2015; Moliterni et al., 2015; Pang et al., 2013; Park et al., 2015; Qu et al., 2015; Ren et al., 2014; Sobkowiak et al., 2014; Song et al., 2013; Sun et al., 2015; Tian et al., 2013; Wang et al., 2013a; Wang et al., 2013b; Wang et al., 2014; Wu et al., 2014; Xin et al., 2013; Xu et al., 2014; Zhang et al., 2012; Zhao et al., 2012; Zhu et al., 2013). Induction of cold-regulated (*COR*) gene expression leads to remodelling of metabolic pathways which results in the accumulation of cryoprotective proteins and hundreds of cryoprotective metabolites (Cook et al., 2004; Guy et al., 2008; Shinozaki and Yamaguchi-Shinozaki, 1996).

The most extensively studied cold-responsive pathway which impacts on the basal level of freezing tolerance (Hannah et al., 2006) and leads to cold acclimation is the CBF/DREB1 (C-REPEAT BINDING FACTOR/DROUGHT RESPONSE ELEMENT BINDING FACTOR1) route (Gilmour et al., 2000; Medina et al., 1999; Park et al., 2015). The CBF1, CBF2 and CBF3 transcription factors (TF) belong to a small family of AP2/ERF (Apetala2/Ethylene-responsive element binding protein) DNA-binding proteins and in response to low temperature activate transcription of more than 100 genes (CBF-regulon), such as *COR15A (COLD-REGULATED15A), COR15B (COLD-REGULATED15B), COR47 (COLD-REGULATED47)* and *KIN1* (Fowler and Thomashow, 2002).

Microarray analysis has shown that the CBF pathway regulates approx. 10% of the cold-responsive transcriptome (Fowler and Thomashow, 2002). Hence, non-CBF transcriptional

activators may regulate the remaining classes of *COR* genes. These additional regulatory pathways have been studied in much less detail, however certain players have been identified, such as ESK1 (ESKIMO1; Xin et al., 2007), SFR (SENSITIVITY TO FREEZING; McKown et al., 1996) and LOS2 (LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 2; Lee et al., 2002).

### 1.1.1 The CBF regulatory pathway

The CBF cold-responsive transcription factors and CBF-regulon of *COR* genes have a major role in freezing tolerance of plants. *CBF* transcription is induced rapidly and transiently by cold. CBF proteins bind to the CRT/DRE (C-repeat/Dehydration-responsive element) regulatory element in the promoters of target genes to stimulate their transcription (Thomashow, 2010). The products of CBF-regulated genes are cryoprotective proteins, enzymes for synthesis of cryoprotective metabolites as well as transcription factors (Fowler and Thomashow, 2002).

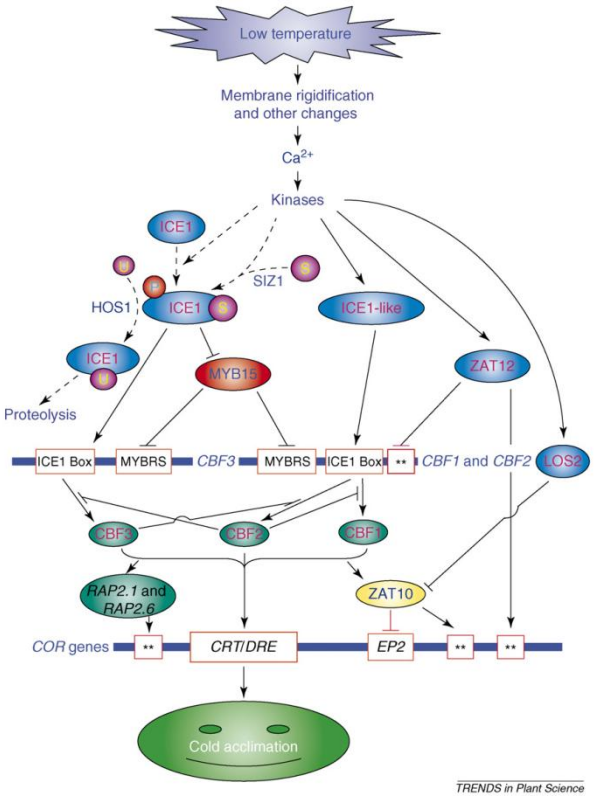


**Figure 1.** Diagram of cold-responsive transcriptional network in arabidopsis (Chinnusamy et al., 2007, *Trends Plant Sci.*)

A number of components of the pathway upstream of CBFs have been identified. One of the most extensively studied is ICE1 (INDUCER OF CBF EXPRESSION), a MYC-like basic helix-loop-helix (bHLH) transcriptional activator that binds to E-box motifs in the *CBF3*

promoter (Figure 1; Chinnusamy et al., 2003). Cold-induced activity of ICE1 is controlled by posttranslational modification events, such as phosphorylation and SUMOylation (Miura et al., 2007; Miura et al., 2011). SUMOylation of ICE1, induced by cold and facilitated by a SUMO E3 ligase SIZ1, leads to activation or stabilisation of ICE1 protein and promotion of *CBF3* expression (Miura et al., 2007). Moreover, ICE1 protein abundance is controlled by phosphorylation and ubiquitination catalysed by the E3 ubiquitin ligase HOS1 (Miura et al., 2011).

In addition, CBF activity is repressed by ZAT12 (Vogel at al., 2005) and MYB15 (Agarwal et al., 2006), and activated by CAMTA (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR; Doherty et al., 2009) and ICE1 (INDUCER OF CBF EXPRESSION1; Fursova et al., 2009). Furthermore, PIF4 and PIF7 (PHYTOCHROME-INTERACTING FACTORS4 and 7) can bind to the E-box motifs in the *CBF* promoters to suppress the cold signalling and freezing tolerance of plants under long day conditions, which indicates that CBF pathway is also regulated by photoperiod (Lee and Thomashow, 2012).

Activity of CBF transcription factors was shown to regulate contents of bioactive gibberellin (GA) and stability of DELLA proteins in cold-treated plants, indicating that CBFs, in addition to their function in cold acclimation, also restrain plant growth and delay the transition to flowering via the DELLA-dependant signalling pathway under low temperature conditions (Achard et al., 2008). In arabidopsis, tobacco and tomato overexpression of *CBFs* led to reduction in levels of bioactive GA, which was correlated with a suppressed growth and late flowering, and could be rescued by exogenous application of GA (Achard et al., 2008; Hsieh et al., 2002; Zhou et al., 2014; Shan et al., 2007).

### 1.1.2 LOS2/MBP1 is a bi-functional locus, involved in cold stress responses

*LOS2/ENO2 (LOS2/ENOLASE2)* was initially identified as a bi-functional locus. *LOS2/ENO2* encodes an enolase in the glycolytic pathway and was shown to be involved in the regulation of *COR* gene expression and in the control of acquired freezing tolerance of plants (Lee et al., 2002). The LOS2/ENO2 protein could bind to the promoter of *ZAT10/STZ*, a zinc finger transcriptional repressor from arabidopsis. *ZAT10/STZ* expression is induced rapidly and transiently by cold and leads to the suppression of *COR* gene expression. The *los2-1* single point mutation was shown to influence LOS2 transcription factor activity and also led to reduction of enolase activity in plants (Lee et al., 2002).

It was further revealed that in plants *LOS2/ENO2* encodes two proteins, in addition to the full length protein which has an enolase activity, also a truncated protein AtMBP1 (*Arabidopsis*

*thaliana* cMyc BINDING PROTEIN) is alternatively translated from the second start codon. AtMBP1 is not active as an enolase, but acts as a transcription factor (Kang et al., 2013). *LOS2*/*ENO2* shares homology with human α-enolase *ENO1*, a bi-functional locus of which the alternatively translated MBP1 acts as a repressor of *cMyc* transcription (Feo *et al*., 2000). In addition to its regulatory role in cold stress responses, arabidopsis MBP1 was shown to function as a positive regulator of ABA signalling and its activity was attenuated by ubiquitin-dependent destabilization, mediated by the E3 ubiquitin ligase AtSAP5 (Kang et al., 2013).

While the role of LOS2 as a transcriptional regulator in arabidopsis was investigated in a number of independent studies, the biological relevance of ENO2 as an enzyme involved in the glycolytic cycle in plants remained unknown. Enolases catalyse the reversible dehydration of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP) in the glycolytic cycle. Three enolase isozymes function in arabidopsis, ENO1, ENO2 and ENO3/ENOc, which share 58-68% similarity at the amino acid level. ENO1 was shown to be localised to plastids and was functionally characterised (Prabhakar et al., 2009). ENO2 and ENO3/ENOc localise in nucleus and cytoplasm of plant cells (Lee et al., 2002; Prabhakar et al., 2009), ENO3/ENOc however does not exhibit enolase activity (Andriotis *et al*., 2010). The impact of ENO2 on glycolysis was not studied in arabidopsis.

### 1.2 Plant hormones control cold stress responses

As most physiological traits in plants, freezing tolerance and cold acclimation are subjected to control by phytohormones. Plant hormones are structurally unrelated compounds that act at extremely low concentrations to regulate all aspects of plant development and to modify growth according to perceived environmental signals (Santner et al., 2009).

Different classes of phytohormones were shown to regulate freezing tolerance of plants by impacting on CBF signalling and also on the expression of non-CBF regulated *COR* genes. Cytokinins (CK), abscisic adic (ABA) and jasmonic acid (JA) were shown to promote freezing tolerance of plants (Jeon et al., 2010; Hu et al., 2013; Lee and Seo, 2015). Components of CK signalling pathway, such as the CK receptors histidine kinases AHK2 and AHK3 (ARABIDOPSIS HISTIDINE KINASE2 and 3) and type A ARR (ARABIDOPSIS RESPONSE REGULATORS) TF, were shown to be involved in the regulation of *COR* gene expression and freezing tolerance (Figure 2). AHKs are positive regulators of CK signalling and may function as negative regulators of cold stress adaptation responses, since plants of *ahk* knockout lines showed elevated freezing tolerance (Jeon et al., 2010; Jeon et al., 2013). Microarray analysis of cold treated *ahk2 ahk3* revealed that the non-CBF regulated cold

transcriptome was differentially regulated in this line as compared to wild-type (Jeon et al., 2013). Other components of CK signalling implicated in cold stress response are type A ARR TF, which were suggested to function as negative regulators of cold signalling in a CBF independent manner. Transcript levels of type A ARRs *ARR5*, *ARR7* and *ARR15* were induced in response to cold and *arr*5, 6 and 7 single knock-out lines showed increased tolerance to freezing under normal conditions and after a cold acclimation period (Jeon et al., 2010; Shi et al., 2012). Transcript levels of *CBFs* were unchanged in ARR overexpressing lines (Jeon et al., 2010; Shi et al., 2012).

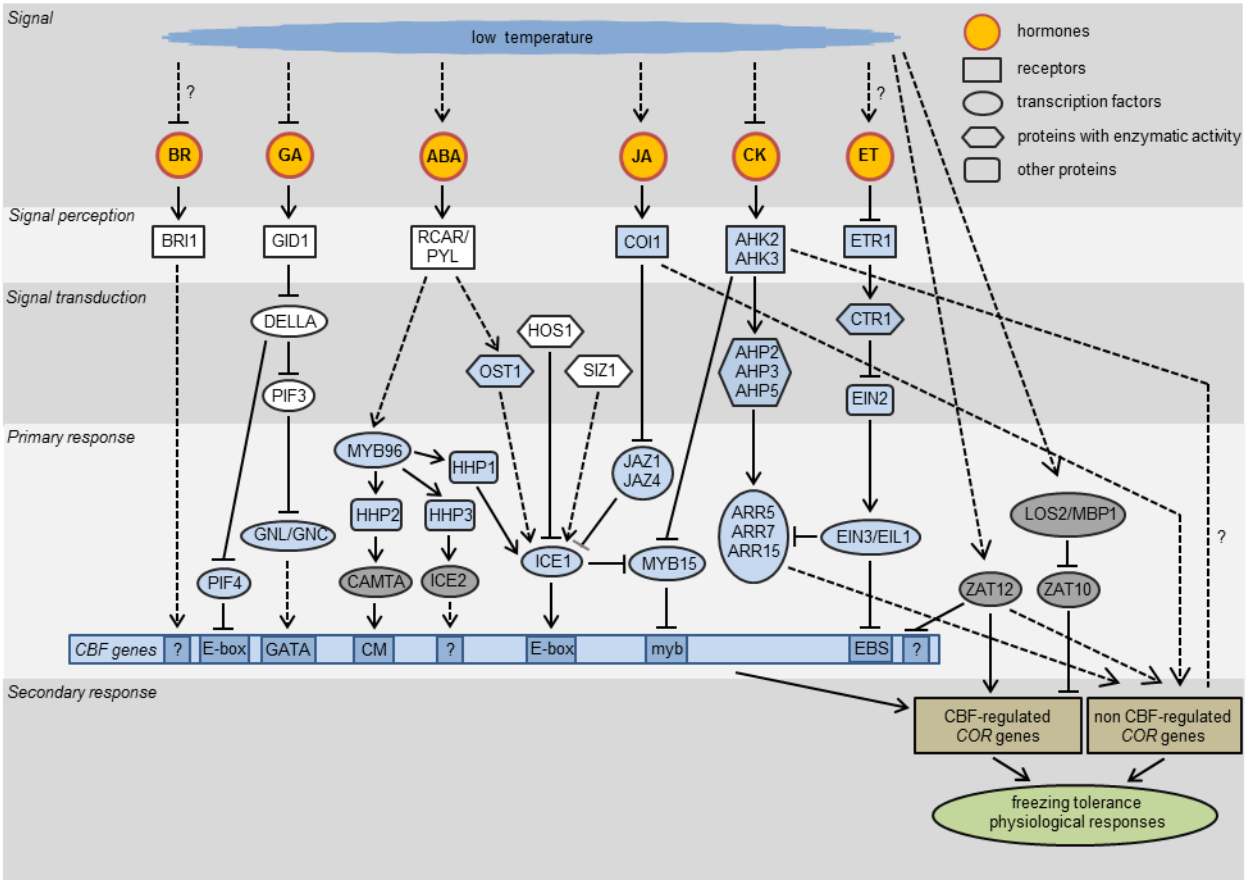


**Figure 2.** Current understanding of the signaling events that confer hormone action in cold adaptation.
In blue colour are TF and components of hormone signaling pathways, which were shown to be regulated by both cold and hormone. In grey colour are TF regulated by cold, but not regulated by hormones. In white colour are components of hormone signaling pathways, which were not shown to be regulated by cold. Arrows and inhibition lines represent activation and suppression processes, respectively. Solid lines mean direct interaction, dotted lines represent indirect interactions.

Jasmonate, a plant hormone mainly known for its role in defence responses, may positively regulate freezing tolerance of plants (Hu et al., 2013). Exogenous application of methyl jasmonate (MeJA) enhanced *CBFs* and CBF regulated gene expression under cold treatment and promoted freezing tolerance in arabidopsis before and after cold acclimation. In

agreement, plants compromised in JA biosynthesis and signalling were hypersensitive to freezing under normal conditions and after cold acclimation (Hu et al., 2013). The repressors of JA signalling, JAZ1 and JAZ4, could physically interact with ICE1 and ICE2 to repress transcriptional activity of these proteins on the *CBF* promoters. Interestingly, microarray analysis of the JA signalling mutant *coi1-1* (*coronatine insensitive1*) following low temperature treatment demonstrated that some of cold-responsive genes of the non-CBF regulon were differently regulated by cold, which indicated that JA may modulate cold acclimation through CBF-independent pathways (Hu et al., 2013). In agreement, endogenous JA production rose rapidly under 4°C treatment (Hu et al., 2013). Therefore, JA may promote basal and acquired freezing tolerance in arabidopsis through modulation of CBF-dependent and CBF-independent cold-responsive pathways.

Cold stress tolerance was considered to be generally ABA-independent in plants, since levels of ABA increase only slightly and transiently in response to cold. However, exogenous application of ABA promotes freezing tolerance and ABA mutants are hypersensitive to freezing (Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999). Recently accumulating evidence suggests that ABA may play a more significant role in cold responses of plants than previously thought. The ABA-regulated TF MYB96 was found to be cold-induced and to control *CBF* induction in cold-adaptation, since it was compromised in a *myb96* knock-out line and was enhanced in a MYB96 over-expressor (Lee and Seo, 2015). Moreover, it was shown that MYB96 regulates expression of the HEPTAHELICAL PROTEIN (HHP) proteins HHP1, HHP2 and HHP3, which interact with ICE1, CAMTA and ICE2 respectively (Chen et al., 2010; Lee and Seo, 2015). Therefore, there is evidence that the MYB96-HHP module controls expression of *CBFs* (Lee and Seo, 2015).

OPEN STOMATA 1 (OST1), a Ser/Thr protein kinase activated by ABA, also regulates *CBF* expression (Ding et al., 2015). OST1 was induced by cold, *ost1* knock-out lines were hypersensitive to freezing and OST1 over-expressing plants were freezing tolerant. OST1 was shown to phosphorylate ICE1, to stabilize the protein and promote its transcriptional activity under cold stress (Ding et al., 2015). Therefore, ABA may impact on *COR* gene expression by controlling *CBF* expression.

Controversial data have been presented regarding a possible role of ethylene (ET) in cold responses of plants. First ET was characterised as a negative regulator of freezing tolerance in arabidopsis (She et al., 2012). Arabidopsis lines compromised in ET signalling showed elevated freezing tolerance. Overexpression of EIN3/EIL1 (ETHYLENE

INSENSITIVE3/EIN3-LIKE1) induced hypersensitivity to freezing in arabidopsis which correlated with supressed expression of *CBF* regulators, whereas in *ein3 eil1* mutant *CBFs* and CBF responsive genes were up-regulated. Furthermore, EIN3 was proposed to function as a repressor of the type A TF *ARR5, ARR7* and *ARR15* and to affect freezing tolerance in an unknown but non-CBF dependant manner (She et al., 2012). ChIP experiments demonstrated that EIN3 directly binds to EIN3 binding sites (EBSs) in the promoters of *CBFs* and type A ARRs to regulate their expression. In support, authors provided evidence that cold stress inhibited ethylene production in plate-grown arabidopsis seedlings (She et al., 2012). However, recently contradictory results on the role of ET in freezing tolerance of arabidopsis were presented and demonstrated that arabidopsis ET deficient *octuple* line was hypersensitive to freezing (Catala et al., 2014). Cold induction of *CBF1*, *CBF2* and target genes in *octuple* plants was significantly lower than in wild-type. Therefore, evidence was provided that ET positively regulates basal and acquired FT in arabidopsis. In support to these findings, authors reported that ET levels increased transiently after cold treatment in soil-grown arabidopsis plants, which was correlated with a transient increase in transcripts of different ET biosynthetic ACS (ACC SYNTHASE) enzymes (Catala et al., 2014).

A number of studies indicated that GA signalling components can affect responses to low temperatures in plants, since GA-insensitive or GA-deficient lines exhibit altered chilling and freezing tolerance. GA-deficient plants showed enhanced freezing tolerance (Achard et al., 2008; Richter et al., 2013; Tanaka et al., 2006), which correlated with elevated levels of *CBF1*, *CBF2* and *COR15A* (Richter at al., 2013). Overexpression of GA-regulated GATA TF GNC and GNL enhanced *CBF2*, *COR15A* and *COR15B* expression and increased survival rates of transgenic seedlings before and after cold acclimation (Richter at al., 2013). Since it has been shown that *GNL* and *GNC* expression is promoted in mutants with a block in GA signalling (Richter at al., 2013), GNC and GNL may act to promote freezing tolerance in the absence of GAs. In such a way, the decrease in levels of bioactive GAs under low temperature activates components of GA signalling pathway not only to contribute to the suppression of growth, but also to modulate levels of *CBF* transcripts to enhance freezing tolerance.

## 2. Brassinosteroids regulate development and stress responses of plants

### 2.1 Brassinosteroid signalling

Brassinosteroids are a class of steroid hormones which regulates many aspects of vegetative and reproductive plant development, such as cell division and elongation, photomorphogenesis, flowering time and response to environmental stresses (Clouse, 2011).

BRs are perceived by the plasma membrane localised receptor-like kinase BRI1 (BRASSINOSTEROID INSENSITIVE1) and through a cascade of phosphorylation-dephosphorylation events the signal is transduced to BR-regulated transcription factors. One of the main effects of BR signal transduction is the inactivation of the BRASSINOSTEROID INSENSITIVE2 (BIN2) kinase and its homologues ASKs (SHAGGY-RELATED PROTEIN KINASES). Inactivation of BIN2 and ASKs leads to activation of two closely related transcription factors which are members of one of the major classes of BR-regulated TFs, BZR1 (BRASSINAZOLE-RESISTANT1) and BES1 (BRI1-EMS SUPPRESSOR1). BZR1 and BES1 activate expression of a set of target genes, affecting numerous cellular processes including expression of BR biosynthetic enzymes (Wang et al., 2002; Yin et al., 2002).

Another important class of BR-regulated TFs is a group of homologous bHLH proteins BEE1-3 (BRASSINOSTEROID ENHANCED EXPRESSION1-3; Friedrichsen et al., 2002) and CES (CESTA; Poppenberger et al., 2011). CES was isolated in a screen for BR over-accumulation phenotypes, is phosphorylated by BIN2 and functions as a positive regulator of BR biosynthesis (Poppenberger et al., 2011). Recently it was demonstrated that CES transcriptional activity and subnuclear localisation are regulated by BRs through the posttranslational modifications (Khan et al., 2014). Inhibition of ASK activity was shown to induce CES compartmentalization in nuclear bodies (Poppenberger et al., 2011), which was further revealed to be controlled by SUMOylatoin, since interfering with SUMOylation inhibited nuclear body association, whereas promoting of SUMOylation induced constitutive nuclear compartmentalization of CES (Khan et al., 2014). Moreover, evidence was provided that SUMOylation of CES is antagonized by phosphorylation. Furthermore, it has been shown that phosphorylation regulates CES transcriptional activity and protein turnover by the proteasome. A model was proposed in which, in a BR-deficient situation, CES is phosphorylated for activation of target gene transcription and to enable further posttranslational modification that control CES protein stability (Khan et al., 2014).

### 2.2 Brassinosteroids are involved in cold stress responses of plants

Brassinosteroids have been shown to confer tolerance to various biotic and abiotic stresses in different species (Kagale et al., 2006), including cold stress (Kagale et al., 2006; Kim et al., 2009; Xia et al., 2009). A protective role of BRs was shown for the chilling sensitive species maize and cucumber (Xia et al., 2009; Singh et al., 2012; Jiang et al., 2013). Maize seedlings treated with the active BR epiBL (epiBrassinolide) were more tolerant to chilling stress as compared to the control, which was demonstrated by a significant increase in plant height, dry

matter accumulation, chlorophyll content, total soluble proteins and starch contents (Singh et al., 2012). BR levels positively correlated with tolerance to chilling stress in cucumber (Xia et al., 2009; Jiang et al., 2013). Photochemical efficiency of PSII was promoted by application of epiBL in cucumber and decreased by application of BR biosynthetic inhibitor brassinazole (BRZ; Asami et al., 2000). Also, epiBL significantly increased $CO_2$ assimilation, activity of Calvin cycle enzymes and antioxidant capacity in cucumber treated with cold (Xia et al., 2009; Jiang et al., 2013).

Enhanced cold stress resistance and increased *COR15A* transcript levels were observed in plants over-expressing the BR biosynthesis gene *DWF4* (Divi and Khrishna, 2010). Moreover, *BRI1* over-expression elevated basal *CBF1-3* expression levels (Kim et al., 2010). In line with this data, application of epiBL enhanced expression of *CBF1*, *COR47* and *COR78* in arabidopsis under low temperature treatment, which indicates that BR can have a positive effect on the *CBF* gene expression and cold tolerance (Kagale at al., 2007). Ion leakage in arabidopsis BR insensitive line *bri1-116* subjected to chilling stress was higher than in wild-type, indicating that BR signalling could promote cold acclimation (Qu et al., 2011).

BR signalling was shown to be required to stimulate induction of pectin methylesterase (PME) activity under chilling stress, an enzyme induced by cold with a proposed role in cell wall modification under low temperature (Qu et al., 2011). In contradiction to the previous studies BR insensitive line *bri1-9* was shown to be more tolerant to chilling stress (Kim et al., 2010), however, under low temperature treatment there was no difference observed in the induction of *CBF* and CBF-regulated genes in *bri1-9* as compared to wild-type (Kim et al., 2010).

## 3. Aims of this study

Freezing tolerance and cold acclimation of plants are controlled by phytohormones and there was evidence that the BRs take part. Therefore, the aim of my work was to gain knowledge about the molecular modes, which confer this ability.

In a first project the role of BRs in the regulation of *COR* gene expression was addressed. For this purpose it was investigated how altering BR activity, either by chemical means or through the use of mutants, impact on *COR* gene expression and freezing tolerance of arabidopsis plants. It was found that BRs participate in the control of the CBF-regulon of *COR* genes and that this activity is conferred, at least partly, by a direct regulatory function of CES and the BEEs in the control of *CBF1* and *CBF3* expression.

The second project of my thesis studied LOS2/ENO2, a factor that is thought to control *COR* gene expression in a CBF-independent manner and had been found to interact with CES in yeast (Poppenberger lab, unpublished results). *LOS2/ENO2* is a bi-functional locus that contributes to cold stress resistance. Full-length LOS2/ENO2 functions as an enolase in the glycolytic pathway, while a truncated product MBP1 is a TF. Although MBP1 TF activity had been investigated in a number of different studies, function of the encoded enolase ENO2 had not been addressed. To do so a phenotypic and biochemical characterisation of three independent *los2/eno2* knock-out mutants was carried out and the impact of LOS2/ENO2 deficiency on vegetative and reproductive development of plants was studied. This showed that ENO2 is required for the synthesis of major building blocks including fatty acids, soluble sugars and lignin and is essential for multiple plant developmental processes. Since *los2/eno2* knock-out lines showed a striking phenotypic resemblances with MBP1 over-expressing lines the question if *MBP1* may control ENO2 transcription arose and was addressed. This revealed that the LOS2/ENO2 locus utilizes an elegant feedback regulatory loop to control its own promoter activity, where MBP1 is translated to repress transcription of *ENO2* and thereby regulates glycolysis. The results of the second project where published in *The Plant Journal* earlier this year.

# II. Materials and Methods

## 1. Materials

### 1.1 Plant materials

**Table 1**. Plant lines used in this study.

| Line | Reference |
|---|---|
| Col-0 | wild type |
| C24 | wild type |
| *ces-D* | Poppenberger et al., 2011 |
| *ces-ko 1* | Poppenberger et al., 2011 |
| *ces-ko 2* | unpublished line |
| *bee1 bee3 haf (tM)* | Crawford and Yanofsky, 2011 |
| *bee1 bee2 bee3 ces-2 (qM)* | unpublished line |
| *los2-1* | Lee et al., 2002 |
| *los2-2* | in this study |
| *los2-3* | in this study |
| *los2-4* | in this study |
| *LOS2p:LOS2-NES in los2-4* | in this study |
| *BRI1oe* | Wang et al., 2001 |
| *bri1-301* | Xu et al., 2008 |
| *bri1-1* | Friedrichsen et al., 2000 |
| *bri1-5 bak1-D* | Noguchi et al., 1999 |
| *ces-Dxbri1-5 bak1-D* | unpublished line |
| *cpd* | Szekeres et al., 1996 |
| *bes1-D* | Yin et al., 2002 |
| *bzr1-1D* | Wang et al., 2002 |
| *askϑ-10* | Rozhon et al., 2010 |
| *askϑ-27* | Rozhon et al., 2010 |

### 1.2 Bacterial strains

*Escherichia coli* BL21 (DE3) (Miroux B and Walker JE, 1996)
*Escherichia coli* XL1 Blue (Bullock et al., 1987)
*Agrobacterium tumefaciens* GV3101 (Koncz and Schnell, 1986)

### 1.3 Enzymes, antibodies, markers and commercial kits

**Table 3**. Enzymes, antibodies, markers and commercial kits used in this study.

| Name | Company |
|---|---|
| GoTaq DNA Polymerase | Promega, Mannheim, Germany |
| DNase I, RNase-free | Thermo Fisher Scientific, Braunschweig |
| RevertAid RT Reverse Transcription Kit | Thermo Fisher Scientific, Braunschweig |
| GeneRuler DNA ladder | Thermo Fisher Scientific, Braunschweig |
| T4 DNA ligase | Thermo Fisher Scientific, Braunschweig |
| Restriction enzymes | Thermo Fisher Scientific, Braunschweig |
| Dual-Luciferase® Reporter Assay | Promega, Mannheim, Germany |
| E.Z.N.A.® RNA Isolation Kit | VWR, Radnor, USA |
| E.Z.N.A.® Gel Extraction Kit | VWR, Radnor, USA |
| E.Z.N.A.® Plasmid Isolation Kit | VWR, Radnor, USA |
| Bradford reagent | Sigma, St. Louis, USA |
| SensiFAST SYBR® Lo-ROX Kit | Bioline, London, UK |
| Glucose Oxidase | Sigma, St. Louis, USA |
| Peroxidase | Sigma, St. Louis, USA |
| Proteinase K | Boehringer, Ingelheim, Germany |
| Anti-GFP | Roche, Base, Switzerland |
| T4 polynucleotide kinase | Thermo Scientific, Waltham, USA |

### 1.4 Vectors

pGWR8 (Rozhon et al., 2010)
pGreenII-0800 (Hellens et al., 2005)
pGEX4T2 (Amersham Pharmacia Biotech, Uppsala, Sweden)

### 1.5 Antibiotics

Kanamycin (final concentration 30μg/ml)
Ampicillin (final concentration 100μg/ml)
Gentomycin (final concentration 30μg/ml)

### 1.6 Media, buffers and solutions

Extraction buffer for enolase activity assay:
DTT 5mM, Polyvinylpyrrolidone 10 0.5%, 100mM TRIS, pH=7.4
Reaction buffer for enolase activity assay:
2-phosphoglycerate 2,5mM, $MgCl_2$ 1mM, 50mM TRIS, pH=7.4
FAA solution for section preparation:
Formaldehyde 5%, Acetic acid 5%, Ethanol 50%
CTAB extraction buffer:
2% CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM TRIS/HCl, pH=8.0
Protoplast lysis buffer:
1mM DTT, 10% glycerol, 1% Triton X-100, 2.5mM Tris-phosphate, pH=7.8
Pollen germination medium:
5 mM MES, pH 5.8, 1 mM KCl, 10 mM CaCl2, 0.8 mM MgSO4, 1.5 mM boric acid, 1 % [w/v] agar, 17 % [w/v] sucrose.
WD solution for protoplast transformation:
8 mM $CaCl_2x2H_2O$, 0.4 M Mannitol
PEG solution:
40 % PEG 4000, 0.4 M Mannitol, 0.1 M $CaCl_2x2H_2O$
MMM solution:
15 mM $MgCl_2$, 0.1 % MES, 0.5 M Mannitol
W5 solution:
154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM Glucose
WI solution for protoplast transformation:
0.5M mannitol, 20mM KCl, 4mM MES, pH=5.7
Extraction buffer 1 for ChIP:
0.4M Sucrose, 10mM Tris, pH8.0, 10mM $MgCl_2$, 5mM β-mercaptoethanol, Protease inhibitors
Extraction buffer 2 for ChIP:
0.25M Sucrose, 10mM Tris-HCl, pH8.0, 10mM $MgCl_2$, 1% Triton X-100, 5mM β-mercaptoethanol, Protease inhibitors
Extraction buffer 3 for ChIP:
1.7M Sucrose, 10mM Tris-HCl, pH8.0, 2mM $MgCl_2$, 0.15% Triton X-100, 5mM β-mercaptoethanol, Protease inhibitors
Nuclei Lysis Buffer for ChIP:

50mM Tris-HCl, pH8.0, 10mM EDTA, 1% SDS, Protease inhibitors

ChIP Dilution Buffer:

1.1% Triton X-100, 1.2mM EDTA, 1% SDS, Protease inhibitors

Elution buffer for ChIP:

1% SDS, 0.1M $NaHCO_3$

Low Salt Wash Buffer for ChIP:

150mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH8.0

High Salt Wash Buffer for ChIP:

500mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH8.0

LiCl Wash Buffer for ChIP:

0.25M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH8.0

TE buffer for ChIP:

1mM EDTA, 10mM Tris-HCl, pH8.0

Extraction buffer for measurement of total Salicylic acid content, 10x

500 mM triethanolamine, 2 mM EDTA, 4 mM CaCl2 set with HCl to pH 7.5

Elution buffer for measurement of Salicylic acid content

2 mM iron(III) chloride; 5 mM HCl

Transformation buffer for chemically competent cells preparation:

10mM PIPES pH=6,7, 15mM $CaCl_2$, 250mM KCl, 55mM $MnCl_2$

### 1.7 Primers used in this study

| Name | | Sequence | |
|---|---|---|---|
| SALK LBa1 | 5' | TGGTTCACGTAGTGGGCCATCG | 3' |
| SAIL LB1 | 5' | GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC | 3' |
| LOS2-fw2 | 5' | TGGCTACTATCACCGTTGTTA | 3' |
| LOS2-rv2 | 5' | GTTCACATTGCCAACAGCCT | 3' |
| LOS2-fw3 | 5' | CATTCCTCTATACAAGCACATT | 3' |
| LOS2-rv3 | 5' | AGCGGCAACATCCATTCCAA | 3' |
| LOS2-fw4 | 5' | CTAAGGCAATCGCAGAGAAG | 3' |
| LOS2-rv4 | 5' | ACCAGGTTTAACATCTCAGACC | 3' |
| ubc-fw1 | 5' | TCAAATGGACCGCTCTTATC | 3' |
| ubc-rv1 | 5' | CACAGACTGAAGCGTCCAAG | 3' |
| los2qp-fw1 | 5' | GGTGCTCCTTGCAGATCCGA | 3' |
| los2qp-rv1 | 5' | CCACAGGTTTGCGGAAGTTG | 3' |
| los2qp-fw2 | 5' | CTAAGGCAATCGCAGAGAAG | 3' |
| los2qp-rv2 | 5' | CGGTTTTGATTTGTCCAGTG | 3' |
| LOS2p-FW2 | 5' | AAGAAGCATCAGGAGAGCGGTTC | 3' |
| LOS2p-RV | 5' | TGATAGTAGCCATGGCGAGTAGAT | 3' |
| LOS2-CDS-fwd | 5' | ATCACCATGGCTACTATCACCGTTGTTAAG | 3' |
| LOS2-CDS-rev | 5' | TCTAGCGGCCGCAGTAGGGTTCCACAGGTTTGCGG | 3' |
| NES-fw | 5' | CTAGCTCTGAAGTTGGCTGGACTCGACATC | 3' |
| NES-rv | 5' | GATGTCGAGTCCAGCCAACTTCAGAGCTAG | 3' |
| COR15A-pPCR-FW | 5' | ACCTCAACGAGGCCACAAAGAAAG | 3' |
| COR15A-pPCR-RV | 5' | CGCTTTCTCACCATCTGCTAATGC | 3' |

| | | | |
|---|---|---|---|
| COR15B-pPCR-FW | 5' | CCTCAACGAAGCCACAAAGAA | 3' |
| COR15B-pPCR-RV | 5' | TTCCTCAGTCGCAGTTTCATT | 3' |
| KIN-pPCR-FW | 5' | AAGAATGCCTTCCAAGCCGGTCAG | 3' |
| KIN-pPCR-RV | 5' | TACACTCTTTCCCGCCTGTTGTGC | 3' |
| COR47-pPCR-FW | 5' | TCCCAGGACACCACGACAA | 3' |
| COR47-pPCR-RV | 5' | AATCCTCTGCTTTCTCGTCGT | 3' |
| COR78-pPCR-FW | 5' | GACGAAGTTACCTATCTCCG | 3' |
| COR78-pPCR-RV | 5' | CTTCCTTTGTCGTCGTTTCC | 3' |
| CBF-pPCR-FW | 5' | TTCGCTGACTCGGCTTGG | 3' |
| CBF1-pPCR-RV | 5' | CATGATTCGTGGTCGTCGT | 3' |
| CBF2-pPCR-RV | 5' | TCAAACATCGCCTCATCGTG | 3' |
| CBF3-pPCR-RV | 5' | GACACATCTCATCTTGAAAATT | 3' |
| CBF1-ChIP-FW | 5' | ACACACGTCAGACAGCGAGT | 3' |
| CBF1-ChIP-RV | 5' | CGGAGGAGAGATAACGGATATG | 3' |
| CBF2-ChIP-FW | 5' | CAGTGATTGACAGCCTTGATA | 3' |
| CBF2-ChIP-RV | 5' | ATTTGGATATTTGTGGGGTCG | 3' |
| CBF3-ChIP-FW | 5' | CCATTGTCCATACCTTCTCT | 3' |
| CBF3-ChIP-RV | 5' | AAAGAGTGGGTATGGTCAAGA | 3' |
| CBF1-luc-FW | 5' | GATTGCTTCTCGAGGTACTATA | 3' |
| CBF1-luc-RV | 5' | TTGGCGTCTTCCATGGATCAGAGTACTCTG | 3' |
| CBF1-luc-FW1 | 5' | TTGCTTCTCGAGGTACTATAAAAAAGTCATTCACA | 3' |
| CES KO fwd | 5' | GGCCATTGATTTCACTCTTCA | 3' |
| CES KO rev | 5' | ACTGACGTCACACACAAAAGA | 3' |
| BEE1 KO fwd | 5' | AACAATTACTTCACTTTCCCGG | 3' |
| BEE1 KO rev | 5' | TTGATTCTGTAGAGACTGGACATAAT | 3' |
| BEE2 KO fwd | 5' | GAAGATCAAAGCAGAGGATGAA | 3' |
| BEE2 KO rev | 5' | TTCACCTGTTTTGCGGATAA | 3' |
| BEE3 KO fwd | 5' | ACCTCTTCTGCTCAAGTTTCCA | 3' |
| BEE3 KO rev | 5' | CGAACTTGCTGCTGTAAGCTT | 3' |
| CBF1 fwd comp | 5' | TCGCTATGTACTATACACGTGTCATTCACAGAGACA | 3' |
| CBF1 rev comp | 5' | TGTCTCTGTGAATGACACGTGTATAGTACATAGCGA | 3' |
| CBF1mt fwd comp | 5' | TCGCTATGTACTATAAAAAAATCATTCACAGAGACA | 3' |
| CBF1mt rev comp | 5' | TGTCTCTGTGAATGATTTTTTTATAGTACATAGCGA | 3' |
| CBF2 fwd comp | 5' | TAGCTGTTTCTTATCCACGTGGCATTCACAGAGACA | 3' |
| CBF2 rev comp | 5' | TGTCTCTGTGAATGCCACGTGGATAAGAAACAGCTA | 3' |
| CBF2mt fwd comp | 5' | TAGCTGTTTCTTATCAAAAAAGCATTCACAGAGACA | 3' |
| CBF2mt rev comp | 5' | TGTCTCTGTGAATGCTTTTTTGATAAGAAACAGCTA | 3' |
| CBF3 fwd comp | 5' | TGTGGAGTCTCGTACCACGTGTCGCGTCACTTCACT | 3' |
| CBF3 rev comp | 5' | AGTGAAGTGACGCGACACGTGGTACGAGACTCCACA | 3' |
| CBF3mt fwd comp | 5' | TGTGGAGTCTCGTACAAAAAATCGCGTCACTTCACT | 3' |
| CBF3mt rev comp | 5' | AGTGAAGTGACGCGATTTTTTGTACGAGACTCCACA | 3' |
| CPD-1 | 5' | CTTGCTCAACTCAAGGAAGAG | 3' |
| CPD-2 | 5' | CTCGTAGCGTCTCATTAACCAC | 3' |
| DWF4-3 | 5' | CCGTTGAAGAGCTTAGGGAAGAG | 3' |
| DWF4-4 | 5' | CATTTCCCAATCGAAGAGTTTC | 3' |
| BR6ox2-1 | 5' | AGCTTGTTGTGGGAACTCTATCGG | 3' |
| BR6ox2-2 | 5' | CGATGTTGTTTCTTGCTTGGACTC | 3' |

# 2. Methods

## 2.1 Plant work

### 2.1.1 Seed sterilisation and plant growth conditions

Seeds were sterilised using the chlorine vapour method (Clough and Bent, 1998), plated on half-strength MS medium and stratified at 4°C for 2 days. Plants were grown on ½ MS plates or in soil in Bright Boy growth chambers (CLF Plant Climatics GmbH, Wertingen, Germany), in long day conditions (16 h light 80 µmol m-2 s-1/ 8 h dark) at 21°C.

### 2.1.2 Plant transformation (Floral dip)

A single colony of the *Agrobacterium* strain was inoculated in 50ml MLB medium containing the appropriate antibiotics and incubated at 28°C and 180prm rotation ON. The overnight culture was diluted in 300ml LB containing the appropriate antibiotics to an $OD_{600}$ of 0.3. The culture was grown at 28°C and 180rpm rotation until the $OD_{600}$ reached 0.6-0.8, centrifuged at 5000rpm for 15min. The bacterial pellet was resuspended in 300ml 5% sucrose, transferred to beakers and 150µl Sylvet L-77 was added (final concentration: 0.05%). Arabidopsis plants were dipped in the suspension for about 30s. On the next day plants were switch to light of half intensity for two days and then plants were grown as usual.

### 2.1.3 Freezing tolerance assay

Plant freezing assays were performed as described previously (Miura et al., 2011) with modifications. For treatments without cold acclimation 3-week-old plants were incubated in a controlled-temperature chamber (Panasonic MIR-154, Panasonic Biomedical, Osaka, Japan) for 30 min at 4°C, then for 1 hour at 0°C, before the temperature was decreased at a rate of 2°C per hour. The final desired sub-zero temperature was maintained for the indicated period of time before the temperature was again increased at the same rate to 4°C. The plants were then kept at 4°C for 1 day before returning them to 21°C.

For the cold acclimation experiments 3-week-old plants were acclimated for 3 days at 4°C in the light. The freezing treatment was then performed in the same manner as for non-acclimated plants with the final freezing temperature of -10°C maintained for 6 h.

### 2.1.4 Electrolyte leakage assay

Electrolyte leakage from fully expanded rosette leaves of 3-week-old plants was assessed as described previously (Gilmour et al., 1988) with modifications. The leaves of $5^{th}$ and $6^{th}$ leave

pairs were placed in tubes containing 100 µl deionised water. An ice chip was added to facilitate nucleation and the tubes were kept at -2°C for 2 h followed by a temperature decrease at a rate of 2°C per hour. Samples were taken out at the indicated temperature points and immediately placed on ice for gradual thawing over-night. On the next day 6 ml of deionised water were added to each tube and the samples were incubated for 5 h at 21°C with gentle shaking, after which the conductivity of the solution was determined with a conductivity meter (GMH 3430, Greisinger electronic, Germany). The tubes were then incubated at 95°C for 3 h, and the conductivity of the solution was measured again. The electrolyte leakage was quantified as a percentage of the conductivity after treatment to the total conductivity.

### 2.1.5 *In vitro* pollen germination

The germination efficiency of pollen grains was assessed in vitro according to Fan et al. (2001). Pollen of fresh, anther-dehisced flowers was transferred to germination medium and was incubated for 20h in a climate-controlled chamber at 21°C, 100% humidity and in low light (30 µmol m-2 s-1). For each line one hundred pollen grains were assessed on four plates and the germination efficiency was calculated. Photos of pollen grains were taken with an Olympus binocular microscope (Olympus, Tokyo, Japan).

## 2.2 Molecular biology methods

### 2.2.1 Extraction of genomic DNA

Approximately 100 mg plant material was ground to a fine powder, 1ml of CTAB extraction buffer was added, after which samples were heated to 65°C for 15 min and centrifuged (5 min, 13000rpm). The clear supernatant was transferred into a fresh tube and mixed with 500µl of chloroform. Samples were centrifuged (5 min, 13000 rpm), the clear supernatant was transferred into a fresh tube and the washing step with chloroform was repeated. After the centrifugation (5 min, 13000 rpm), the supernatant was transferred into a fresh tube and an equal volume 2-propanol was added, after which samples were centrifuged (5 min, 13000 rpm). The pellet was washed with 500µl of 70% ethanol, air dried for several minutes and dissolved in 200 ml distilled water.

### 2.2.2 PCR and agarose gel electrophoresis

The polymerase chain reaction (PCR) was performed with 2µl template, 5µl PCR GoTaq buffer, 200µM dNTPs, 1µl forward and reverse primer (5µM stock), 0.1 µl 5U/µl GoTaq polymerase (Promega, Madison, USA). The PCR conditions used as follows: 94°C final

denaturation step for 2min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C to 60°C for 30 sec and extension at 72°C for 1min/kb, final extension step at 72°C for 10 min. The PCR products were visualised on 1% agarose gel.

### 2.2.3 RNA extraction and cDNA synthesis

Total mRNA was extracted with a Plant RNA Kit (Omega Bio-Tek, Norcross, USA) and treated with DNaseI to digest traces of DNA. First strand cDNA was synthesized from 1 µg RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instruction.

### 2.2.4 Quantitative PCR (qPCR)

For qPCR analysis plants were grown on agar plates vertically or in soil depending on the experiment. qPCRs were performed with the SensiFAST SYBR Lo-ROX Kit (Bioline, London, UK) using the Mastercycler Realplex (Eppendorf, Hamburg, Germany).

### 2.2.5 Cloning

DNA fragments were PCR-amplified, digested with corresponding restriction enzymes and ligated with T4 DNA ligase (Thermo Fisher Scientific, Braunschweig, Germany) into vector backbone. All constructs were confirmed by DNA sequencing.

For generation of the *los2-4* complementation lines a 1.7 kb fragment of the *LOS2/ENO2* promoter and the *LOS2/ENO2* coding sequence were amplified by PCR and cloned into the binary plant expression vector pGWR8 (Rozhon et al., 2010). For generation of the nuclear export signal the NES-fw and NES-rv oligonucleotides were hybridised and treated with T4 polynucleotide kinase (Thermo Scientific, Waltham, USA). The double-stranded NES fragment was cloned to the C-terminal part of the *LOS2p:LOS2* sequence. For transient protoplast transformation double-stranded NES and YFP tag were cloned to the C-terminal part of the *LOS2p:LOS2* sequence.

For the reporter plasmids generation *CBF1* promoter fragment was PCR amplified with the primer pair CBF1-luc-FW/RV to obtain a fragment containing G-box. The primer pair CBF1-luc-FW1/RV was used to replace the G-box sequence with AAAAA sequence and generate *mCBF1* promoter fragment. The obtained PCR fragments were cloned into the pGreenII-0800-LUC vector (Hellens et al., 2005).

### 2.2.6 Preparation of competent cells and transformation

For *E.coli* XL-1 Blue strain chemically competent cells were prepared. A single colony of

*E.coli* cells was inoculated into 50 ml MLB and grown overnight at RT with shaking. On the next day overnight culture was diluted in 300 mL MLB to an $OD_{600}$ of 0.2 and incubated at RT with shaking to an $OD_{600}$ of 0.5. Then cells were chilled on ice for 10 min, centrifuged (10min, 2500g, 4°C) and resuspended in 80 ml ice-cold transformation buffer. After incubation on ice for 10 min, cells were centrifuged again and resuspended in 20 ml ice-cold transformations buffer. 1.5ml DMSO was added to cells to reach the final concentration of 7%, and competent cells were stored in aliquots at -80°C.

To obtain *E.coli* cells carrying corresponding vector, 100µL of competent cells was mixed with 1µL of plasmid DNA and kept for 10-15 min on ice. The cells were heat-shocked at 42°C for 1 min, after which 1mL of MLB medium was added to a tube and samples were incubated at 37°C for 1h with rotation at 550rpm. Then cells were centrifuged at 4000g for 1min and plated on MLB (solid) plates with appropriate antibiotics. The plates were incubated at 37°C overnight for colonies to grow.

To prepare electrocompetent *A. tumefaciens* cells, a single colony was inoculated to MLB containing the appropriate antibiotics and incubated on a shaker for two days. The overnight culture was diluted to $OD_{600}$ of 0.2 with 300 mL of LB containing the antibiotics and incubated on a shaker at 28°C, until the $OD_{600}$ reached 0.8. The culture was chilled on ice for 15min, spinned down (20min, 5000xg), and the pellet was washed three times with 30mL of ice cold 10% glycerol, after which the pellet was resuspended in 2 ml 10% ice cold glycerol. Competent cells were stored in aliquots at -80°C.

To obtain *A. tumefaciens* cells carrying corresponding vector, 1µL of plasmid DNA was mixed with 50µL of competent cells in a chilled electroporation cuvette, cuvette was placed in electroporation apparatus and a pulse of 2.5kV, 25µF, 200ohms, 5ms was applied. Immediately 1 mL of MLB was added to cuvettes, and samples were incubated for 1h at 28°C on a shaker. Then cells were centrifuged (4000g, 1 min) and plated on MLB plates containing the appropriate antibiotics. The plates were incubated at 28°C overnight for colonies to grow.

### 2.2.7 PEG-mediated protoplast transformation

For transformation of arabidopsis cell suspension culture protoplasts, culture 3-5 days after subcultivation was used. 4mL of culture was washed with 10 mL WD solution. Cells were resuspended in 10mL WD supplemented with 100mg Cellulase and 25mg Macerozyme, and incubated at RT in the dark for 5h with gentle shaking. Protoplasts were centrifuged (100g, 2 min), washed once with 10ml WD solution and once with 4mL W5, afterwards protoplasts were suspended in 2ml of W5 and incubated on ice for 30min. After the incubation, W5 was

removed and cells were resuspended in 1ml of MMM solution. 200 µL of cells was mixed with 20µg of plasmid DNA and 200µL PEG was added slowly to each tube. Tubes were incubated at RT for 30min, then cells were washed with W5 twice, resuspended in 300 µL of K3 solution and incubated at 21°C for 16-24h prior to microscopy.

For isolation of protoplasts from leaves of arabidopsis plants, 12-day-old seedlings of *haf bee1 bee3* line grown on agar plates were used. Protoplasts were isolated from plants and transiently transformed using a PEG-mediated transformation protocol according to Yoo et al., 2007. Seedlings were cut with razor blade in small strips of approximately 1mm width, immediately transferred in 0.5M mannitol solution and incubated for 30min to allow plasmolysis. Then mannitol was replaced with 10mL WD solution supplemented with 100mg Cellulase and 25mg Macerozyme, and cells were incubated in the dark at 21°C with gentle shaking. Afterwards the mixture was filtered through one layer of miracloth, centrifuged (100g, 3 min) and the pellet was washed twice with 0.5M mannitol, 0.2M $CaCl_2$ solution. Protoplasts were centrifuged (100g, 2 min), washed once with 10ml WD solution and once with 4mL W5, suspended in 2ml of W5 and incubated on ice for 30min. After the incubation, W5 was removed and cells were resuspended in 1ml of MMM solution. 200µL of cells was mixed with corresponding plasmid DNA and 200µL PEG was added slowly to each tube. Tubes were incubated at RT for 30min, cells were washed with W5 twice, resuspended in 300µL of WI solution and incubated at 21°C for 16-24h prior to luciferase activity assay.

### 2.2.8 Luciferase assay

Luciferase assays were performed using a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) and a Lumat LB9501 luminometer (Berthold, Bad Wildbach, Germany) according to Yoo et al., 2007. After 16h incubation period following transformation, protoplasts were centrifuged (100g, 2min), supernatant was removed and samples were frozen in liquid nitrogen. To each tube 100µl lysis buffer was added, samples were vortexed and centrifuged (13000rpm, 4°C, 10min). Fluorescence was recorded for each sample after mixing 10µl of supernatant with 100µl LARII, and then after adding to this mixture 100µl of Stop and Glo reagent.

### 2.2.9 Chromatin immunoprecipitation (CoIP)

Plants were grown in soil for three weeks, and harvested untreated or after treatment at 4°C in light for 3h. 1.5g of plant material was rinsed twice with 40mL of bidistilled water. Plants were then submerged into 37mL 1% formaldehyde, and each tube covered with nylon mesh. Tubes were placed in exsiccator and vacuum was applied for 10min. 2.5mL 2M glycine was

added to quench crosslinking, and vacuum was applied again for 5min. Plants were washed with 40mL bidistilled water, after the second wash water was removed as far as possible. Plant material was ground to fine powder and mixed with 30mL of Extraction buffer 1, vortexed and kept at 4°C until solution was homogeneous. Samples were filtered twice through Miracloth. The extract was centrifuged (4000rpm, 20min, 4°C), pellet was resuspended in 1mL of Extraction buffer 2 and centrifuged (13000rpm, 10min, 4°C). Supernatant was removed and pellet was resuspended in 300µL Extraction buffer 2, obtained mixture was carefully layered on 300µL of Extraction buffer 3. Tubes were spinned (13000rpm, 1h), supernatant was removed and pellet resuspended in 500µL of cold Nuclei Lysis Buffer. Samples were sonicated for 4h using ultrasonic bath (P4500, qteck, Bergen, Germany) to obtain DNA fragments of approximately 200-400bp. After sonication samples were centrifuged (10 min, 13000 rpm, 4°C) and supernatant was mixed with 1.8mL ChIP Dilution Buffer. To reduce nonspecific background supernatant was pre-cleared by incubation with 75µL of Protein A Agarose/Salmon Sperm DNA for 30min at 4°C with agitation, then agarose was pelleted by centrifugation and each sample was divided in two parts. A control part was incubated with 60µL of Protein A Agarose/Salmon Sperm DNA, whereas to the second part the immunoprecipitating antibody was added (5µL of anti-GFP). Samples were incubated overnight at 4°C with rotation. On the next day 60µL of Protein A Agarose/Salmon Sperm DNA was added to each tube and samples were incubated for 4h at 4°C with rotation to collect the antibody/histone complex. On the next day agarose was pelleted by gentle centrifugation (1000rpm, 4°C, 1min), the pellet was washed for 3-5min with 1mL of each of the following buffers: a) Low Salt Immune Complex Wash Buffer (one wash); b) High Salt Immune Complex Wash Buffer (one wash); c) LiCl Immune Complex Wash Buffer (one wash); d) TE Buffer (two washes). The protein A/antibody/histone/DNA complex was eluted with 500µL freshly prepared Elution buffer (1 % SDS, 0.1 M $NaHCO_3$), 20µL 5M NaCl was added to the eluate and histone-DNA crosslinks were reverted by heating at 65°C for 4h. Then 10µL 0.5 MEDTA, 20µL 1M Tris and 2µL 10mg/mL Proteinase K was added to each tube and samples were incubated for 1hour at 45°C. To purify DNA, phenol/chloroform extraction method was used. 400µL of phenol/chloroform mixture (Sigma) was added to each tube, samples were vortexed and centrifuged (13000 rpm, 10min). Upper phase was mixed with 1mL of 96% ethanol to precipitate DNA and 20µg of glycogen was added to each tube to visualise pellets. Samples were centrifuged (13000 rpm, 5 min), pellets were washed with 500µL of 70% ethanol, air-dried and dissolved in 100µL water.

## 2.3 Biochemistry methods

### 2.3.1 Enolase activity assays

Enolase activity was determined spectrophotometrically as described previously (Van Der Straeten et al., 1991). Plant material was ground to a fine powder and 200µL extraction buffer was added to each sample. Tubes were centrifuged for 10min at 13000rpm. Reactions were performed using 1mL of reaction buffer and 50µL of plant extract. The increase in absorbance at 230nm was recorded for 20min after adding the protein extract. The linear part of the graph was used for calculation of enzymatic activity. Phosphoenolpyruvate (PEP) was used to establish a calibration curve.

### 2.3.2 Measurement of soluble sugars and starch

Soluble sugars were extracted from 50mg of plant material (leaves of 3-week-old plants) with 1ml of Extraction buffer (1% pyridine in water), by heating at 90°C for 30min. To the extract 50µl Internal Standard was added (xylose 500 mg/l in water), samples were centrifuged and supernatant evaporated in the vacuum. For the derivatisation, tubes were cooled to 0°C and 100µl ice cold derivatisation reagent (10% benzoyl chloride in pyridine) was added, samples were incubated at 0°C overnight. To hydrolyze the surplus of benzoyl chloride, 10µl water was added to each tube and samples were incubated at 25°C for 30min. Samples were then washed with 800µl toluene, 800µl 2M phosphoric acid, 800µl 0.5M sodium phosphate buffer pH 7.0 and 800µl water and supernatant was evaporated in the valcuum. The residue was dissolved in 80% ACN, centrifuged and injected into the HPLC system equipped with a nucleosil C18 100-5 200 x 4.6mm column. Elution started with 35% eluent A (10% ACN) and 65% eluent B (100% ACN). Within 15min eluent B was linearly increased to 70% and within further 21min to 100% and then kept at 100% for another 3 minutes prior reducing it to the starting conditions within 1 min. The column was finally equilibrated for 5min with 35% A and 65% B before the next sample was injected. For quantification the UV absorbance was recorded at 230nm.

Starch was quantified as previously described (Rose et al., 1991). Leaves of 3-week-old plants were ground in liquid nitrogen and extracted three times with 80% ethanol at 75°C. The pellet was dried and heated at 50°C with 0.1 N NaOH for 30min, then 900µl 0.1N acetic acid was added to adjust the pH to 5.1. To each sample 200U of α-amylase and 2U of amyloglucosidase were added and incubated for 2h at 37°C following 20min incubation at 50-55°C. Glucose was analyzed with glucose oxidase/peroxidase/o-dianisidine solutuion.

### 2.3.3 Salicylic acid content determination

Total and free salicylic acid was measured as described previously (Rozhon et al., 2005) except that a Nucleodur 100-5 NH2 125x4 mm column and an eluent consisting of 8.5% ACN containing 25mM formic acid set with NaOH to pH 4.0 were used.

For extraction of Free salicylic acid 50mg plant material was mixed with 1ml 90% methanol, 20µl internal standard (5-FSA, 1 mg/l) was added to each sample. Tubes were incubated at 25°C for at least 1h, centrifuged (13000rpm, 5min) and supernatant was evaporated in the vacuum. 1ml Extraction buffer was added to each tube, samples were incubated for 10min at 25°C and 1000rpm. Then 50µl hydrochloric acid 4 mol/l was added to each tube, samples were centrifuged for (13000rpm, 1min) and supernatant was loaded on an equilibrated SPE column (equilibrated twice with 1ml AcCN 100% and twice with 10mM hydrochloric acid).

Total salicylic acid was extracted from 10 to 20mg plant material with 1ml Extraction buffer, 50µl internal standard was added to each tube. Samples were incubated at 95°C and 1000rpm for 1 to 2h, centrifuged for 5min at 13000rpm, the supernatant was mixed with 50µl hydrochloric acid 4 mol/l and incubated at 95°C for 1 to 2h. Samples were then centrifuged (1min, 13000rpm) and supernatant was loaded on an equilibrated SPE column.

The SPE columns were washed with 1ml 10mM hydrochloric acid, and samples were eluted with with 500µl elution buffer (2mM iron(III) chloride in 5mM HCl). 5µl DTT and 5µl bipyridine were added to the eluates and samples were injected into the HPLC system equipped with Nucleodur 100-5 NH2 125x4 mm column. An eluent consisting of 8.5% acetonitril containing 25mM formic acid set with NaOH to pH 4.0 were used.

### 2.3.4 Lignin measurements

Lignin was quantified using the acetyl bromide method optimized for small amounts of plant tissue (Van Acker et al., 2013). Primary inflorescence stems of 6-week-old plants were used. Purified cell wall residues (CWR) were obtained with a series of extraction steps at near boiling temperatures with water (98°C), ethanol (76°C), chloroform (59°C), and acetone (54°C), for 30min each. 5mg of the dried CWR were dissolved in 0.1ml of freshly made 25%acetyl bromide in glacial acetic acid and 4µl 60% perchloric acid. The solution was incubated for 30min at 70°C with shaking and centrifuged (16000g, 15min). The supernatant was mixed with 0.2ml of 2M sodium hydroxide and 0.5ml glacial acetic acid, and the pellet was washed with 0.5ml glacial acetic acid. The supernatant and the washing phase were combined. After 20min incubation at RT, the absorbance at 280nm was measured with

Jenway 7315 spectrophotometer (Bibby Scientific, Stone, UK).

### 2.3.5 Quantification of sinapoyl malate

Sinapoyl malate was extracted from 10mg of plant material with 500µl extraction buffer (20% acetonitrile, 20mM sodium phosphate pH 7.0). 10µl internal standard (5-fluorosalicylic acid 250 mg/l) were added to each sample and heated at to 60°C and for 1h. Samples were then centrifuged (15000g, 5min). The supernatant was transferred into a new tube, acidified with 10µl 20% formic acid and centrifuged. The obtained clear supernatant was analysed by HPLC using a LC-10 system (Shimadzu, Kyoto, Japan) equipped with a Symmetry 3.5µm C18 100 x 4.6 mm column (Waters, Milford, MA). A constant flow rate of 1 ml/min was maintained with a gradient starting with 100% eluent A (0.1% formic acid in 10% ACN). Within 8min eluent B (80% ACN) was linearly raised to 25% and within further 7min to 100% prior reducing it to the starting conditions within 0.5min. The column was finally equilibrated for 6.5min with 100% A before injection of the next sample. Fluorescence detection was performed at an excitation wavelength of 335nm and an emission wavelength of 450nm.

### 2.3.6 Analysis of fatty acid composition

Quantification of fatty acids was adapted from Durst et al., 1975. Fatty acids were extracted from 20 mg plant material with 300 µl of Extraction buffer (9/1 mixture of 96% ethanol and 3M aqueous potassium hydroxide) at 60°C overnight. On the next day 400µl 0.5M HCl and 500 µl n-hexane were added to each tube. The organic phase was collected and evaporated in the vacuum. Then 200 µl freshly prepared derivatisation reagent (1 mg/ml 4-bromophenacyl bromide and 0.2 mg/ml 18-crown-6 dissolved in pure acetonitril) and 1 mg $K_2CO_3$ were added to each tube and samples were incubated at 60°C for 30 min. 25 µl of the supernatant was directly injected into the HPLC system equipped with a LiChrospher 60 RP-select B 5 µm 125x4 mm column. Elution was performed at a flow rate of 1 ml/min with a gradient starting with 20% A (1 mM acetic acid in 10% ACN) and 80% B (1 mM acetic acid in 100% ACN). The concentration of B was linearly raised to 85% after 8 min and finally to 94% after another 7 min. Subsequently, B was decreased to the starting conditions within 0.5 min and the column equilibrated for 6.5 min prior injection of the next sample. The absorbance at 260 nm was recorded.

## 2.4 Histochemical methods

### 2.4.1 Microscopy

For images of the adaxial leaf epidermis the second leaf pair of 4-week-old plants was shock-

frozen in liquid nitrogen and visualized with a Hitachi TM3000 scanning electron microscope (Hitachi High-Tech, Tokyo, Japan). The flowers and hypocotyls were fixed on a metal support rack and visualized without treatment. The YFP signal in transformed arabidopsis protoplast was analysed with Olympus BX-61 fluorescence microscope (Olympus, Tokyo, Japan).

### 2.4.2 Section preparation and staining

Stem sections were made from primary inflorescence stems of 6-week-old plants 2-3 mm above the rosette. Tissue fixation and embedding was performed as described previously (Beeckman et al., 2000). Briefly: the stems were harvested and fixed in FAA solution (45% ethanol, 5% acetic acid and 5% formalin in water), the fixed plant material was dehydrated in a graded ethanol series (2 h each in 30%, 50%, 70% and 100% ethanol), infiltrated and embedded with Technovit 7100 embedding kit (Heraeus Kulzer, Wehrheim, Germany). Sectioning was performed with a Leica RM 2065 microtome (Leica Mikrosysteme, Wetzlar, Germany). Sections were stained for 5 min in 0.02% aqueous Toluidine blue O (Sigma-Aldrich, St. Louis, USA), rinsed with water and analyzed with an Olympus BX-61 microscope (Olympus, Tokyo, Japan)

# III. Results

## 1. Brassinosteroids control cold stress responses of plants

### 1.1 Brassinosteroid signalling positively regulates basal freezing tolerance

To investigate if BRs govern the basal expression of *CBFs* and targeted *COR* genes mRNA levels of the genes were determined in the BR biosynthetic mutant *cpd* (Szekeres et al., 1996), the two BR signalling defective mutants *bri1-1* (Friedrichsen et al., 2000) and *bri1-301* (Xu et al., 2008), and the BR hyper-signalling line *35S:BRI1-GFP (BRI1oe;* Wang et al., 2001) by qPCR.

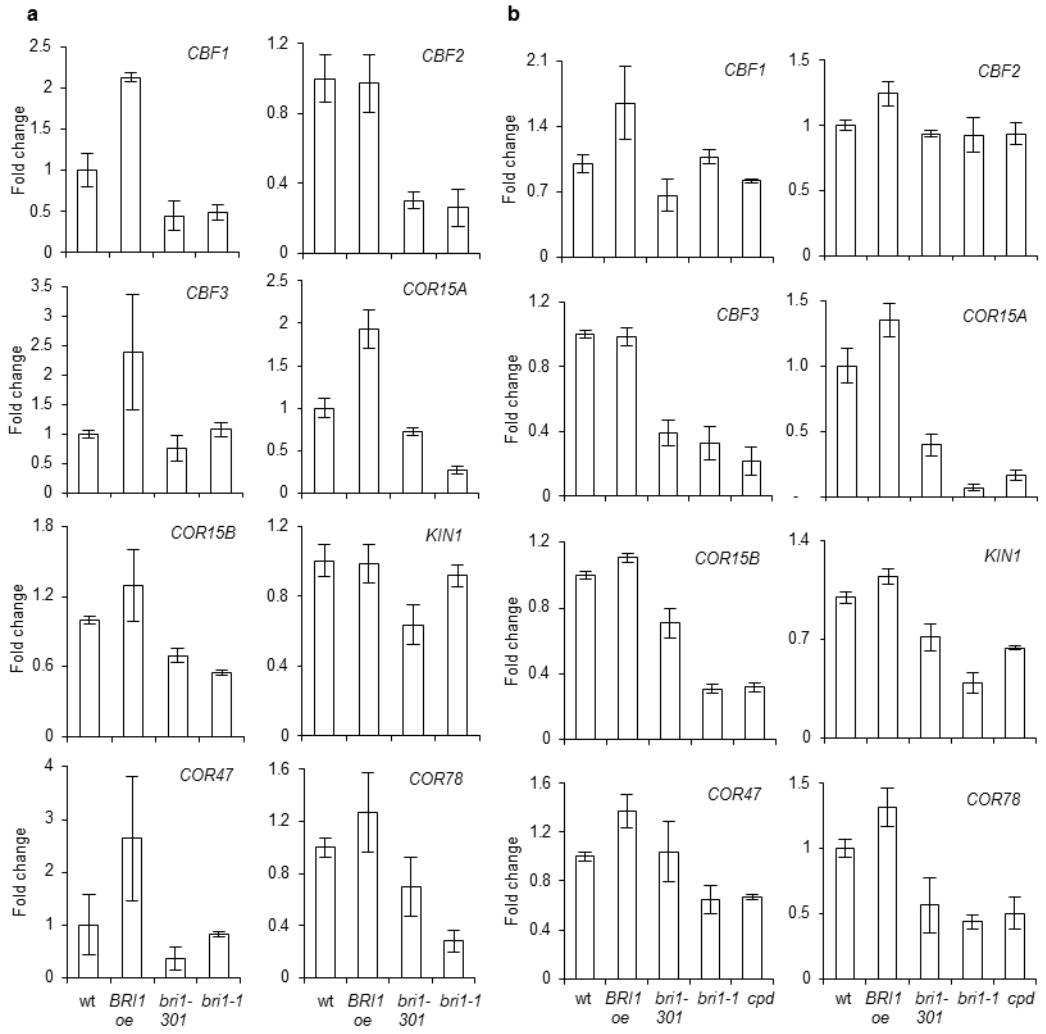


**Figure 3.** BR signalling positively regulates *COR* gene expression.
Steady-state transcript levels of *CBFs*, and indicated down-stream target genes were determined in 3-week-old soil-grown plants (**a**) and seedlings (**b**) of wild-type, *35S:BRI1-GFP* (*BRI1oe*) and the BR-deficient mutants *bri1-301*, *bri1-1* and *cpd* by qPCRs. Values are the means from 3 independent biological replicates each measured in 4 technical repeats; error bars show the SD of biological replicates.

As demonstrated in Figure 3a,b, BR-deficiency repressed *CBF* transcription both in the adult (Figure 3a) and seedling stage (Figure 3b). Moreover, the down-stream targets *COR15A*, *COR15B*, *COR47*, *COR78* and *KIN1* were all strongly reduced in BR-deficient mutant backgrounds (Figure 3a,b). On the contrary, in *BRI1oe* plants, in which BR signalling is promoted, there was an increase in the transcript levels of *CBFs* and CBF-regulated genes (Figure 3a,b).

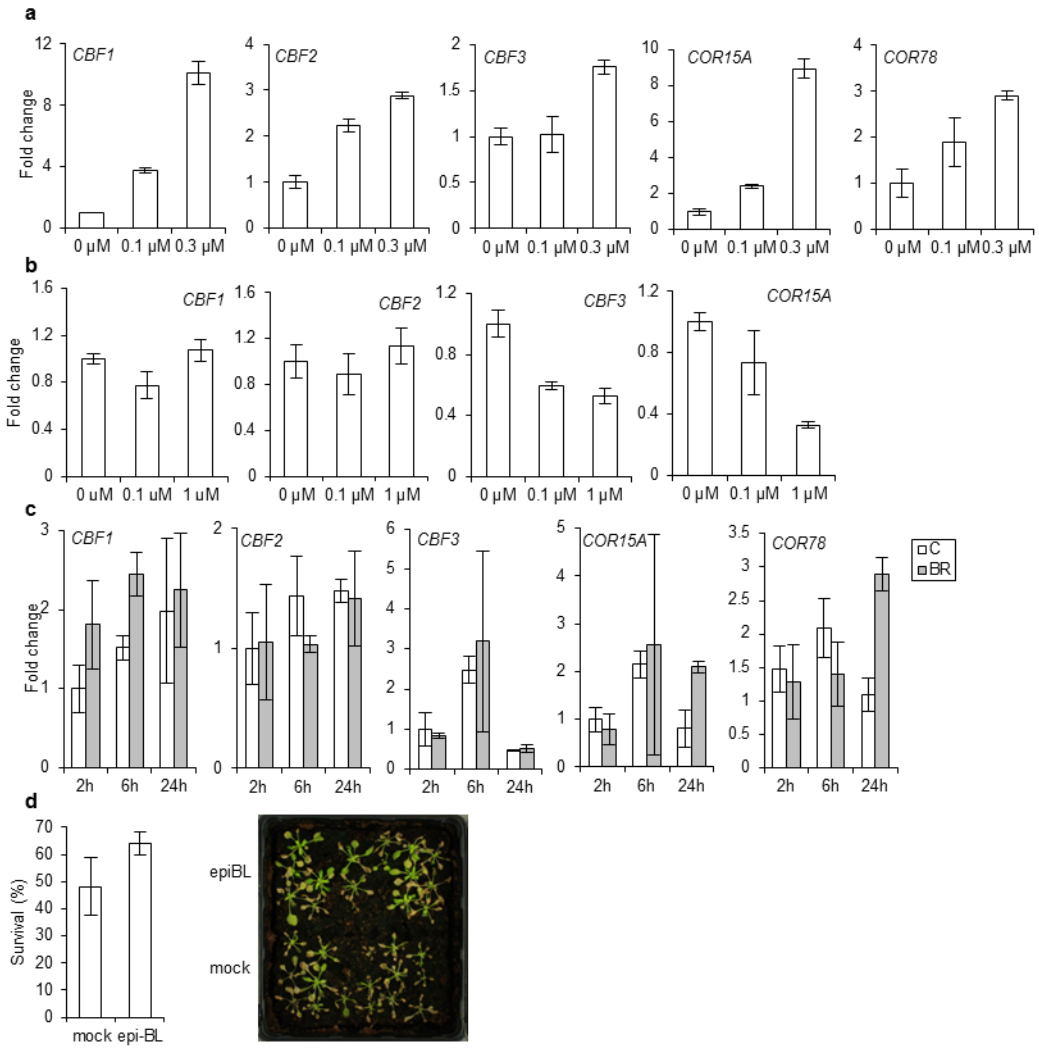


**Figure 4.** Application of epiBL promotes freezing tolerance in arabidopsis.
(**a,b**) Transcript levels of *CBFs* and *COR* genes were determined by qPCRs in 10-day-old wild-type arabidopsis seedlings grown on ½ MS media supplemented with different concentrations of epiBL (**a**) or Brz (**b**). Values are the means from 3 independent biological replicates each measured in 4 technical repeats; error bars show the SD of biological replicates.
(**c**) Effect of epiBL treatment on expression of *CBFs* and *COR* genes. Three-week-old soil-grown wild-type plants were sprayed with mock or 10µM epiBL solution, plant material was collected 2h, 6h and 24h after treatment. Values are the means from 3 independent biological replicates each measured in 4 technical repeats; error bars show the SD of biological replicates.
(**d**) Three-week-old soil-grown arabidopsis plants were sprayed with mock or 10µM epiBL solution twice a day for two days and freezing tolerance assay was performed. Quantification (left) and representative plants (right) are shown.

In agreement, also external application of BR or the BR biosynthesis inhibitor brassinazole produced equivalent changes in *CBF* expression (Figure 4a,b). Moreover, treatment with epiBL affected *COR* gene expression and enhanced freezing tolerance of soil-grown 3-week-old arabidopsis plants (Figure 4c,d).

To assess if the changes in *CBF* and *COR* gene expression also impacted on the basal freezing tolerance of BR signalling mutants, freezing tolerance assays were performed. For this purpose 3-week-old, soil-grown plants were treated with either -6°C or -8°C for 4h and were then moved back to 21°C. Survival was assessed after 2 weeks of recovery as the ability to form new leaves. Wild-type Columbia-0 (Col-0) showed survival rates of approx. 45% following exposure to -6°C and approx. 15% following exposure to -8°C (Figure 5a), whereas the BR hyper-response line *BRI1oe* had clearly increased survival rates (approximately 70% and 30%, respectively). Importantly, the BR signalling deficient mutants were both strongly impaired in their constitutive freezing tolerance (Figure 5a). In agreement also electrolyte leakage of acclimated *bri1-301* plants was significantly increased (Figure 5b).

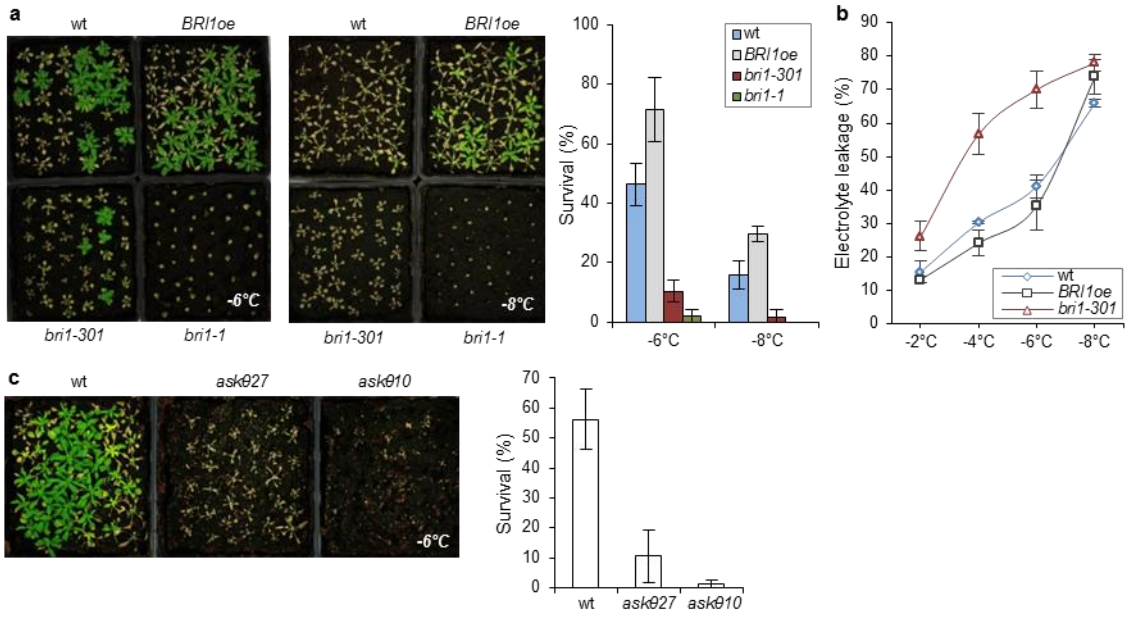


**Figure 5.** BR signalling positively regulates the basal freezing tolerance of plants.
(**a**) Freezing tolerance of non-acclimated *BRI1oe*, *bri1-301* and *bri1-1* plants as compared to wild-type. Plants were grown in soil in long day (LD) growth conditions at 21°C. After 3 weeks they were treated with -6°C or -8°C for 4 h. Survival was assessed after 2 weeks of recovery at 21°C. Representative plants of each line and the quantification of the results, the values being the means of 3 independent experiments; error bars show the SD.
(**b**) Electrolyte leakage in leaves of non-acclimated plants of wild-type, *BRI1oe* and *bri1-301* plants grown in the same conditions as in **a** and **b** and treated with the indicated temperatures. Values are means of 3 independent experiments; the error bars show the SD.
(**c**) Freezing tolerance of non-acclimated *askθ27* and *askθ10* plants as compared to wild-type. Quantification (right) and phenotypes of the representative plants (left) are shown.

To investigate at which step in the BR signalling pathway the regulatory effects occur the survival rates of two independent lines over-expressing ASKθ, a BIN2 homologue (Rozhon et al., 2010), were assessed following -6°C treatment. Both lines were hypersensitive to freezing (Figure 5c), indicating that BR function in freezing tolerance is conferred down-stream of ASKs. Taken together, these results indicate that BR signalling promotes freezing tolerance in arabidopsis under nonacclimated conditions.

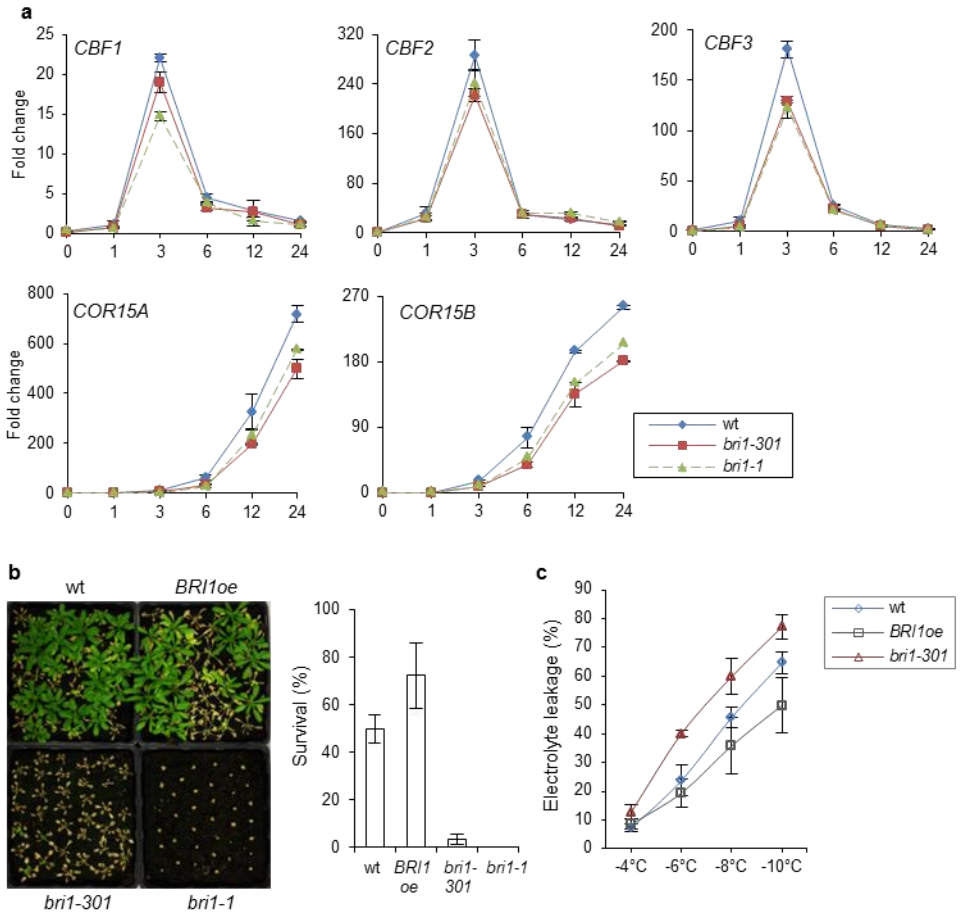


**Figure 6.** BR signalling enables cold acclimation.
(**a**) Transcript levels of *CBF* and *COR* genes in wild-type, *bri1-301* and *bri1-1* treated with 4°C for the indicated periods of time (h). Plants were grown on ½ MS plates in LDs at 21°C for 12 days, were transferred to 4°C, samples were collected at the given time points and mRNA levels were determined by qPCRs. Values are the means from 3 independent biological replicates each measured in 4 technical repeats; error bars are the SD.
(**b**) Freezing tolerance of *BRI1oe*, *bri1-301* and *bri1-1* plants as compared to wild-type after cold acclimation. 3-week-old plants grown in LDs at 21°C were acclimated for 3 days at 4°C and then treated with -10°C for 6h. Survival was scored after 2 weeks of recovery at 21°C. Pictures of representative plants (left) and the quantified results (right) are shown. Values are the means of 3 independent experiments; the SD is shown.
(**c**) Electrolyte leakage in acclimated plants of wild-type, *BRI1oe* and *bri1-301*. Plants were grown and acclimated as in **b** and ion leakage was measured in detached leaves following exposure to the indicated sub-zero temperatures. Values are the means of 3 independent experiments; the SD is shown.

### 1.2 Brassinosteroid signalling impacts on the cold acclimation

To investigate if BRs also participate in the cold acclimation process we treated BR signalling mutants with 4°C and quantified mRNA levels of *CBFs*, *COR15A* and *COR15B* in a time-course manner by qPCR analysis. Importantly, the transient induction of *CBF* expression, which took place in wild-type plants, was attenuated in BR-signalling mutants (Figure 6a). Consistent with this finding, levels of *COR15A* and *COR15B* were decreased in *bri1-301* and *bri1-1* at all time points tested. This resulted in an even more pronounced decrease in mRNA levels following cold treatment as compared to untreated conditions (Figure 6a).

To assess if the decreased induction of *CBF* expression and their targets also impacted on the acquired freezing tolerance of BR signalling-defective mutants, freezing tolerance assays following cold acclimation were carried out. Three-week-old, soil-grown plants of *BRI1oe*, *bri1-1*, *bri1-301* and wild-type were acclimated for three days at 4°C and were then treated with -10°C for 6h. The result is given in Figure 6b and shows that the freezing tolerance of acclimated *bri1-1* and *bri1-301* plants was strongly decreased as compared to wild-type, whereas freezing tolerance of *BRI1oe* plants was increased (Figure 6b). Therefore, there is evidence that BR signalling contributes to the cold-induced increase of *CBF* expression and their targets and contributes to the acquired freezing tolerance of plants.

### 1.3 CES and homologues are positive regulators of basal freezing tolerance

To identify BR-regulated TFs that control *CBF* expression, a candidate gene approach was employed. Since the BES1/BZR1 protein family is best characterized, first *CBF1-3* mRNA levels were determined by qPCR in the dominant *bes1-D* (Yin et al., 2002) and *bzr1-1D* (Wang et al., 2002) mutants. However, no significant change was found (Figure 7a). Moreover, freezing tolerance and ion leakage assay showed no difference in freezing tolerance of *bzr1-1D* as compared to wild-type and *bes1-D* being hypersensitive (Figure 7b,c), excluding BES1 and BZR1 as activators of *CBF* expression.

To investigate if the CES/BEE family of BR-regulated transcription factors may be involved steady-state levels of *COR* gene transcripts in 3-week-old plants of the dominant *ces-D* line were assessed (Poppenberger et al., 2011). The result showed that all three *CBFs* were up-regulated in adult *ces-D* plants (Figure 8a), whereas in *ces-D* seedlings only *CBF1* expression was strongly increased (Figure 8b). Moreover transcript levels of CBF-regulated genes were dramatically up-regulated in *ces-D* (Figure 8a,b).

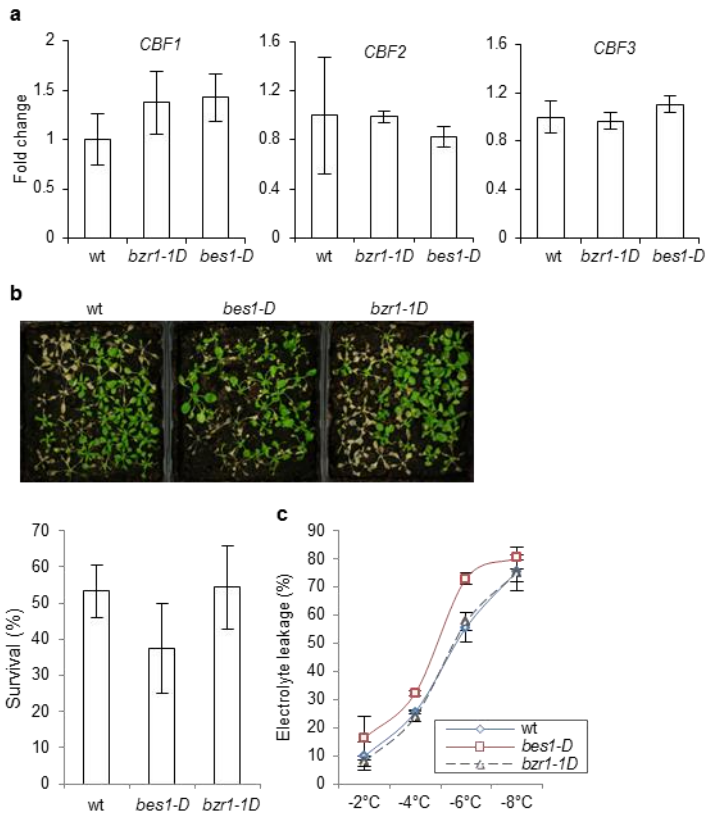


**Figure 7.** Freezing tolerance of *bzr1-1D* and *bes1-D* lines.
(**a**) Transcript levels of *CBF1*, *CBF2* and *CBF3* in *bzr1-1D* and *bes1-D* as compared to wild-type. Steady-state mRNA levels of *CBFs* in 12-day-old, non-acclimated *bzr1-1D* and *bes1-D* plants were determined by qPCR.
(**b**) Freezing tolerance of non-acclimated *bzr1-1D* and *bes1-D* lines as compared to wild-type. Plants were grown in soil in LD growth conditions at 21°C for 3 weeks and were treated with -6°C for 4h. Shown are representative plants of each line and a quantification of the results, the values being the means of 3 independent experiments; error bars are the SD.
(**c**) Electrolyte leakage in leaves of non-acclimated plants of wild-type, *bzr1-1D* and *bes1-D* grown in the same conditions as in **b** and treated with the indicated temperatures. Values are means of 3 independent experiments; error bars are the SD.

Since in Col-0 BEE1 and BEE3 act redundantly with CES in floral organ development (Crawford and Yanofsky, 2011), it was speculated that BEE1 and BEE3 may also complement for a loss of CES function in cold responses. Therefore, a quadruple mutant *bee1 bee2 bee3 ces-2* (*qM*) line was generated in the Poppenberger lab by introducing *ces-2* into the *bee1 bee2 bee3* triple mutant background (Friedrichsen et al., 2002) by crossing. In this mutant expression of all four genes was strongly compromised (Poppenberger lab, unpublished data). In addition also the *haf/ces-3 bee1 bee3* mutant (Crawford and Yanofsky, 2011) was included in the analysis (*tM*). Cold-responsive gene transcripts were analyzed both in seedlings and adult plants of the *tM* and *qM* lines. As shown in Figure 8a transcript levels of *CBF1* and *CBF3* were reduced by approx. 5-fold in adult *tM* and *qM* plants, whereas in seedlings a significant reduction was detectable only for *CBF3* (Figure 8b). This correlated with a reduction of all assessed down-stream *COR* genes in both developmental settings (Figure 8a,b).

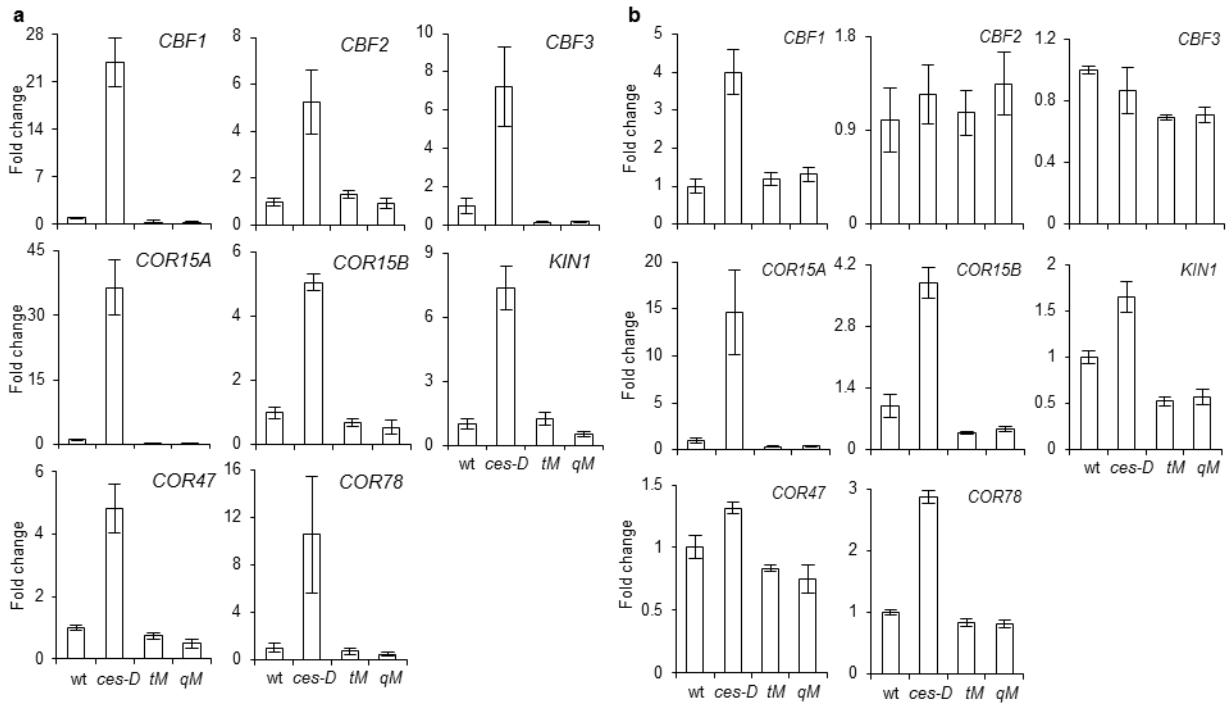


**Figure 8.** CES and homologues promote *COR* gene expression.
(**a, b**) Steady-state mRNA levels of *CBFs* and *COR* genes in 3-week-old soil-grown (**a**) and 12-day-old seedlings (**b**) of non-acclimated *ces-D, tM* and *qM* plants determined by qPCR. The means of 3 independent biological replicates each measured in 4 technical repeats are shown; error bars are the SD.

To assess if the changes in *CBF* and *COR* gene expression in *CES* mutant lines may correlate with altered basal freezing tolerance, freezing stress experiments and electrolyte leakage assays were performed. The results showed that *ces-D* plants had a strongly increased freezing tolerance, being highly resistant against -6°C treatment (Figure 9a), which was also correlated with significantly reduced electrolyte leakage (Figure 9b). Importantly, in support the *tM* and *qM* exhibited a reduced resistance against freezing damage, which was correlated with enhanced electrolyte leakage in both mutant lines (Figure 9a,b). Therefore, there was evidence that the CES/BEE bHLH subfamily acts as a promoter of the CBF transcriptional cascade and of the basal freezing tolerance of plants.

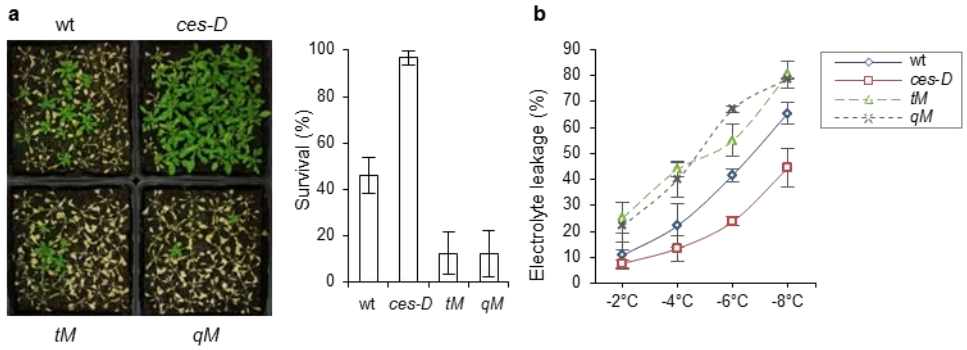


**Figure 9.** CES and homologues promote basal freezing tolerance.
(**a**) Freezing tolerance of non-acclimated *ces-D*, *tM* and *qM* lines as compared to wild-type. Plants were grown in soil in LD growth conditions at 21°C for 3 weeks and were treated with -6°C for 4h. Survival was assessed following 2 weeks of recovery at 21°C. Shown are representative plants of each line (left) and a quantification of the results (right), the values being the means of 3 independent experiments; error bars are the SD.
(**b**) Electrolyte leakage in leaves of non-acclimated plants of wild-type, *ces-D*, *tM* and *qM* grown in the same conditions as in **a** and treated with the indicated temperatures. Values are means of 3 independent experiments; error bars are the SD.

### 1.4 CES and homologues promote cold acclimation

To test whether CES and the BEEs may also act to enable plants a further increase in freezing tolerance during cold acclimation, I treated 12-day-old seedlings of wild-type, *ces-D* and the *qM* with 4°C, harvested samples in a time-course manner and analysed gene expression by qPCR. As shown in Figure 10a, the induction of all *CBFs*, but in particular of *CBF1*, was more pronounced in *ces-D* than in wild-type, whereas it was attenuated in the *qM* plants. This correlated with a more pronounced increase in mRNA levels of *COR15A* and *COR15B* in *ces-D*, and a reduction in the *qM* line (Figure 10a).

To determine whether this altered induction of *CBF* and *COR* gene expression also impacted on the freezing tolerance of *CES* mutants following cold adaptation, 3-week-old soil-grown plants were acclimated at 4°C for 3 days, treated with -10°C for 6h and allowed to recover for 2 weeks at 21°C before survival rates were determined. The results showed that also in this experimental set-up the *ces-D* mutant was more resistant to freezing stress than wild-type, whereas the *tM* and *qM* line were hypersensitive (Figure 10b). Electrolyte leakage assays performed following cold acclimation confirmed that *ces-D* was less affected by the treatment whereas the *tM* and *qM* were hypersensitive (Figure 10c).

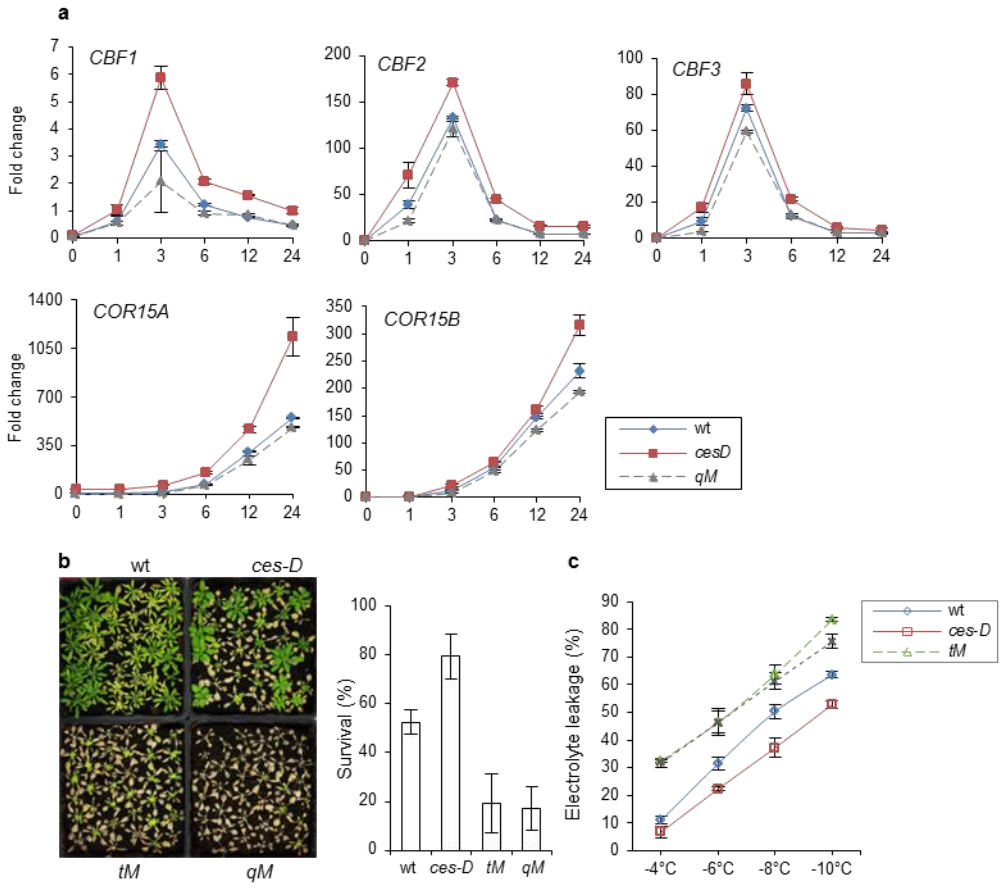


**Figure 10.** CES and homologues promote the acquired freezing tolerance of plants.
(**a**) Transcript levels of *CBF* and *COR* genes in 12-day-old plants of wild-type, *ces-D* and *qM* plants treated with 4°C for the indicated periods of time (h). The means of 3 independent biological replicates each measured in 4 technical repeats are shown; error bars are the SD.
(**b**) Freezing tolerance of the *ces-D, tM* and *qM* plants. 3-week-old plants, grown in LD growth conditions at 21°C, were acclimated for 3 days at 4°C and then treated with -10°C for 6h. Survival was scored after 2 weeks of recovery at 21°C. Pictures of representative plants (left) and the quantified results (right) are shown. Values are the means of 3 independent experiments; the SD is shown.
(**c**) Electrolyte leakage in detached leaves of acclimated plants of wild-type, *ces-D, tM* and *qM*. Plants were grown and acclimated as in **b** before electrolyte leakage assays were performed. Values are means of 3 independent biological experiments.

### 1.5 CES directly binds to the *CBFs* promoters *in planta*

Since there was evidence that CES acts to control *CBF* expression, it seemed possible that *CBFs* are direct targets of CES. A search for CES binding sites in the *CBF* promoters demonstrated that all three promoters contained G-box motifs. To investigate if CES can bind to these regulatory elements chromatin immuno-precipitation assays were performed with *35S:CES-YFP* expressing plants before and after cold-treatment. The result demonstrated that CES was enriched on all promoters both in untreated conditions as well as also following cold-treatment, with the strongest enrichment on the *CBF1* promoter, which also further increased in response to cold (Figure 11a,b).

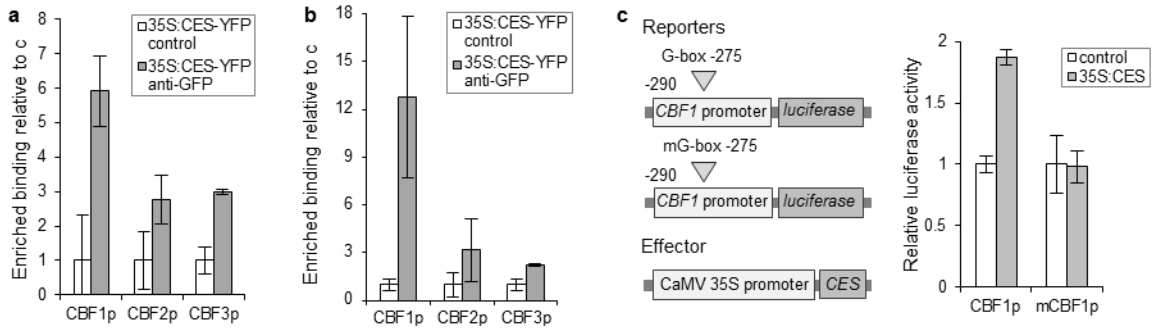


**Figure 11.** CES directly binds to the promoters of *CBFs* and activates their expression.
(**a,b**) ChIP of CES-YFP followed by qPCR of DNA fragments containing the G-box motif in the *CBF1* (G-box: -275), *CBF2* (G-box: -251) and *CBF3* (G-box: -2275) promoter. Leaves of 3-week-old *35S:CES-YFP* plants grown in soil in LDs, either untreated (**a**) or treated with 4°C for 3h (**b**), were used. 5srRNA was utilized for normalization. Values are fold enrichment of CES-bound DNA containing the G-box motif in immuno-precipitated samples relative to the total input DNA. The means of three biological replicates with the SDs are shown.
(**c**) Luciferase transactivation assays in arabidopsis protoplasts. The LUC reporter constructs illustrated were transiently expressed in protoplasts either alone (control) or with CES as an effector. The results are presented as the means of 3 independent biological replicates with the SDs.

To confirm that CES directly binds to the G-box motif in the *CBF1* promoter luciferase (LUC) transactivation assays with a fragment of the *CBF1* promoter containing the G-box, and one in which the G-box was mutated, were performed in arabidopsis protoplasts. Mutating the G-box eliminated CES activation of *CBF1p:LUC* expression showing that CES directly binds to the G-box in the *CBF1* promoter *in vivo* (Figure 11c).

### 1.6 BR-induced expression of *CBF1* and *CBF3* is controlled by CES

The results presented in this study suggested a model in which BRs control the basal and acquired freezing tolerance of plants in a CES and BEE controlled manner. To verify this hypothesis wild-type, *tM* and *qM* lines were treated with epiBL and expression of *CBF1*, *CBF3* and *COR15A*, which were most responsive to epiBL application, were tested. As shown in Figure 12a *CBF1* and *CBF3* were unresponsive to epiBL treatment in the knock-out lines providing evidence that the CES/BEE subfamily of bHLH proteins regulates the BR induction of these genes. *COR15A* induction by epiBL was also impaired in the mutants, however, some response remained, indicating that additional factors of BR signalling may also contribute to the BR control of *COR15A* expression.

To test if CES is sufficient to alleviate the freezing hypersensitivity of BR signalling defective plants, *ces-D* was introduced into *bri1-5 bak1-1D*, a weak BR signalling mutant (Li et al., 2002), and freezing tolerance was assessed. In this line freezing tolerance was restored to a significant extent (Figure 12b).

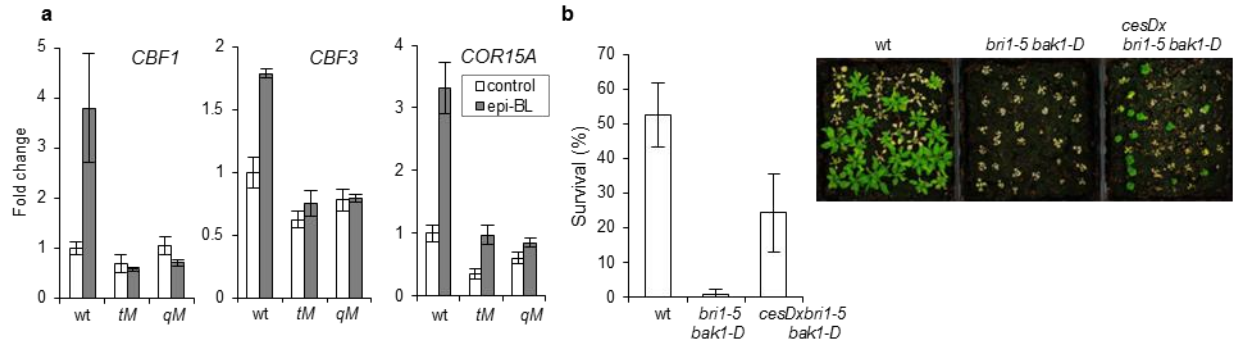


**Figure 12.** CES and homologues control BR-induction of *CBF1, CBF3* and *COR15A* expression.
(**a**) Transcript levels of *CBF1, CBF3* and *COR15A* were assayed in 10-day-old wild-type seedlings grown on ½ MS media supplemented with 250nM epiBL. Values are the means of 3 biological replicates each measured in 4 technical repeats; error bars indicate the SDs.
(**b**) *ces-D* rescues freezing sensitivity of *bri1-5 bak1-D*. Shown are representative plants of each line (right) and a quantification of the results (left), the values are the means of 3 independent experiments; error bars are the SD.

### 1.7 SUMOylation positively regulates CES activity in cold responses of plants

CES activity is altered by SUMOylation, which is induced in response to an activation of BR signalling (Khan et al., 2014). Since protein SUMOylation plays a central role in cold responses (Miura et al., 2007), it was important to investigate if the SUMOylation state of CES impacts on its activity in *COR* gene expression and freezing tolerance. For this purpose freezing tolerance was analysed in plant lines expressing CES wild-type (35S:CES-YFP, line 32) or mutant versions, in which SUMOylation is either impaired (K72R, line 411) or enhanced (S75A+S77A, line 310; Khan et al., 2014). Freezing tolerance in *35S:CES-YFP* and *S75A+S77A* was enhanced as compared to wild-type, whereas it was not significantly changed in the *K72R* plants (Figure 13a,b).

Moreover, non-SUMOylated CES was unable to activate *COR15A* expression, whereas the constitutively SUMOylated version was highly active *in planta* (Figure 13c). Therefore, there is evidence that SUMOylation increases CES activity in *COR15A* expression and cold responses of plants.

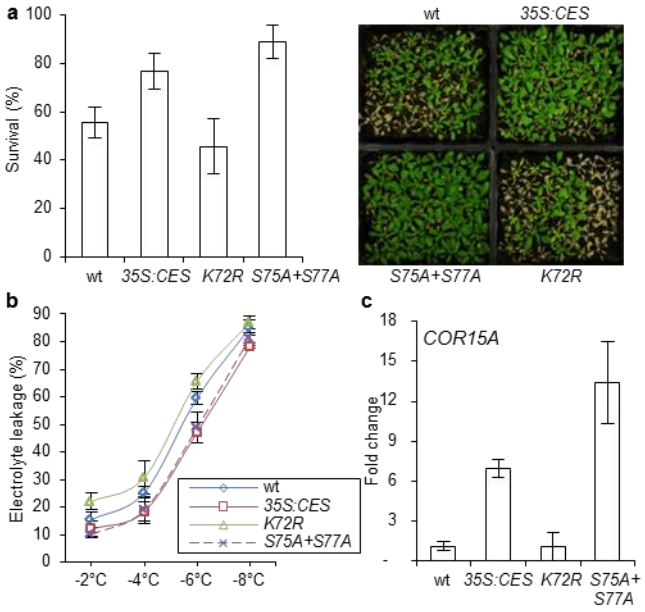


**Figure 13.** Posttranslational modifications of CES impact on freezing tolerance.
(**a**) Freezing tolerance of plants over-expressing either the wild-type CES protein (35S:CES-YFP), a mutant impaired in SUMOylation (K72R) or a mutant constitutively SUMOylated (S75A+S77A). Plants were grown in soil in LD growth conditions at 21°C for 3 weeks and were treated with -6°C for 4 h.
(**b**) Electrolyte leakage in leaves of non-acclimated plants of wild-type, *35S:CES*, *K72R* and *S75A+S77A* plants grown in the same conditions as in **a** and treated with the indicated temperatures. Values are means of 3 independent experiments; the error bars show the SD.
(**c**) Transcript level of *COR15A* in *35S:CES*, *K72R* and *S75A+S77A* lines. Values are the means of 3 biological replicates each measured in 4 technical repeats; error bars indicate the SDs.

### 1.8 Brassinosteroid biosynthesis gene transcripts are suppressed under cold

In cold responses protein SUMOylation plays a key role. Over-all SUMOylation of proteins drastically increases in response to cold stress and SUMOylation of ICE1 enhances its activity in *CBF3* transcription (Miura et al., 2007). How ICE1 SUMOylation is induced is currently unknown although a phospho-deficient mutant of ICE1 is more readily SUMOylated (Miura et al., 2011) and also in CES de-phosphorylation promotes SUMOylation (Khan et al., 2014). Therefore, it is possible that in response to cold BR signaling is activated to alter the phosphorylation state of TFs, which induces SUMOylation and activates them in *CBF* expression. This would reduce BR biosynthesis, since when BR signaling is activated BR biosynthetic gene expression is repressed. To test this hypothesis I assessed transcript levels of key BR biosynthesis enzymes in response to 2h, 6h and 12h of cold treatment. The result presented in Figure 14 shows that transcript levels of *CPD* (*CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM)*, *DWF4* (*DWARF4*) and *Br6ox2* (*BRASSINOSTEROID-6-OXIDASE2*) were repressed under cold treatment, indicating that BR levels may decrease in response to cold.

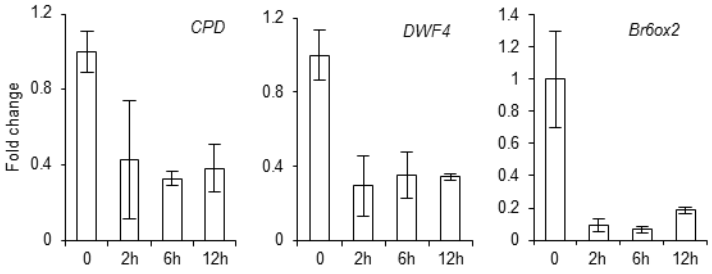


**Figure 14.** Impact of cold on the expression level of BR biosynthetic genes.
Transcript levels of *CPD, DWF4* and *Br6ox2* were determined in 3-week-old soil-grown wild-type plants by qPCRs. Plants were treated with 4°C in the continuous light. Values are the means from 3 independent biological replicates each measured in 4 technical repeats; error bars show the SD of biological replicates.

## 2. LOS2/ENO2 activity is essential for the development of plants and is feedback-regulated by AtMBP1

The results presented above provided evidence that BRs regulate cold stress responses, at least partially, in a CBF-dependent manner. The second project of my thesis dealt with the characterization of LOS2/ENO2, since in a yeast-two-hybrid screen LOS2 was identified as a CES interacting protein (Poppenberger lab, unpublished results). To be able to address the question if BRs via CES may also impact on LOS2 activity and thus CBF-independent cold responses in arabidopsis an initial characterisation of LOS2 loss-of-function plants was carried out.

### 2.1 Identification of *los2/eno2* knock-out lines

Three independent mutant lines, predicted to harbour insertions in *LOS2/ENO2* in the Col-0 wild-type background, were identified in the SALK and SAIL T-DNA insertional mutant collections and were obtained from NASC. The location of the insertions was determined by PCR and is shown in Figure 15a. The alleles were numbered according to the position of their T-DNAs, with *los2-2* (SALK_021737) mutated in intron one (insertion down-stream of nucleotide 320, with a 12 bp deletion), *los2-3* (SALK_077784) mutated in exon six (insertion down-stream of nucleotide 1350) and *los2-4* (SAIL_208_B09) mutated in exon ten (insertion down-stream of nucleotide 2376, with a 28 bp deletion). For comparison the *los2-1* mutation (G325S), which is a single point mutation that lies in exon eight (Lee et al., 2002), is shown.

Quantitative real-time PCR analysis using primers that bind to the 3' region of the mRNA confirmed that *LOS2/ENO2* transcript abundance was severely reduced in plants homozygous for *los2-2* and *los2-3*, and was also decreased in the *los2-4* mutant, albeit to a lower extent

(Figure 15b). To investigate the effect of the mutations on ENO2 activity we measured enolase activity in 2-week-old plants of the mutants and compared it to wild-type. As shown in Figure 15c enolase activity in *los2-2* was reduced to about 20%, while in *los2-3* and *los2-4* it was reduced to about 6% of that in wild-type. Since we noted that enolase activity is highest in buds and flowers, we also measured floral tissues, which confirmed a strong reduction in *los2-3* and *los2-4* and a weaker reduction in *los2-2* (Figure 15d). Enolase activity in *los2-1* was also reduced, however to a smaller extent as compared to its wild-type background C24 (Figure 15e). Therefore, although some transcript is produced in *los2-4* it appears not to be functional. Both *los2-3* and *los2-4* severely compromise enolase activity, whereas *los2-1* and *los2-2* have smaller effects.

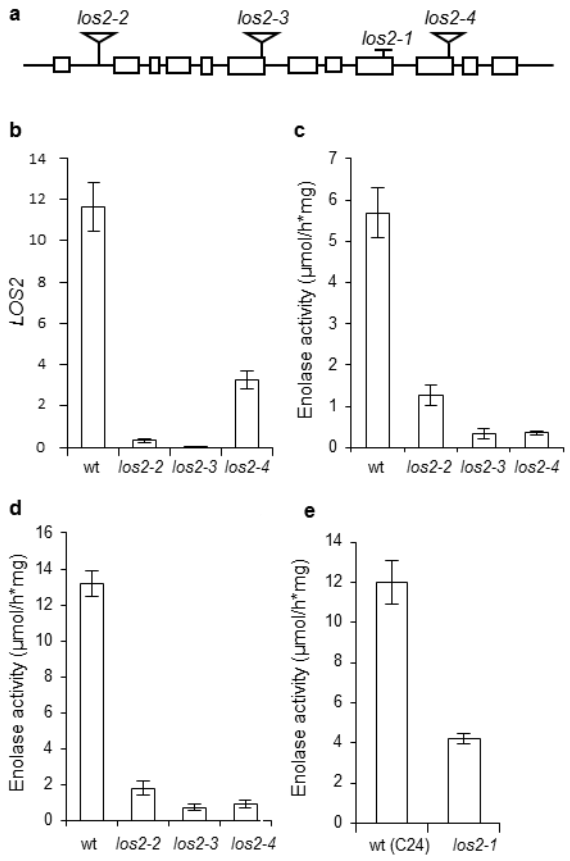


**Figure 15.** Characterization of *los2/eno2* knock-out alleles.
(**a**) Intron-exon organization of the *LOS2/ENO2* gene. The position of the T-DNA insertions of the identified *los2/eno2* mutant alleles and the position of the *los2-1* single point mutation, as previously given (Lee et al., 2002), is indicated.
(**b**) *LOS2/ENO2* transcript abundance in wild-type and *los2/eno2* knock-out lines as determined by qPCR. *LOS2/ENO2* expression was analyzed in 2-week-old seedlings of the indicated lines and is shown in comparison to wild-type. The error bars are the SD calculated from three independent biological repeats each measured in four technical repeats.
(**c**) Enolase activity in crude extracts of *los2/eno2* mutant plants. Enolase activity was measured in 2-week-old seedlings of the lines shown. Values with the mean SD calculated from three independent biological replicates are shown.
(**d**) Enolase activity in crude extracts of *los2/eno2* mutant plants. Enolase activity was measured in floral tissues of 6-week-old plants of the lines shown. Values with the mean SD calculated from three independent biological replicates are shown.
(**e**) Enolase activity in crude plant extracts in C24 and *los2-1* lines. Enolase activity was determined in floral tissues of 6-week-old plants. Values are the mean SD calculated from three independent biological replicates.

## 2.2 LOS2/ENO2 is essential for shoot and root growth

The identified *los2/eno2* mutant lines were subjected to a comprehensive, progressive phenotypic analysis over their life spans to investigate the function of LOS2/ENO2 in the different stages of plant development. Plants homozygous for *los2-2, los2-3* and *los2-4* did not produce seeds and thus segregating progeny from plants heterozygous for the insertions were used. Homozygous *los2/eno2* seeds germinated normally and in early seedling

development no morphological differences between the mutants and wild-type were obvious. However, starting between eight and ten days past germination pronounced developmental defects of *los2/eno2* mutant plants became apparent. Twelve-day-old seedlings of *los2-3* and *los2-4* had much shorter roots as well as smaller, pale green leaves with shorter petioles than wild-type (Figure 16a). *los2-2* exhibited very similar phenotypic features, however, they were less marked, correlating with an attenuated reduction of enolase activity as compared to the other two lines.

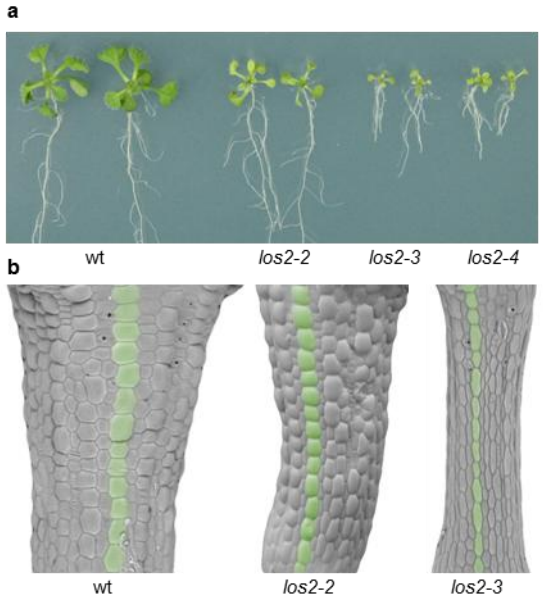


**Figure 16.** Characterization of *los2/eno2* knock-out alleles.
(**a**) Phenotypes of 12-day-old seedlings of wild-type and *los2/eno2* mutants grown on MS media in LD growth conditions.
(**b**) Scanning electron micrographs of 14-day-old hypocotyls of the indicated lines. One representative protruding cell file is marked in green.

The impaired growth of *los2/eno2* mutants indicated an essential function of LOS2/ENO2 in cell development. To address if cell shape or size may be altered, the epidermis of hypocotyls of *los2/eno2* mutant plants was analysed using scanning electron microscopy. Interestingly, while the length of epidermal hypocotyl cells appeared not to be changed, their width was reduced (Figure 16b). To quantify this observation we measured cell length and width and calculated the mean length:width ratio. The results showed that, whereas this ratio was approx. 1.2 in wild-type, it was significantly changed in the *los2/eno2* knock-out lines, with 1.57 in *los2-2* (n=50; p-value $7.18*10^{-5}$), and 2.64 in *los2-3* (n=50; p-value $8.56*10^{-17}$). Correspondingly, whereas *los2/eno2* hypocotyl length was not notably altered the diameter was clearly reduced (Figure 16b). Therefore, while primary elongation growth of *los2/eno2* hypocotyls was not defective, secondary radial growth was strongly compromised.

The growth defects induced by *LOS2/ENO2* mutation extended to adult development. Adult *los2-3* and *los2-4* plants were characterized by severe dwarfism. This adult phenotype was less pronounced in *los2-2* (Figure 17a), but also in this line was still clearly visible. *los2-3* and

*los2-4* plants were much smaller in size and showed early senescence. Their leaves were characterized by reduced size, epinastic growth and leaf reticulation and/or chlorosis (Figure 17a).

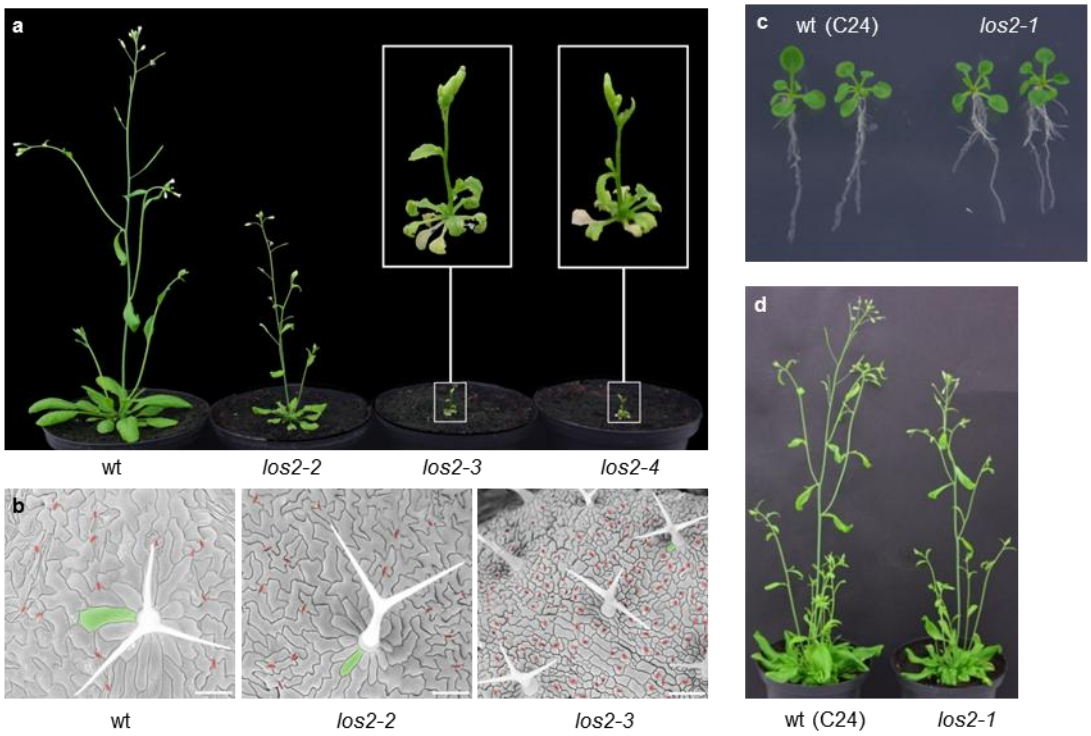


**Figure 17.** *los2/eno2* mutants are impaired in adult development.
(**a**) Adult phenotypes of *los2/eno2* knock-out mutants. Representative 5-week-old plants are shown.
(**b**) Scanning electron micrographs of the adaxial epidermis of leaves of the indicated lines. Fully expanded leaves of 4-week-old plants of wild-type, *los2-2* and *los2-3* are shown. A representative basal trichome cell is highlighted in green. Stomata are marked in red (scale bars = 100 µm).
(**c**) Phenotypes of 12-day-old wild-type and homozygous *los2-1* seedlings grown on agar plates.
(**d**) Adult phenotypes of 5-week-old wild-type and *los2-1* plants grown in LD growth conditions.

To investigate the cellular basis of the development defects we analysed the adaxial leaf epidermis of *los2/eno2* leaves using electron microscopy and compared the second pair of fully expanded true leaves of *los2-2* and *los2-3* with those of wild-type. Figure 17b shows that leaf epidermal pavement cells had a reduced size in *los2-2* mutant plants and were extremely small in *los2-3* plants. Moreover, the puzzle-like shape of pavement cells was lost in *los2-3*. Interestingly, the size of stomatal guard cells appeared unaffected (Figure 17b). The size of trichomes and their basal cells was also reduced (Figure 17b). *los2-1* plants did not show any constitutive developmental defects in early seedling development (Figure 17c), however exhibited slightly reduced growth in adult stages (Figure 17d), confirming previous reports

(Lee et al., 2002). Thus, in *los2/eno2* mutants reduced organ size correlates with reduced cell size providing evidence that LOS2/ENO2 is essential for cell expansion.

### 2.3 *los2-ko* mutants are impaired in reproductive development

*los2/eno2* mutation also effected floral morphogenesis. As shown in Figure 18a buds and flowers of *los2/eno2* knock-out plants were smaller and did not progress through flower development normally. In *los2-3* and *los2-4* petals were smaller and pale green and the buds did not fully open. In some buds both petals and sepals turned necrotic. When siliques did develop, they were smaller and did not contain seeds (Figure 18a); thus the plants were sterile.

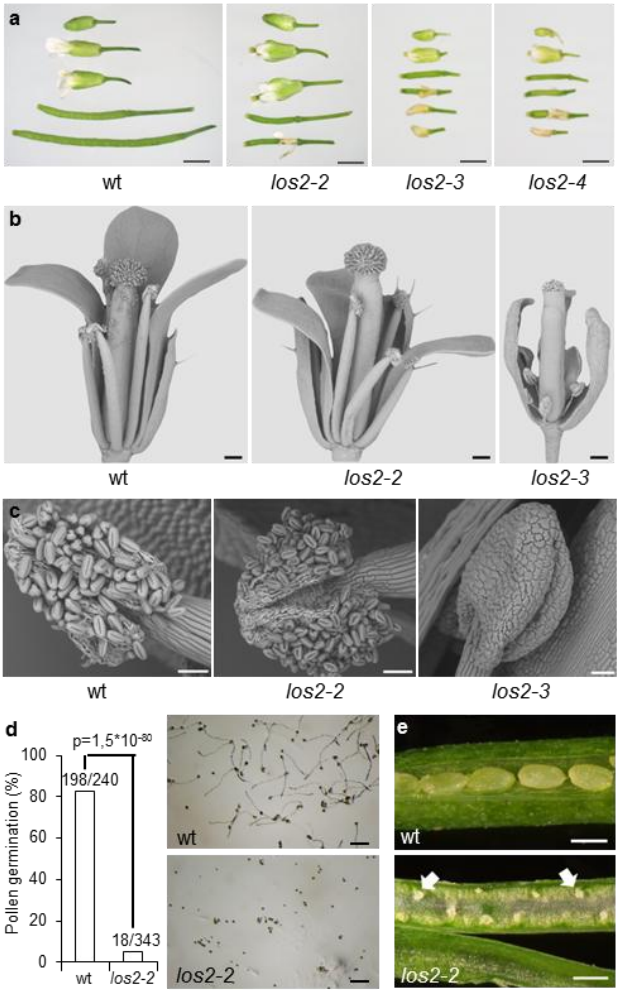


**Figure 18.** *los2/eno2* mutants are compromised in reproductive organ development and male gametophyte function.
(**a**) Flower and silique morphology of wild-type and *los2/eno2* knock-out plants. Siliques and flowers in comparable developmental stages for each line are shown (scale bar = 2 mm).
(**b**) Scanning electron micrographs of wild-type, *los2-2* and *los2-3* flowers. Representative flowers of 5-week-old plants in the same developmental stage are shown (scale bar = 0.2 mm).
(**c**) Scanning electron micrographs of wild-type, *los2-2* and *los2-3* anthers in the same developmental stage (scale bar = 400 µm).
(**d**) *In vitro* pollen germination and pollen tube growth. Percentage of germination was calculated from one hundred plated pollen grains, analyzed in four repeats. The p-value was calculated and is shown (scale bar = 200 µm).
(**e**) Opened siliques of wild-type and *los2-2* plants. Arrows indicate aborted embryos (bar = 500 µm).

To investigate the basis of the morphological defects of floral organs and for sterility in more detail electron microscopy was utilized. The imaging revealed that *los2-2* and *los2-3* flowers were strongly deformed and, most strikingly, that anther development was severely compromised. Anthers of *los2-3* remained in a stage of pre-anthesis being unable to initiate elongation (Figure 18b) and moreover did not produce pollen (Figure 18c). In addition, the

female organs were deformed, with in particular the stigma being strongly distorted (Figure 18b). In *los2-2* pollen grains were produced, however, their size was clearly reduced (Figure 18c).The ability of *los2-2* pollen to germinate *in vitro* was investigated. While around 80% of wild-type pollen grains germinated, only 5% of *los2-2* pollen germinated *in vitro* (Figure 18d). In those pollen that did germinate pollen tube length was markedly reduced as compared to pollen from wild-type plants (Figure 18d). Reduced pollen germination and impaired pollen tube elongation also correlated with abolished seed set in the weaker *los2-2* allele (Figure 18e). Thus, female and male organ development and male gametophyte function require LOS2/ENO2 and consequently defects in *LOS2/ENO2* lead to reduced fertility.

**2.4 LOS2/ENO2 is essential for vascular development and secondary growth of stems**

Although *los2-3* and *los2-4* knock-out lines were extremely dwarfed, they did develop inflorescence-like structures with very short stems that reached a final height of approx. 2 cm (Figure 17a). To investigate vascular development in *los2/eno2* stems cross-sections from the basal stem part (just above the soil line) of 6-week-old wild-type and *los2-3* plants were stained with toluidine blue O and analysed by light microscopy.

As shown in Figure 19a in the wild-type the typical tissue pattern of secondary stems was developed, characterized by concentric layers of epidermis, cortex and endodermis, followed by a vascular system laid out close to the stem periphery, and a parenchymatous pith prominently present in the centre (Sanchez et al., 2012). Cells of the outmost tissue layers were undifferentiated, the xylem was disconnected and showed severely reduced lignification; moreover it was composed mainly of large tracheids, lacking interspersed parenchyma cells. The vascular bundles were oriented closer to the centre than in wild-type and showed defects in the ordered development of radial cell files that differentiate into xylem and phloem. In wild-type these ordered cell division and differentiation events arise from procambial-cell activity (Sanchez et al., 2012); in line, also the development and differentiation of cambial cells was impaired in *los2-3* (Figure 19b).

In addition to a distorted vascular structure, *los2-3* plants were compromised in the development of pith parenchyma cells in the stem centre, which had a reduced size, showed an irregular shape and appeared collapsed. Moreover, *los2-3* also produced irregular, collapsed xylem elements (Figure 19c), which are indicative of secondary cell wall defects (Brown et al., 2005). *los2-2* showed similar defects (Figure 19d), however they were less marked, correlating with a higher over-all enolase activity in *los2-2* plants. In summary, there

is evidence that LOS2/ENO2 is required for oriented cell division and differentiation events in vascular tissues, for the structural integrity of cell walls and for secondary growth of stems.

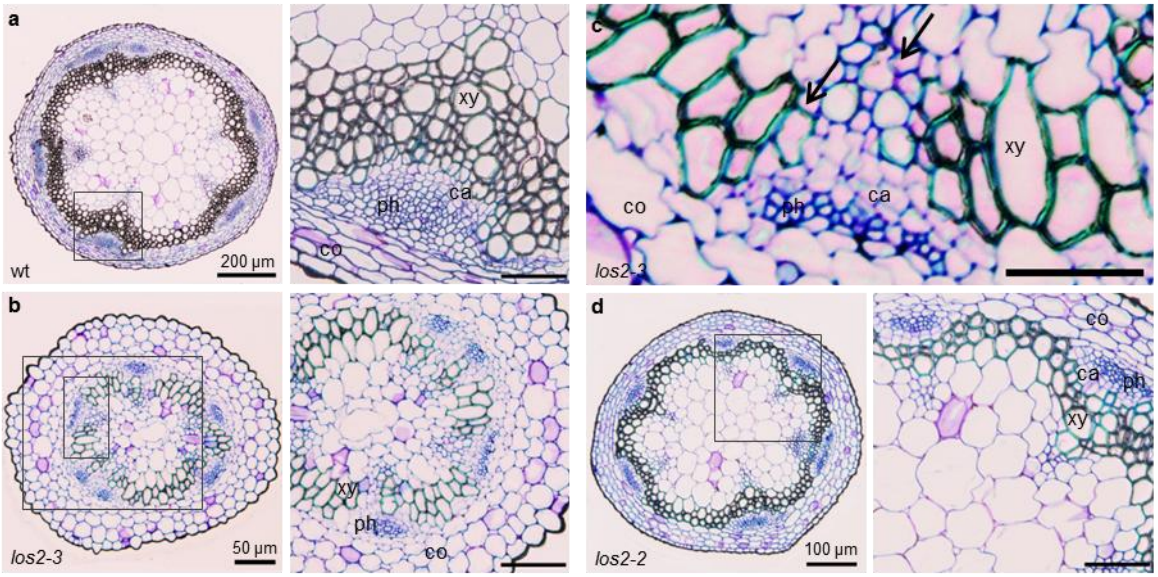


**Figure 19.** *los2/eno2* mutants are defective in vascular tissue organization.
(**a**) Representative transverse section of an inflorescence stem of a 6-week-old wild-type plant stained with Toluidine Blue. *Left*: whole stem section. *Right*: magnified view of the boxed area on the left, to display a close-up of a vascular bundle (scale bar = 50 µm). ca, cambium; co, cortex; ph, phloem; xy, xylem.
(**b**) Representative transverse section of an inflorescence stem of a 6-week-old *los2-3* plant stained with Toluidine Blue. *Left*: whole stem section. *Right*: magnified view of the boxed area on the left (scale bar = 50 µm).
(**c**) Magnified view of the area marked with the smaller square in **b**, highlighting a *los2-3* vascular bundle. Arrows indicate collapsed xylem cells (scale bar = 25 µm).
(**d**) Representative transverse section of an inflorescence stem of a 6-week-old *los2-2* plant stained with Toluidine Blue. *Left*: whole stem section. *Right*: magnified view of the boxed area on the left, to display a close-up of a vascular bundle (scale bar = 50 µm).

### 2.5 LOS2/ENO2 impacts on the synthesis of lignin, sinapoyl malate and salicylic acid

The histological analysis of *los2-2* and *los2-3* stems provided evidence that *los2/eno2* mutants contained decreased amounts of the cell wall polymer lignin. Since enolase activity is required for PEP synthesis (Plaxton, 1996), which, via the shikimate pathway, is converted to chorismate, a direct precursor of phenylalanine (Vermerris and Nicholson, 2006), phenylpropanoid synthesis could be affected in *los2/eno2* mutants.

To test this hypothesis the amount of lignin in inflorescence stems of wild-type and *los2/eno2* knock-out plants was analysed. As shown in Figure 20a the lignin content was decreased by approx. 40% in *los2-2* and approx. 65% in *los2-3* and *los2-4*. Secondly, the abundance of sinapoyl malate was assessed, which is a member of another class of important phenylpropanoids, the sinapoyl esters (Vermerris and Nicholson, 2006). Sinapoyl malate

contents were significantly decreased in 3-week-old plants of *los2/eno2* knock-out lines (Figure 20b). The *los2-1* mutant, like *los2-2*, exhibited smaller changes in lignin abundance (Figure 20c), but no changes in sinapoyl malate contents (Figure 20d). Therefore, major developmental defects in those *los2/eno2* mutants that are strongly impaired in enolase activity, correlate with impaired production of the phenylpropanoids lignin and sinapoyl malate.

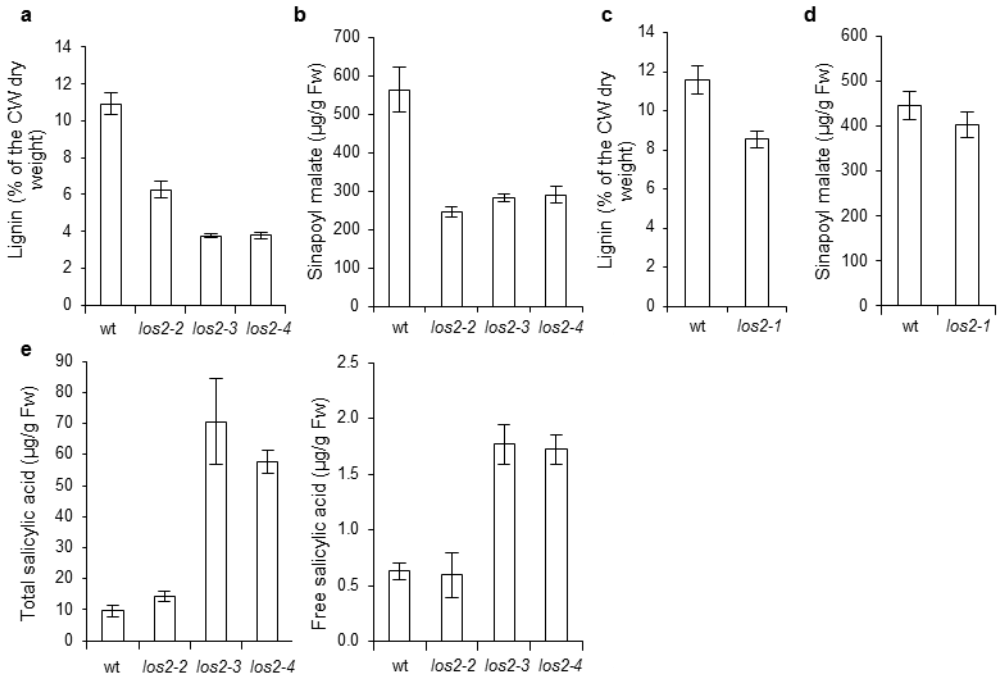


**Figure 20.** LOS2/ENO2 impacts on the secondary metabolite composition of plants.
(**a**) Lignin contents in the primary inflorescence stems of 6-week-old wild-type and *los2/eno2* mutant plants. Values are given as percentage of dry cell wall material.
(**b**) Sinapoyl malate contents in leaves of 3-week-old wild-type and *los2/eno2* knock-outs.
(**c**) Lignin contents in the primary inflorescence stems of 6-week-old wild-type and *los2-1* plants. Values are given as percentage of dry cell wall material.
(**d**) Sinapoyl malate contents in leaves of 3-week-old wild-type and *los2-1* plants grown in LD growth conditions.
(**e**) Total and free salicylic acid levels in leaves of 3-week-old wild-type and *los2/eno2* knock-out mutant plants. The SD of three biological replicates is shown.

Another important product of chorismate is the plant hormone salicylic acid (SA; Vermerris and Nicholson, 2006). To investigate if SA levels were altered in *los2/eno2* mutants, free and total SA levels were measured using HPLC. Whereas SA concentrations were not affected in *los2-2*, they were strongly increased in *los2-3* and *los2-4* plants (Figure 20e). Hence, LOS2/ENO2 plays a complex role in impacting on chorismate-dependent biosynthetic pathways acting both as a promoter of lignin and sinapoyl malate production and as a repressor of SA biosynthesis.

## 2.6 LOS2/ENO2 affects fatty acid composition and soluble sugar contents

A different class of essential building blocks that are formed from PEP is fatty acids (Rawsthorne, 2002). We analysed fatty acid composition in *los2* knockout lines. While we did not observe significant differences in the contents of saturated fatty acids 16:0 (palmitic acid) and 18:0 (stearic acid), unsaturated fatty acids 16:1, 16:3 and 18:1 were significantly reduced and 18:3 was significantly increased in the strong *los2-3* and *los2-4* alleles (Figure 21a).

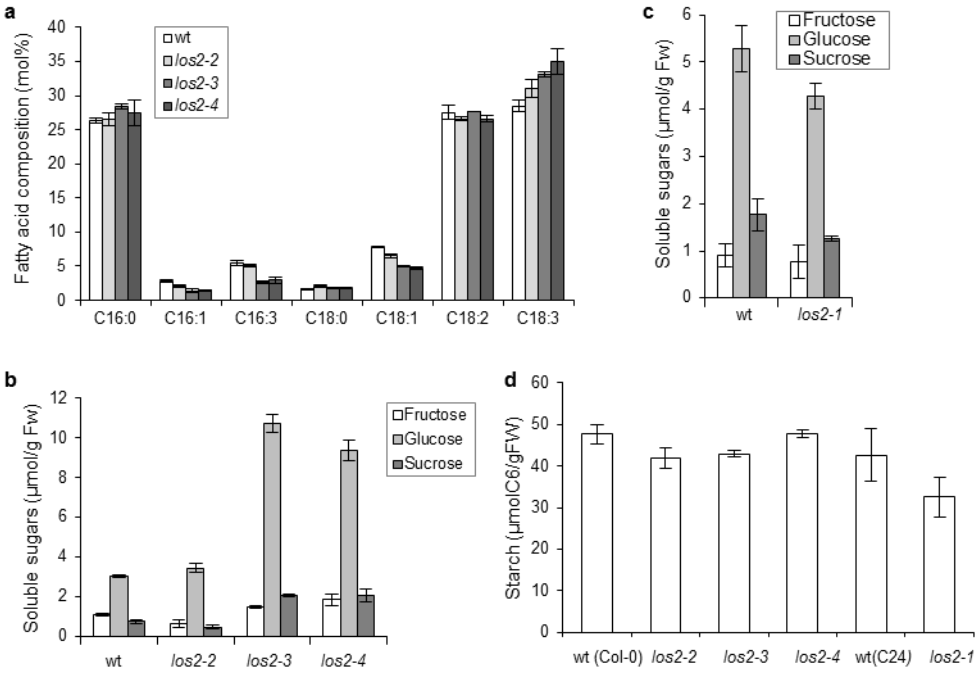


**Figure 21.** LOS2/ENO2 impacts on the fatty acid and soluble sugar contents.
(**a**) Fatty acid composition of leaves of 3-week-old wild-type and *los2* knock-out plants. The SD of three biological replicates is shown.
(**b**) Soluble sugar content in leaves of 3-week-old wild-type and *los2/eno2* knock-out lines grown in soil. Results are shown as the mean ± SD of four biological replicates.
(**c**) Soluble sugar content in leaves of 3-week-old wild-type and *los2-1* grown in LD growth conditions. Results are shown as the mean ± SD of four biological replicates.
(**d**) Starch content in leaves of 3-week-old *los2/eno2* knock-out lines. Plants were grown in LD conditions. Results are shown as the mean ± SD of four biological replicates (each was a pool of five plants).

With a proposed role of *LOS2* in cold signalling (Lee et al., 2002) it was possible that, in addition to fatty acid composition, mutations in *LOS2/ENO2* also alter soluble sugar contents, since both fatty acid (Uemura et al., 1995) and soluble sugar contents impact on cold resistance of plants (Gilmour et al., 2000). Therefore, carbohydrate levels in the leaves of *los2/eno2* plants were examined. In a weak allele *los2-2* there was no significant change in fructose, glucose and sucrose level, however lines with a stronger phenotype *los2-3* and *los2-4* exhibited slight increase in the level of fructose and more pronounced elevation in glucose

and sucrose levels (Figure 21b). The *los2-1* line exhibited only minor changes in contents of soluble sugars and starch (Figure 21c). There was no significant change in the starch content of *los2* knockout lines observed (Figure 21d).

### 2.7 AtMBP1 represses *LOS2/ENO2* promoter activity

Recently it was shown that over-expression of YFP fusions of full-length *LOS2*/*ENO2* and of the shorter *AtMBP1* ORF induces constitutive developmental defects, which bear a striking resemblance to *los2/eno2* knock-out plants: they develop normally as seedlings, but are compromised in adult development resulting in dwarf plants with reduced fertility (Kang et al., 2013). Thus, it was possible that *AtMBP1* over-expression may result in repression of ENO2 enolase activity. To test this hypothesis, enolase activity in leaves and floral tissues of *35S:LOS2-YFP* and *35S:AtMBP1-YFP* plants was assessed. The result demonstrated that in both lines enolase activity was strongly decreased (Figure 22a,b).

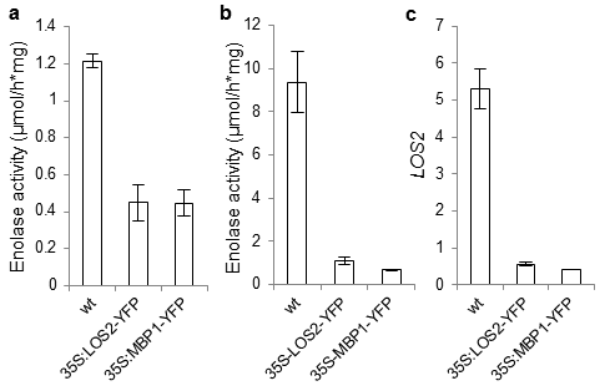


**Figure 22.** AtMBP1 represses *LOS2/ENO2* promoter activity.
(**a**) Enolase activity in crude extracts of *35S:LOS2-YFP* and *35S:MBP1-YFP* mutant plants. Enolase activity was measured in leaves of 5-week-old plants of the lines shown. Values with the mean SD calculated from three independent biological replicates are shown.
(**b**) Enolase activity in crude extracts of *35S:LOS2-YFP* and *35S:MBP1-YFP* mutant plants as compared to wild-type. Enolase activity was measured in floral tissues of 5-week-old plants of the lines shown. Values with the mean SD calculated from three independent biological replicates are shown.
(**c**) *LOS2/ENO2* transcript abundance in wild-type and *35S:LOS2-YFP* and *35S:MBP1-YFP* lines as determined by qPCR. *LOS2/ENO2* expression was analyzed in leaves of 5-week-old plants of the indicated lines and is shown in comparison to wild-type. The error bars are the SD calculated from three independent biological repeats each measured in four technical repeats.

Since human MBP1 is known to act as a transcriptional repressor that also controls its own promoter activity (Feo et al., 2000) we investigated if in *AtMBP1* over-expressing lines *LOS2/ENO2* promoter activity may be repressed. For this purpose qPCR analysis was performed utilizing primers that span the 3' UTR region of the gene to specifically detect endogenous *LOS2/ENO2* mRNA abundance (and not measure *LOS2/AtMBP1* transgene abundance, which is increased in these lines; Kang et al., 2013). The result showed that

transcription of endogenous *LOS2/ENO2* was strongly impaired in *LOS2/ENO2* and *AtMBP1* over-expressing plants (Figure 22c).

Therefore, there is evidence that AtMBP1 negatively regulates *LOS2/ENO2* promoter activity. The fact that over-expression of LOS2-YFP does not complement for the reduction in endogenous LOS2/ENO2 activity indicates that the YFP-tag may impair ENO2 function whereas it does not significantly impact on AtMBP1 activity.

### 2.8 Expression of a LOS2-NES fusion protein partially restores enolase activity in *los2-4* and recovers growth defects

Since reduced enolase activity clearly correlates with the growth defects of different *los2/eno2* knock-out as well as LOS2 and AtMBP1 over-expressing plants there was evidence that the constitutive developmental defects are a consequence of impaired ENO2 function. However, given the fact that the mutated locus also contains a transcription factor, it was important to test if the effects seen in the knock-outs are not caused by a loss of AtMBP1 function. For this purpose LOS2 was fused to a nuclear export signal (NES; LALKLAGLDI, Wen et al., 1995) and to YFP and its localization was assessed in protoplasts.

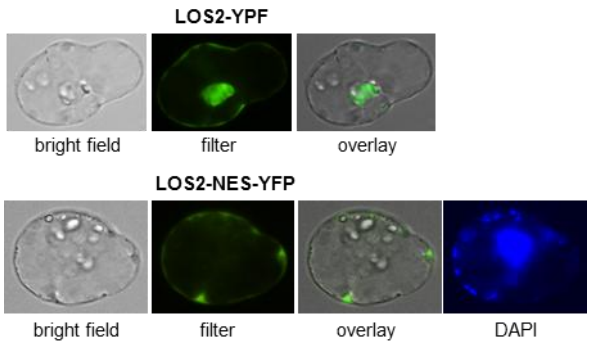


**Figure 23.** Expression of a *LOS2-NES-YFP* construct in protoplasts and *los2-4* plants.
LOS2-YFP and LOS2-NES-YFP were transiently expressed in arabidopsis protoplasts and their localisation was analysed using fluorescence microscopy. DAPI staining was used to show the nucleus.

While LOS2-YFP is present in the nucleus and in the cytoplasm (Lee et al., 2002; Figure 23), the NES interfered with the nuclear localization of the fusion protein (Figure 23). The LOS2-NES construct (without the YFP tag) was then introduced into *los2-4* under *LOS2* promoter control and homozygous lines expressing the transgene were selected (Figure 24a; please note that the primers chosen do not detect endogenous *LOS2/ENO2* mRNA in the *los2-4* lines since one primer spans the region deleted in *los2-4*). To investigate if restricting nuclear localization of LOS2/ENO2 also impaired AtMBP1 transcriptional activity, *ZAT10* expression in wild-type, *los2-4* and the complementation lines was compared. *ZAT10* is negatively regulated by AtMBP1 and thus its expression is enhanced in *los2-1* (Lee et al., 2002). In

agreement *ZAT10* expression in *los2-4* was elevated as compared to wild-type. This effect was not released in the complementation lines (Figure 24b) confirming that AtMBP1 activity is not restored by the transgene.

Enolase activity in the complementation lines was re-established to some extent (Figure 24c), however, not to wild-type levels, which correlated with the only partially restored *LOS2/ENO2* expression levels (Figure 24a). Nevertheless, this partial recovery of ENO2 enolase function in *los2-4* was sufficient to recover growth defects of *los2-4*. Lines with a higher enolase activity (36 and 11) were significantly larger in the adult stage, with larger leaves and also reduced growth repression of the inflorescences (Figure 24d), whereas in line 1, in which enolase activity was only weakly restored, the phenotypes were still more pronounced. Thus, the rescue of phenotypes correlated with the recovery of enolase activity providing further evidence that a loss of enolase function in *los2/eno2* knock-out lines accounts for major aspects of the developmental defects revealed.

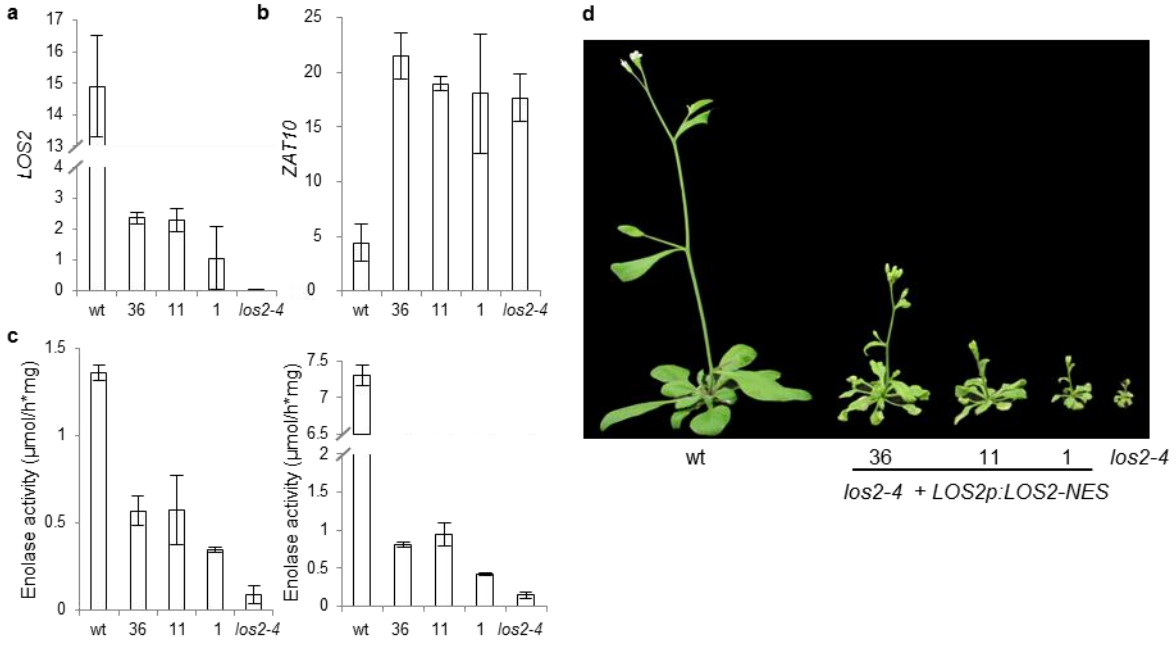


**Figure 24.** Growth repression of *los2-4* is partially complemented by LOS2-NES expression.
(**a**) *LOS2/ENO2* transgene abundance in *LOS2p:LOS2-NES* expressing *los2-4* plants as determined by qPCR. *LOS2/ENO2* expression was analyzed in leaves of 5-week-old plants of the indicated lines. The error bars are the SD calculated from three independent biological repeats each measured in four technical repeats.
(**b**) *ZAT10* transcript level in the lines shown as determined by qPCR. *ZAT10* expression was analyzed in leaves of 5-week-old plants of the indicated lines.
(**c**) Enolase activity in crude extracts of *LOS2p:LOS2-NES* expressing *los2-4* plants as compared to *los2-4* and wild-type. Enolase activity was measured in leaves (left) and floral tissues (right) of 5-week-old plants of the lines shown. Values with the mean SD calculated from three independent biological replicates are shown.
(**d**) Adult phenotypes of wild-type, *LOS2p:LOS2-NES* expressing *los2-4* plants and *los2-4* lines. Representative, 4-week-old plants are shown.

# IV. Discussion

## 1. BRs promote basal and acquired freezing tolerance of plants

This study aimed to investigate a function of the plant hormones BRs in the freezing tolerance of plants. My results demonstrate that BRs promote constitutive and acquired freezing tolerance in arabidopsis. Plants deficient in BR signalling or production were hypersensitive to freezing with and without cold-acclimation, whereas plants with enhanced BR signalling showed a slight increase in their ability to tolerate freezing stress.

Plants with defects in BR activity had reduced steady-state transcript levels of *CBFs* as well as CBF target genes. Furthermore, induction of *CBF*s and CBF-regulated genes was attenuated in BR-signalling mutant lines following cold temperature treatment. On the contrary, in a line with enhanced BR signalling levels of *CBF* transcripts were elevated under untreated conditions and after cold treatment.

It has previously been shown that application of epiBL can enhance expression of *CBF* family members in arabidopsis under low temperature treatment (Kagale et al., 2006). Here I show that epiBL induces the expression of all members of the *CBF* family as well as members of CBF-regulon under normal conditions. In agreement, inhibiting BR biosynthesis with brassinazole suppresses expression of *CBF3* and *COR15A* in arabidopsis. Therefore, there is evidence that BRs promote basal and acquired freezing tolerance by modulating the expression of the *CBFs*.

These data indicate that BR-induced adaptive response to cold is at least partially mediated by CBF cold-responsive pathway. It is however likely that also additional non-CBF regulated cold-responsive signalling pathways contribute to the BR-induced freezing tolerance of plants and this warrants future investigation. Similarly, ET and JA signalling pathways have been shown to impact on freezing tolerance of plants by affecting the activity of CBFs, as well as non-CBF transcriptome.

## 2. CES and homologues promote freezing tolerance of plants

In this study we demonstrated that the BR-regulated transcription factor CES is a positive regulator of the basal and acquired freezing tolerance in arabidopsis. Plants overexpressing CES had a dramatically increased ability to tolerate freezing temperatures with or without cold acclimation, whereas knockout lines of CES and homologues were hypersensitive to freezing. These phenotypes correlated with elevated transcript levels of *CBFs* and CBF target genes in *ces-D* and repressed levels of transcript levels of *CBFs* and CBF regulon genes in the knockout lines under normal conditions and following low temperature treatment. I show that

*CBF1* may be the primary CES target among all *CBFs*, since CES most strongly impacts on *CBF1* expression and is most strongly enriched on the *CBF1* promoter.

It was reported previously that overexpression of *CBF1* results in stunted growth and delayed flowering (Gilmour et al., 2004). This was shown to be mediated through a DELLA-dependent mechanism. Overexpression of *CBF1* led to accumulation of DELLA proteins possibly by a decrease in levels of bioactive GAs (Achard et al., 2008). Interestingly, in *ces-D* constitutive up-regulation of *CBF1* led to significantly increased freezing tolerance, but did not result in a dwarf phenotype. This result indicates that the cold-induced *CBF1*-mediated mechanism for DELLA-regulated growth inhibition may not function in *ces-D* plants. Regulation of GA biosynthesis by BRs may be a possible reason for this observation. BRs induce GA biosynthesis (Unterholzner et al., 2015) to stimulate degradation of the DELLA transcriptional repressors, and in *ces-D* BR biosynthesis is promoted (Poppenberger et al., 2011), which may interfere with effects imposed by CBF1 on GA biosynthesis.

Overexpression of *CBFs* enhances freezing tolerance of plants (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Gilmour et al., 2000; Vogel et al., 2005) and is considered to be one of the possible strategies to improve plants' cold tolerance. However, stunted growth and delayed flowering imposed by *CBF* overexpression are undesirable agronomic traits. Therefore, it may be important to uncover molecular mechanisms which prevent the *CBF1*-mediated DELLA-regulated growth inhibition in *ces-D*. Ultimately it may provide knowledge and means for generating plants exhibiting enhanced cold tolerance mediated by *CBF* overexpression, but being still able to maintain their full agronomical potential.

## 3. BR-induced expression of *CBF1* and *CBF3* is controlled by CES

Previously it was shown that BRs promote cold tolerance in different plant species (Kagale et al., 2006; Kim et al., 2009; Xia et al., 2009), however the molecular control of this process had remained unclear. This study provides evidence that BR-induced alterations in steady-state levels of *CBFs* require activity of CES and its' homologs, the bHLH transcription factors BEEs. When introduced into the weak BR signalling-deficient line *bri1-5 bak1-1D*, *ces-D* could partially rescue its freezing-sensitivity. Moreover, after application of epiBL in wild-type plants transcripts of *CBF1* and *CBF3* were elevated, whereas in *ces bee* knock-out plants transcript levels of *CBF1* and *CBF3* remained unchanged. However, induction of *COR15A* expression after epiBL treatment in the *tM* line was only partially suppressed, which may indicate that additional players are involved in the control of *COR* gene expression.

Therefore, there is evidence that BRs promote freezing tolerance by modulating the activity of CES and homologues.

## 4. Posttranslational modifications of CES affect freezing tolerance of plants

Recently it was shown that CES undergoes posttranslational modifications such as SUMOylation and phosphorylation, which regulate CES subnuclear localisation, transcriptional activity and protein turnover (Khan et al., 2014). It was reported previously that low temperature treatment induced accumulation of SUMO-conjugated proteins in arabidopsis (Miura et al., 2007) and that posttranslational modifications play a significant role in the regulation of cold-responsive transcriptional activators (Miura et al., 2007; Miura et al., 2011). Mutants defective in SUMOylation showed reduced *COR* gene expression and freezing tolerance (Miura et al., 2007).

ICE1, a regulator of *CBF3* transcription, is phosphorylated and SUMOylated in response to cold, which stabilises ICE1 protein and enhances its transcriptional activity. Similar posttranslational modifications occur in CES, when BR signalling inhibits CES phosphorylation and promotes SUMOylation, which may facilitate CES activity on *CBF* promoters. In support, plants of *S75A+S77A* line, overexpressing constitutively SUMOylated CES, displayed enhanced tolerance to freezing and elevated transcript levels of *COR15A*, suggesting that SUMOylation may promote CES activity in regulation of *COR* gene expression.

Interestingly, there is evidence that BR signalling inhibits CES activity in BR biosynthesis (Khan et al., 2014). This study shows that expression of key BR biosynthetic enzymes *CPD, DWF4* and *Br6ox2* is repressed under cold treatment, and this result is in line with previously published microarray data which indicated that transcript levels of *CPD* were reduced after cold treatment in arabidopsis (Lee et al., 2005) and mung-bean (Huang et al., 2006). Therefore, it is possible that in response to cold CES promotes *CBFs* expression, as well as represses BR biosynthesis and this will have to be investigated further.

## 5. LOS2/ENO2 activity is essential for the development of plants

The results presented above provided evidence that BRs regulate cold stress responses, at least partially, in a CBF-dependent manner. However, a yeast-two-hybrid screen for the CES interacting proteins identified LOS2/ENO2 (Poppenberger lab, unpublished results), which was previously shown to regulate *COR* gene expression in CBF-independent manner (Lee et al., 2002). Therefore, it is possible that BRs may also impact on CBF-independent cold responses in arabidopsis by modulating LOS2 activity.

Addressing this hypothesis is a complicated task, since the *LOS2/ENO2* locus encodes not only the transcription factor MBP1, but also an enolase ENO2, which is essential for cytosolic PEP provision (Andriotis et al., 2010; this study). PEP serves as a precursor for many secondary metabolites, such as fatty acids and soluble sugars, which are essential for the development of freezing tolerance in plants. Therefore, in order to further study the role of the LOS2/ENO2 locus in cold response, it was important to characterise the role of the enolase ENO2 in plant growth and development, to be able to dissect the effects imposed by transcriptional activity of MBP1 from those caused by ENO2.

This study showed that compromised function of ENO2 leads to multiple growth defects in plants. Three independent *los2/eno2* knock-out lines were identified, in which enolase activity was decreased to different extents. The degree of phenotypes correlated with a reduction in enolase activity, with milder growth defects in *los2-2* and severe growth retardation in *los2-3* and *los2-4*. The developmental defects of *los2/eno2* knock-out plants were partially rescued by expression of a LOS2/ENO2 version compromised in the transcription factor activity, providing evidence that major developmental defects of the knock-out lines were caused by a lack of enolase activity.

Moreover, overexpression of MBP1 in *35S:MBP1-YFP* and *35S:LOS2-YFP* lines caused a strong reduction in enolase activity in these lines and led to phenotypes resembling those of *los2/eno2* knock-out lines, confirming the observation that the growth defects of the mutant lines were due to compromised enolase function, but not due to the altered transcription factor activity of the *LOS2/ENO2* locus. Interestingly, over-expression of LOS2-YFP did not complement for the reduction in endogenous LOS2/ENO2 activity which indicates that the YFP-tag may impair ENO2 function whereas it does not impact on AtMBP1 activity.

In agreement with a function of the enolase in providing essential metabolites in plants, I show that plants deficient in ENO2 activity display reduced levels of phenylpropaniod compounds lignin and synapoyl malate, as well as altered levels of fatty acids, sugars and salicylic acid.

This study provides evidence that AtMBP1 represses *LOS2/ENO2* promoter activity, in analogy to human MBP1 which represses activity of cMyc (Feo et al., 2000; Hsu et al., 2008) that directly controls expression of α-enolase *ENO1* as well as that of other glycolytic enzymes (Osthus et al., 2000). It is to be revealed weather a repressive action of MBP1 on its own promoter serves to control activity of ENO2 in glycolysis or in the regulation of *COR* gene expression in response to cold.

LOS2/ENO2 was characterised as a transcription factor essential for the cold and ABA responses in arabidopsis. The results of this study suggest that the function of LOS2/ENO2 as a glycolytic enzyme in arabidopsis is vital for the growth of plants. To determine function of *LOS2/ENO2* locus in cold stress responses more precisely, plant lines exhibiting wild-type levels of enolase activity but lacking MBP1 transcriptional activity should be used in the freezing tolerance experiments. This may allow to correlate freezing tolerance phenotypes of *mbp1* plants with altered *COR* gene transcripts and to exclude a possibility that altered soluble sugar and fatty acid composition caused by compromised enolase function may impact on the freezing tolerance phenotypes of the *los2/eno2* plants.

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## List of publications

*Research papers:*

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**Eremina, M**, Rozhon, W, Yang, S, and Poppenberger, B. (2015) ENO2 activity is required for the development and reproductive success of plants, and is feedback-repressed by AtMBP-1. *Plant J.* 81(6):895-906.

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**Eremina**, **M**, Rozhon, W, and Poppenberger, B. (2016) The hormonal control of cold stress responses in plants. *Cell Mol Life Sci.* 73:797-810.

# Appendix

**Eremina M**, Rozhon W, Yang S and Poppenberger B (2015) ENO2 activity is required for the development and reproductive success of plants, and is feedback-repressed by AtMBP-1. *Plant J.* 81(6):895-906.

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# ENO2 activity is required for the development and reproductive success of plants, and is feedback-repressed by AtMBP-1

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## SUMMARY

**Enolases are key glycolytic enzymes that are highly conserved in prokaryotic and eukaryotic organisms, and are among the most abundant cytosolic proteins. In this study we provide evidence that activity of the enolase ENO2 is essential for the growth and development of plants. We show that Arabidopsis plants with compromised ENO2 function, which were generated by mutating the *LOS2/ENO2* locus, have severe cellular defects, including reduced cell size and defective cell differentiation with restricted lignification. At the tissue and organ level *LOS2/ENO2*-deficient plants are characterized by the reduced growth of shoots and roots, altered vascular development and defective secondary growth of stems, impaired floral organogenesis and defective male gametophyte function, resulting in embryo lethality as well as delayed senescence. These phenotypes correlate with reduced lignin and increased salicylic acid contents as well as altered fatty acid and soluble sugar composition. In addition to an enolase the *LOS2/ENO2* locus encodes the transcription factor AtMBP-1, and here we reveal that this bifunctionality serves to maintain the homeostasis of ENO2 activity. In summary, we show that in plants enolase function is required for the formation of chorismate-dependent secondary metabolites, and that this activity is feedback-inhibited by AtMBP-1 to enable the normal development and reproductive success of plants.**

**Keywords: LOS2, glycolysis, lignification, secondary growth, Arabidopsis, bifunctional.**

## INTRODUCTION

Enolases are metalloenzymes that, as part of the glycolytic pathway, catalyse the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate (PEP; Canback *et al.*, 2002), which is required for ATP production, and in addition serves as a precursor for the synthesis of aromatic compounds and secondary metabolites (Vermerris and Nicholson, 2006). Whereas in other eukaryotic organisms, such as mammals, enolase function in development is well defined (Díaz-Ramos *et al.*, 2012), the significance of enolase activity in the different stages of a plant's life cycle has not yet been elucidated.

Three enolase isozymes are present in *Arabidopsis thaliana* (Arabidopsis), ENO1, ENO2 and ENO3/ENOc, which share 56–68% similarity at the amino acid level; however, ENO3/ENOc does not exhibit enolase activity (Andriotis *et al.*, 2010). ENO1 is localized in plastids, is not expressed in photosynthetic tissues, and was shown to be required for trichome and root hair development (Prabhakar *et al.*, 2009; Andriotis *et al.*, 2010).

ENO2 is encoded by the *LOS2* (LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 2)*/ENO2* locus, is highly expressed throughout plant development, and is on average 10-fold more abundant than *ENO1* and *ENO3* in all tissues and organs (Andriotis *et al.*, 2010). Interestingly, this gene encodes two proteins. In addition to full-length ENO2, a truncated version, AtMBP-1 (*A. thaliana* cMyc binding protein), is also alternatively translated from a second start codon (Kang *et al.*, 2013). *LOS2*/*ENO2* shares homology with human α-enolase *ENO1*, a bifunctional locus of which the alternatively translated MBP-1 acts as a repressor of *cMyc* transcription (Feo *et al.*, 2000). Arabidopsis AtMBP-1 is proposed to act as a transcription factor in abscisic acid signalling (Kang *et al.*, 2013). Moreover, the *LOS2* locus was suggested to be required for abiotic

stress responses, as a single point mutation in the 5′ region of the gene, *los2-1*, impaired cold-responsive gene expression, resulted in hypersensitivity to freezing stress (Lee *et al.*, 2002) and enhanced salt sensitivity (Barkla *et al.*, 2009).

Whereas the function of *LOS2* in stress-responsive gene expression was addressed in a number of independent studies (Lee *et al.*, 2002; Barkla *et al.*, 2009; Kang *et al.*, 2013), the biological relevance of ENO2 as an enzyme of the glycolytic pathway is unknown. In this work we show that plants deficient in *LOS2/ENO2* expression are strongly compromised in overall enolase activity. Such plants exhibit severe developmental defects, including reduced shoot and root growth, with defective vascular development and secondary growth, impaired floral organogenesis, and male gametophyte function resulting in sterility, as well as early senescence. These phenotypes correlate with reduced lignin and sinapoyl malate contents, increased salicylic acid levels, and altered fatty acid and soluble sugar composition, and can be partially restored by the expression of a LOS2/ENO2 version that lacks the transcription factor bifunctionality, providing evidence that ENO2 deficiency results in growth defects. In addition, we reveal that *LOS2/ENO2* promoter activity is repressed by AtMBP-1, and present a model in which the alternative translation of AtMBP-1 serves to repress the transcription of *ENO2*, thereby governing ENO2 glycolytic function.

## RESULTS

### Identification of *los2/eno2* knock-out lines

To characterize the function of LOS2/ENO2 in plant growth and development, a reverse-genetics approach was chosen. Three independent mutant lines, predicted to harbour insertions in *LOS2/ENO2* in the Columbia-0 (Col-0) wild-type background, were identified in the SALK and SAIL T-DNA insertional mutant collections, and were obtained from Nottingham Arabidopsis Stock Centre (NASC). The location of the insertions was determined by PCR and is shown in Figure 1(a). The alleles were numbered according to the position of their T-DNAs, with *los2-2* (SALK_021737) mutated in intron 1 (insertion downstream of nucleotide 320, with a 12-bp deletion), *los2-3* (SALK_077784) mutated in exon 6 (insertion downstream of nucleotide 1350) and *los2-4* (SAIL_208_B09) mutated in exon 10 (insertion downstream of nucleotide 2376, with a 28-bp deletion). For comparison, the *los2-1* mutation (G325S), which is a single point mutation that lies in exon 9 (Lee *et al.*, 2002), is shown.

Quantitative real-time PCR (qPCR) analysis using primers that bind to the 3′ region of the mRNA confirmed that *LOS2/ENO2* transcript abundance was severely reduced in plants homozygous for *los2-2* and *los2-3*, and was also decreased in the *los2-4* mutant, albeit to a lower extent

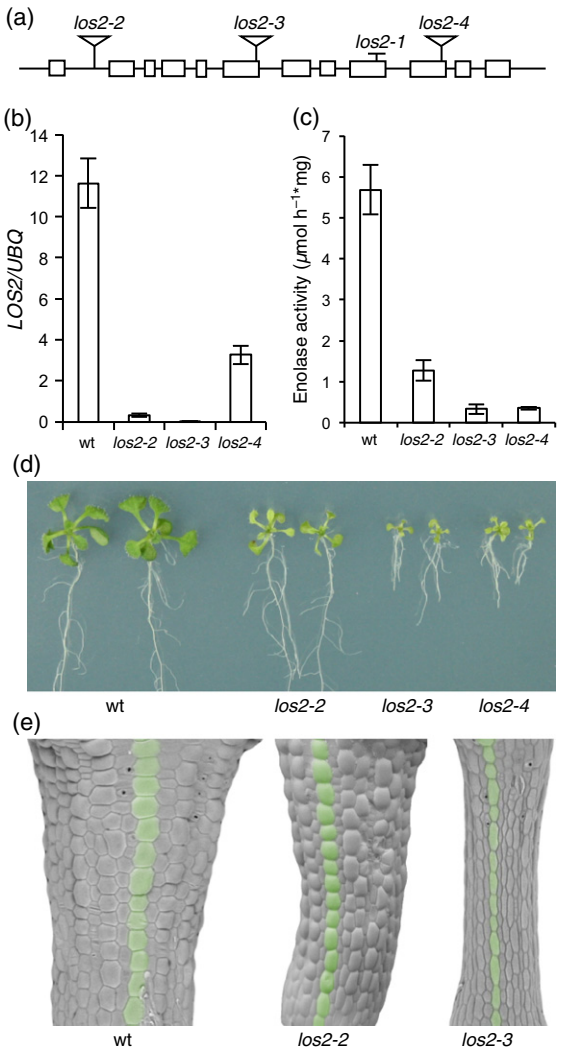


**Figure 1.** Characterization of *los2/eno2* knock-out alleles.
(a) Intron–exon organization of the *LOS2/ENO2* gene. The position of the T-DNA insertions of the identified *los2/eno2* mutant alleles and the position of the *los2-1* single point mutation, as previously given (Lee *et al.*, 2002), is indicated.
(b) *LOS2/ENO2* transcript abundance in wild type and *los2/eno2* knock-out lines, as determined by qPCR. LOS2/ENO2 expression was analysed in 2-week-old seedlings of the indicated lines, and is shown in comparison with the wild type. UBIQUITIN (UBQ) was used as an internal control. The error bars are the SDs calculated from three independent biological repeats, each measured in four technical repeats.
(c) Enolase activity in crude extracts of *los2/eno2* mutant plants. Enolase activity was measured in 2-week-old seedlings of the lines shown. Values with the mean SD calculated from three independent biological replicates are shown.
(d) Phenotype of 12-day-old seedlings of wild type and *los2/eno2* mutants grown on MS media under long-day (LD) growth conditions.
(e) Scanning electron micrographs of 14-day-old hypocotyls of the indicated lines. One representative protruding cell file is marked in green.

(Figure 1b). To investigate the effect of the mutations on ENO2 activity we measured the enolase activity in 2-week-old plants (the same tissues as used for qPCRs) of the

mutants and compared it with the wild type. As shown in Figure 1(c), enolase activity in *los2-2* was reduced to about 20%, whereas in *los2-3* and *los2-4* it was reduced to about 6% of that in the wild type. As we noted that enolase activity is highest in buds and flowers, we also measured floral tissues, which confirmed a strong reduction in *los2-3* and *los2-4*, and a weaker reduction in *los2-2* (Figure S1a). Enolase activity in *los2-1* was also reduced; however, it was reduced to a smaller extent as compared with its wild-type background C24 (Figure S1b). Therefore, although some transcript is produced in *los2-4* it appears not to be functional. Both *los2-3* and *los2-4* severely compromise enolase activity, whereas *los2-1* and *los2-2* have smaller effects.

**LOS2/ENO2 is essential for shoot and root growth**

The identified *los2/eno2* mutant lines were subjected to a comprehensive, progressive phenotypic analysis over their life spans to investigate the function of LOS2/ENO2 in the different stages of plant development. Plants homozygous for *los2-2*, *los2-3* and *los2-4* did not produce seeds, and thus segregating progeny from plants heterozygous for the insertions were used.

Homozygous *los2/eno2* seeds germinated normally, and in early seedling development no morphological differences between the mutants and the wild type were obvious; however, starting between 8 and 10 days after germination, pronounced developmental defects of *los2/eno2* mutant plants became apparent. Twelve-day-old seedlings of *los2-3* and *los2-4* had much shorter roots as well as smaller, pale green leaves, with shorter petioles, than the wild type (Figure 1d). The *los2-2* plants exhibited very similar phenotypic features; however, these were less marked, and correlated with an attenuated reduction of enolase activity compared with the two other lines. In contrast, *los2-1* seedlings did not show any constitutive developmental defects, neither in early nor in late seedling development (Figure S1c), confirming previous reports (Lee *et al.*, 2002).

The impaired growth of *los2/eno2* mutants indicated an essential function of LOS2/ENO2 in cell development. To address whether cell shape or cell size may be altered, the epidermis of hypocotyls of *los2/eno2* mutant plants was analysed using scanning electron microscopy. Interestingly, although the length of epidermal hypocotyl cells appeared not to change, the width was reduced (Figure 1e). To quantify this observation we measured cell length and width and calculated the mean length/width ratio. The results showed that, whereas this ratio was approximately 1.2 in the wild type, it was significantly changed in the *los2/eno2* knock-out lines: 1.57 in *los2-2* ($n = 50$; $P = 7.18 \times 10^{-5}$) and 2.64 in *los2-3* ($n = 50$; $P = 8.56 \times 10^{-17}$). Correspondingly, whereas *los2/eno2* hypocotyl length was not notably altered, the diameter was clearly reduced (Figure 1e). Therefore, whereas the primary elongation growth of *los2/eno2* hypocotyls was not defective, secondary radial growth was strongly compromised.

The growth defects induced by *LOS2/ENO2* mutation extended to adult development. Adult *los2-3* and *los2-4* plants were characterized by severe dwarfism. This adult phenotype was less pronounced in *los2-2* (Figure 2a) and *los2-1* (Figure S1d), but remained clearly visible in these lines. *los2-3* and *los2-4* plants were much smaller in size and showed early senescence. Their leaves were characterized by reduced size, epinastic growth and leaf reticulation, and/or chlorosis (Figure 2a). To investigate the cellular basis of these development defects we analysed the adaxial leaf epidermis of *los2/eno2* leaves using electron microscopy and compared the second pair of fully expanded true leaves of *los2-2* and *los2-3* with those of the wild type. Figure 2(b) shows that leaf epidermal pavement cells had a reduced size in *los2-2* mutant plants and were extremely small in *los2-3* plants. Moreover, the puzzle-like shape of pavement cells was lost in *los2-3*. Interestingly, the size of stomatal guard cells appeared unaffected (Figure 2b). The size of trichomes and their basal cells was also reduced (Figure 2b). Thus, in *los2/eno2* mutants reduced organ size correlates with reduced cell size, providing evidence that LOS2/ENO2 is essential for cell expansion.

**LOS2/ENO2 is required for reproductive development and fertility**

*los2/eno2* mutation also affected floral morphogenesis. As shown in Figure 3(a) buds and flowers of *los2/eno2* knock-out plants were smaller and did not progress through flower development normally. In *los2-3* and *los2-4*, the petals were smaller and pale green and the buds did not fully open. In some buds both petals and sepals turned necrotic. When siliques did develop they were smaller and did not contain seeds (Figure 3a), and thus the plants were sterile.

To investigate the basis of the morphological defects of floral organs and for sterility in more detail we used electron microscopy. The imaging revealed that *los2-2* and *los2-3* flowers were strongly deformed and, most strikingly, that anther development was severely compromised. Anthers of *los2-3* remained in a stage of pre-anthesis, being unable to initiate elongation (Figure 3b), and moreover did not produce pollen (Figure 3c). In addition, the female organs were deformed, in particular with the stigma being strongly distorted (Figure 3b). In *los2-2* plants pollen grains were produced; however, their size was clearly reduced (Figure 3c).

We investigated the ability of *los2-2* pollen to germinate *in vitro*. Whereas around 80% of wild-type pollen grains germinated, only 5% of *los2-2* pollen germinated *in vitro* (Figure 3d). In pollen that did germinate, the pollen tube

 

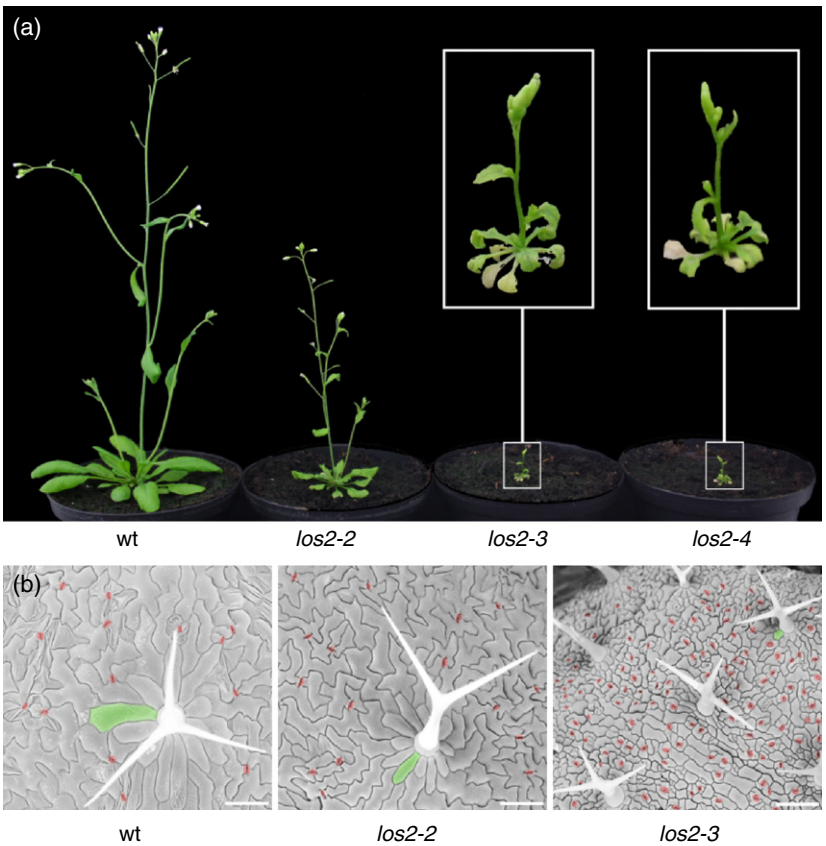


**Figure 2.** *los2/eno2* mutants are impaired in adult development.
(a) Adult phenotypes of *los2/eno2* knock-out lines. Representative, 5-week-old plants of wild-type and *los2/eno2* mutants grown under long-day (LD) growth conditions are shown.
(b) Scanning electron micrographs of the adaxial epidermis of leaves of the indicated lines. Fully expanded leaves of 4-week-old plants of wild type, *los2-2* and *los2-3* are shown. A representative basal trichome cell is highlighted in green and stomata are marked in red (scale bars: 100 μm).

length was markedly reduced as compared with pollen from wild-type plants (Figure 3d). Reduced pollen germination and impaired pollen tube elongation also correlated with abolished seed set in the weaker *los2-2* allele (Figure 3e). Thus, female and male organ development and male gametophyte function require LOS2/ENO2, and consequently defects in *LOS2/ENO2* reduce fertility.

### LOS2/ENO2 is essential for vascular development and secondary growth of stems

Although *los2-3* and *los2-4* knock-out lines were extremely dwarfed, they did develop inflorescence-like structures with very short stems that reached a final height of approximately 2 cm (Figure 2a). To investigate vascular development in *los2/eno2* stems, cross-sections from the basal stem part (just above the soil line) of 6-week-old wild-type and *los2-3* plants were stained with toluidine blue O and analysed by light microscopy. In Figure 4 it is shown that *los2-3* stems had a severely reduced diameter (please note the different bar sizes) and, more interestingly, that drastic defects in vascular tissue composition occurred.

At 6 weeks after germination the wild type had established the typical tissue pattern of secondary stems (Sanchez *et al.*, 2012), characterized by concentric layers of epidermis, cortex and endodermis, followed by a vascular system laid out close to the stem periphery, and a parenchymatous pith prominently present in the centre (Figure 4a). In *los2-3* stems this concentric organization of differentiated tissues was lost (Figure 4b). Cells of the outmost tissue layers were undifferentiated, strongly enlarged and misshapen. The xylem remained disconnected and showed severely reduced lignification; moreover, it was composed mainly of large tracheids lacking interspersed parenchyma cells. The vascular bundles were oriented closer to the centre than in the wild type, and showed defects in the ordered development of radial cell files that differentiate into xylem and phloem. In the wild type these ordered cell division and differentiation events arise from procambial cell activity (Sanchez *et al.*, 2012); in accordance, also the development and differentiation of cambial cells was impaired in *los2-3* (Figure 4b).

In addition to a distorted vascular structure, *los2-3* plants were compromised in the development of pith parenchyma cells in the stem centre, which had a reduced size, an irregular shape and appeared collapsed. Moreover, *los2-3* also produced irregular, collapsed xylem elements (Figure 4c), which are indicative of secondary cell wall defects (Brown *et al.*, 2005). *los2-2* showed similar defects (Figure 4d); however, they were less marked, correlating with a higher overall enolase activity in *los2-2* plants. In summary, there is evidence that LOS2/ENO2 is required for oriented cell division and differentiation events in vascular

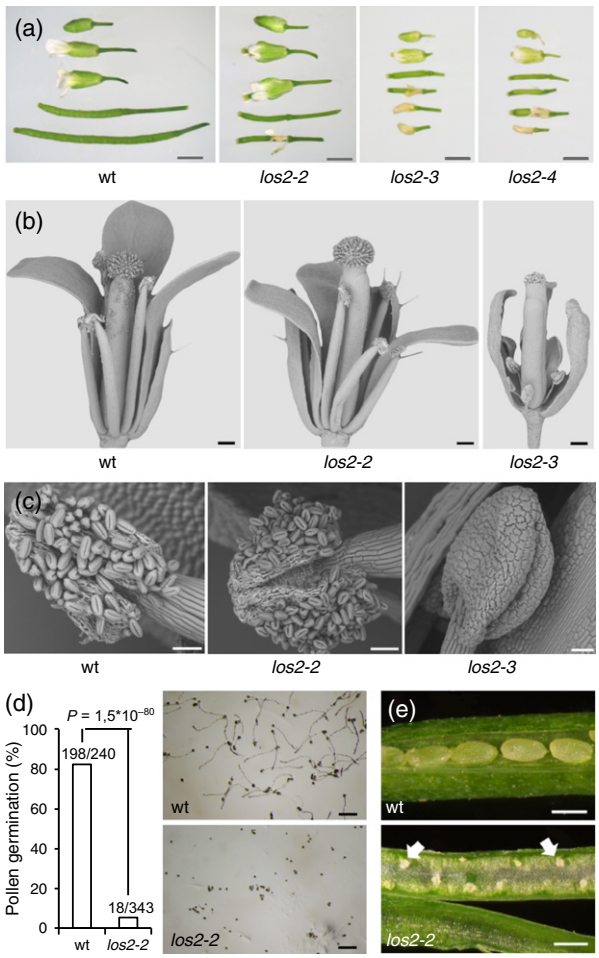


**Figure 3.** *los2/eno2* mutants are compromised in reproductive organ development and male gametophyte function.
(a) Flower and silique morphology of wild-type and *los2/eno2* knock-out plants. Siliques and flowers in comparable developmental stages for each line are shown (scale bar: 2 mm).
(b) Scanning electron micrographs of wild-type, *los2-2* and *los2-3* flowers. Representative flowers of 5-week-old plants in the same developmental stage are shown (scale bar: 0.2 mm).
(c) Scanning electron micrographs of wild-type, *los2-2* and *los2-3* anthers of flowers at the same developmental stage (scale bar: 400 μm).
(d) *In vitro* pollen germination and pollen tube growth. Pollen harvested from freshly anther-dehisced flowers were incubated for 20 h on solid pollen germination media. Percentage of germination was calculated from 100 plated pollen grains, analysed in four repeats. The *P*-value was calculated and is shown (scale bar: 200 μm).
(e) Opened siliques of wild-type and *los2-2* plants. Arrows indicate aborted embryos (scale bar: 500 μm).

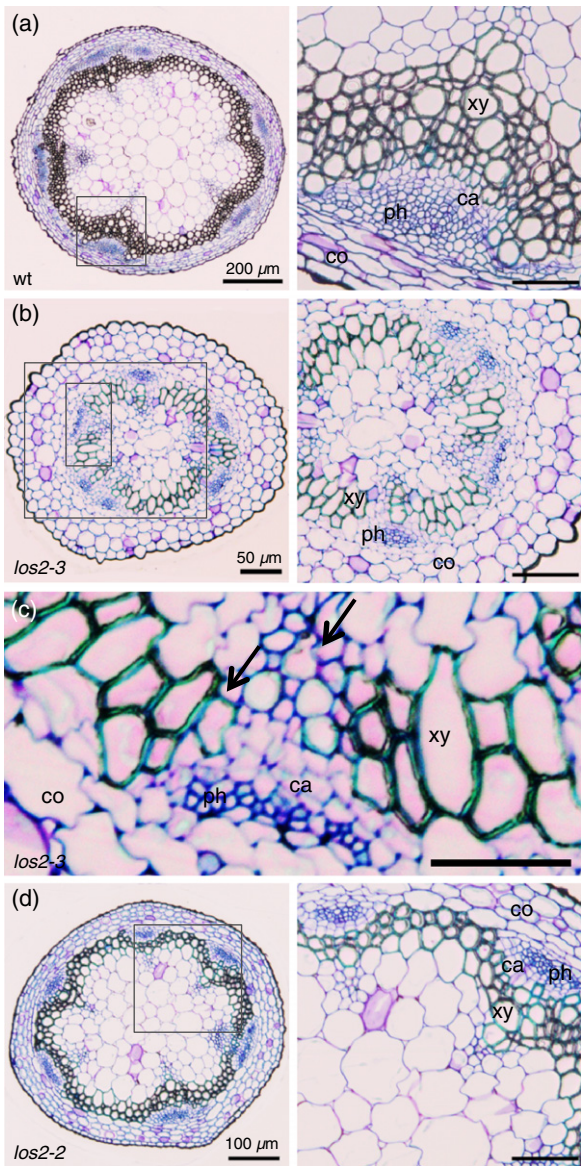


**Figure 4.** *los2/eno2* mutants are defective in vascular tissue organization.
(a) Representative transverse section of an inflorescence stem of a 6-week-old wild-type plant stained with Toluidine Blue. Left: whole stem section. Right: magnified view of the boxed area on the left, displaying a close-up of a vascular bundle (scale bar: 50 μm); ca, cambium; co, cortex; ph, phloem; xy, xylem.
(b) Representative transverse section of an inflorescence stem of a 6-week-old *los2-3* plant stained with Toluidine Blue. Left: whole stem section. Right: magnified view of the boxed area on the left (scale bar: 50 μm).
(c) Magnified view of the area marked with the smaller square in (b), highlighting a *los2-3* vascular bundle. Arrows indicate collapsed xylem cells (scale bar: 25 μm).
(d) Representative transverse section of an inflorescence stem of a 6-week-old *los2-2* plant stained with Toluidine Blue. Left: whole stem section. Right: magnified view of the boxed area on the left, displaying a close-up of a vascular bundle (scale bar: 50 μm).

tissues, for the structural integrity of cell walls and for the secondary growth of stems.

### LOS2/ENO2 impacts on the synthesis of lignin, sinapoyl malate and salicylic acid

The histological analysis of *los2-2* and *los2-3* stems provided evidence that *los2/eno2* mutants contained decreased quantities of the cell wall polymer lignin. In support, *los2/eno2* phenotypes such as dwarfing, male sterility and defective xylem development closely resemble phenotypes of Arabidopsis mutants defective in monolignol

production (Li *et al.*, 2010). As enolase activity is required for PEP synthesis (Plaxton, 1996; Voll *et al.*, 2009), which, via the shikimate pathway, is converted to chorismate, a direct precursor of phenylalanine (Vermerris and Nicholson, 2006), we speculated that phenylpropanoid synthesis could be affected in *los2/eno2* mutants.

To test this, we first analysed the quantity of lignin in inflorescence stems of wild-type and *los2/eno2* knock-out plants. As shown in Figure 5(a), the lignin content was decreased by approximately 40% in *los2-2* and approximately 65% in *los2-3* and *los2-4*. Secondly, we assessed the abundance of sinapoyl malate, a member of another class of important phenylpropanoids, the sinapoyl esters (Vermerris and Nicholson, 2006). Sinapoyl malate is the major sinapoyl ester in leaves and plays a role in the protection against UV radiation (Landry *et al.*, 1995). We measured sinapoyl malate contents in 3-week-old plants of mutants and wild type using HPLC and found that in *los2/eno2* knock-out lines sinapoyl malate contents were significantly decreased (Figure 5b). The *los2-1* mutant, like *los2-2*, exhibited smaller changes in lignin abundance (Figure S2a), but no changes in sinapoyl malate content (Figure S2b). Therefore, major developmental defects in the *los2/eno2* mutants that are strongly impaired in enolase activity correlate with the impaired production of the phenylpropanoids lignin and sinapoyl malate.

Another important product of chorismate is the plant hormone salicylic acid (SA; Vermerris and Nicholson, 2006). To investigate whether SA levels were altered in the *los2/eno2* mutants we measured free and total SA levels using HPLC. Whereas SA concentrations were not affected in *los2-2*, they were strongly increased in *los2-3* and *los2-4* plants (Figure 5c). Hence, LOS2/ENO2 plays a complex role in impacting on chorismate-dependent biosynthetic pathways, acting both as a promoter of lignin and sinapoyl malate production and as a repressor of SA biosynthesis.

### LOS2/ENO2 affects fatty acid composition and soluble sugar contents

A different class of essential building blocks formed from PEP is fatty acids (Rawsthorne, 2002). To assess whether their composition was altered in *los2/eno2* mutants we performed fatty acid measurements using HPLC. Although we did not observe significant differences in the contents of the saturated fatty acids 16:0 (palmitic acid) and 18:0 (stearic acid), the unsaturated fatty acids 16:1, 16:3 and 18:1 were significantly reduced, and 18:3 was significantly increased, in the strong *los2-3* and *los2-4* alleles (Figure 5d).

With a proposed role of *LOS2* in cold signalling (Lee *et al.*, 2002), we were interested whether in addition to fatty acid composition mutations in *LOS2/ENO2* also alter soluble sugar contents, as both fatty acid (Uemura *et al.*,

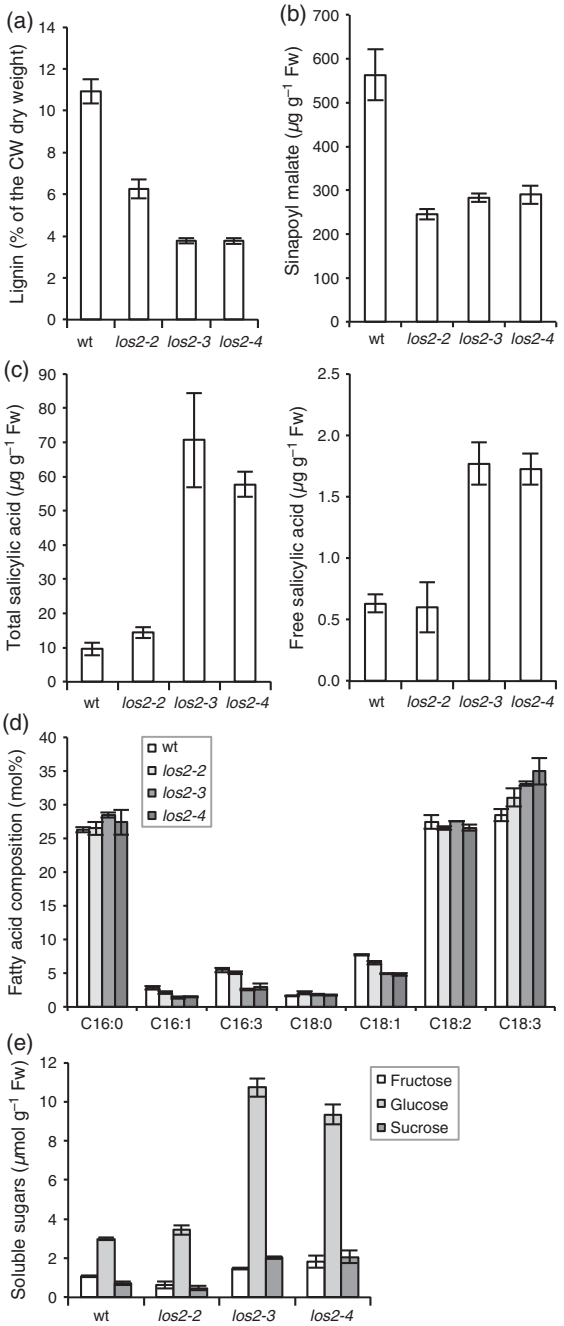


**Figure 5.** LOS2/ENO2 impacts on the secondary metabolite composition of plants.
(a) Lignin contents in the primary inflorescence stems of 6-week-old wild-type and *los2/eno2* mutant plants. Values are given as percentages of dry cell wall material.
(b) Sinapoyl malate contents in leaves of 3-week-old wild-type and *los2/eno2* knock-outs.
(c) Total and free salicylic acid levels in leaves of 3-week-old wild-type and *los2/eno2* knock-out mutant plants. The SDs of three biological replicates are shown.
(d) Fatty acid composition of leaves of 3-week-old wild-type and *los2* knock-out plants. The SDs of three biological replicates are shown.
(e) Soluble sugar content in leaves of 3-week-old wild-type and *los2/eno2* knock-out lines grown in soil. Results are shown as means ± SDs of four biological replicates.

1995) and soluble sugar contents impact on cold resistance in plants (Gilmour *et al.*, 2000). Thus, we measured the carbohydrates glucose, fructose and sucrose as well as starch in leaves of *los2/eno2* knock-out plants using HPLC. Whereas in *los2-2* and *los2-1* no significant changes were found (Figures 5e and S2c), the level of fructose, glucose and sucrose was elevated in *los2-3* and *los2-4* plants (Figure 5e). No significant changes in starch contents were detected (Figure S2d).

### AtMBP-1 represses *LOS2/ENO2* promoter activity

Recently it was shown that the overexpression of YFP fusions of full-length *LOS2/ENO2* and of the shorter *AtMBP-1* open reading frame (ORF) induces constitutive developmental defects, which bear a striking resemblance to *los2/eno2* knock-out plants: they develop normally as seedlings but are compromised in adult development, resulting in dwarf plants with reduced fertility (Kang *et al.*, 2013; Figure S3). Thus, we speculated that *AtMBP-1* overexpression may result in the repression of ENO2 enolase activity. To test this we measured enolase activity in leaves and floral tissues of *35S:LOS2-YFP* and *35S:AtMBP-1-YFP* plants, and found that in both lines enolase activity was strongly decreased (Figure 6a,b).

Since human MBP-1 is known to act as a transcriptional repressor that also controls its own promoter activity (Feo *et al.*, 2000) we investigated if in *AtMBP-1* over-expressing lines *LOS2/ENO2* promoter activity may be repressed. For this purpose we performed qPCR analysis using primers that span the 3′ untranslated region (UTR) of the gene to specifically detect endogenous *LOS2/ENO2* mRNA abundance (and not measure *LOS2/AtMBP-1* transgene abundance, which is increased in these lines; Kang *et al.*, 2013). The result showed that indeed transcription of endogenous *LOS2/ENO2* was strongly impaired in *LOS2/ENO2* and *AtMBP-1* overexpressing plants (Figure 6c).

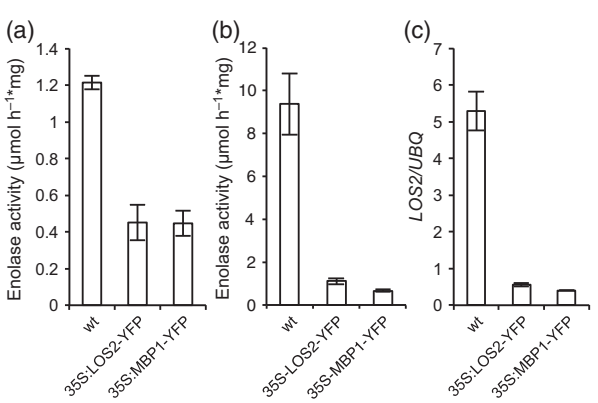


**Figure 6.** AtMBP-1 represses *LOS2/ENO2* promoter activity.
(a, b) Enolase activity in crude extracts of *35S:LOS2-YFP* and *35S:MBP1-YFP* plants. Enolase activity was measured in leaves (a) and floral tissues (b) of 5-week-old plants. Means values and SDs calculated from three independent biological replicates are shown.
(c) *LOS2/ENO2* transcript abundance in the wild type and in *35S:LOS2-YFP* and *35S:MBP1-YFP* plants as determined by qPCR. *LOS2/ENO2* expression was analysed in leaves of 5-week-old plants. UBIQUITIN (UBQ) was used as an internal control. The error bars are SDs calculated from three independent biological repeats, each measured in four technical repeats.

Therefore, there is evidence that AtMBP-1 negatively regulates *LOS2/ENO2* promoter activity. The fact that the overexpression of LOS2-YFP does not complement the reduction in endogenous LOS2/ENO2 activity indicates that the YFP-tag may impair ENO2 function, but does not significantly impact on AtMBP-1 activity.

### Expression of a LOS2-NES fusion protein partially restores enolase activity in *los2-4* and recovers growth defects

As reduced enolase activity clearly correlates with growth defects of different *los2/eno2* knock-out lines as well as LOS2 and AtMBP-1 overexpressing plants, there was evidence that the constitutive developmental defects are a consequence of impaired ENO2 function; however, given the fact that the mutated locus also contains a transcription factor, it was important to test whether the effects seen in the knock-outs are not actually caused by a loss of AtMBP-1 function. For this purpose LOS2 was fused to a nuclear export signal (NES; LALKLAGLDI, Wen *et al.*, 1995) and to YFP, and its localization was assessed in protoplasts. Whereas LOS2-YFP is present in the nucleus and in the cytoplasm (Lee *et al.*, 2002; Figure S4a), the NES interfered with the nuclear localization of the fusion protein (Figure S4a). The LOS2-NES construct (without the YFP tag) was then introduced into *los2-4* under *LOS2* promoter control and homozygous lines expressing the transgene were selected (Figure 7a; please note that the primers chosen do not detect endogenous *LOS2/ENO2* mRNA in the *los2-4* lines as one primer spans the region deleted in *los2-4*). To investigate whether restricting nuclear localization of LOS2/ENO2 also impaired AtMBP-1 transcriptional activity, we compared *ZAT10* expression in the wild type, *los2-4* and in complementation lines. *ZAT10* is negatively regulated by AtMBP-1, and thus its expression is enhanced in *los2-1* (Lee *et al.*, 2002). In agreement, *ZAT10* expression in *los2-4* was elevated compared with the wild type. This effect was not released in the complementation lines (Figure 7b), confirming that AtMBP-1 activity is not restored by the transgene.

When enolase activity was measured in the complementation lines we found that it was re-established to some extent (Figures 7c and S4b), but not to wild-type levels, correlating with the partially restored *LOS2/ENO2* expression levels only (Figure 7a). Nevertheless, this partial recovery of ENO2 enolase function in *los2-4* was sufficient to recover the growth defects of *los2-4*. Lines with a higher enolase activity (36 and 11) were significantly larger in the adult stage, with larger leaves and also with reduced growth repression of the inflorescences (Figure 7d), whereas in line 1, in which enolase activity was only weakly restored, the phenotypes were still more pronounced.

Thus the rescue of phenotypes correlated with the recovery of enolase activity, providing further evidence that a loss of enolase function in *los2/eno2* knock-out lines accounts for major aspects of the developmental defects revealed.

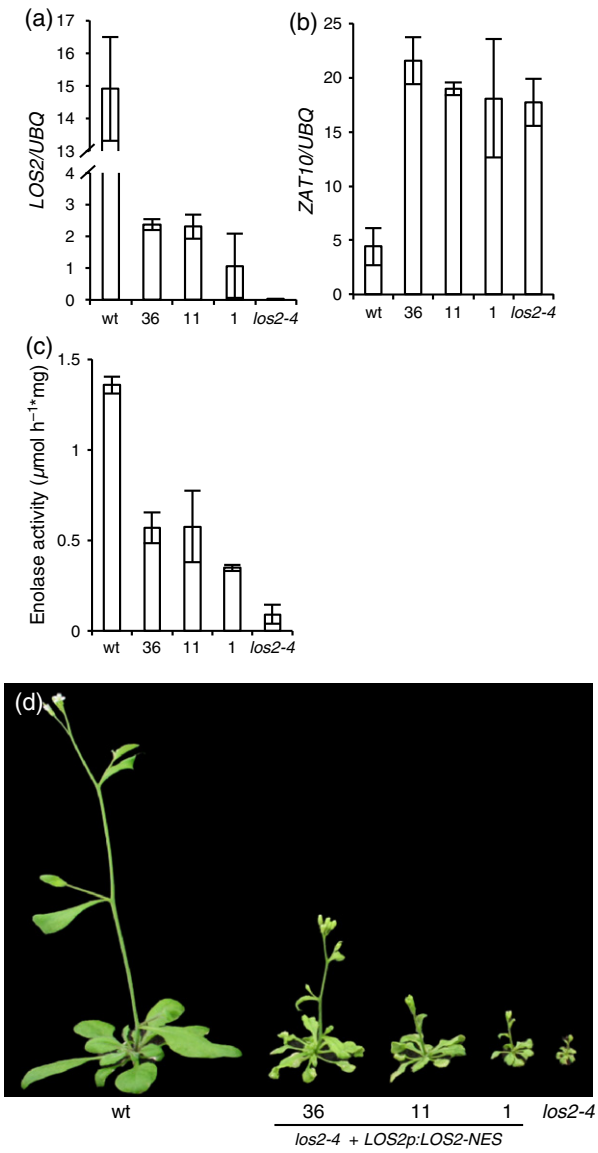


**Figure 7.** Growth repression of *los2-4* is partially complemented by *LOS2p:LOS2-NES* expression.
(a) *LOS2/ENO2* transgene abundance in *LOS2p:LOS2-NES*-expressing *los2-4* plants, as determined by qPCR. *LOS2/ENO2* expression was analysed in leaves of 5-week-old plants of the indicated lines. UBIQUITIN (UBQ) was used as an internal control. The error bars are SDs calculated from three independent biological repeats, each measured in four technical repeats.
(b) *ZAT10* transcript level in the lines shown. *ZAT10* expression was determined by qPCR in leaves of 5-week-old plants. *UBQ* was used as an internal control. The error bars are SDs calculated from three independent biological repeats, each measured in four technical repeats.
(c) Enolase activity in crude extracts of *LOS2p:LOS2-NES*-expressing *los2-4* plants, as compared with *los2-4* and the wild type. Enolase activity was measured in leaves of 5-week-old plants of the lines shown. Mean values with SDs calculated from three independent biological replicates are shown.
(d) Adult phenotypes of the wild type, *los2-4* and *LOS2p:LOS2-NES*-expressing *los2-4* lines. Representative 4-week-old plants are shown.

## DISCUSSION

Glycolysis is a central metabolic pathway in which sugars are converted to pyruvate (Canback *et al.*, 2002). A key intermediate of glycolysis is PEP, which is produced by enolases (Canback *et al.*, 2002), and, in addition to being used for pyruvate production, a precursor for fatty acid biosynthesis, is also fed into the shikimate pathway to synthesize essential aromatic amino acids and phenylpropanoids in plants (Figure 8).

In agreement with the key biochemical function of enolases in generating essential building blocks in prokaryotic and eukaryotic organisms (Díaz-Ramos *et al.*, 2012), we here show that plants with defective enolase function show severe growth defects, including impaired vascular development and secondary growth, defective anthesis, defective pollen tube elongation and defective female reproductive organ development, resulting in sterility. The plants were generated by T-DNA insertional mutagenesis of *LOS2/ENO2*, which is the most strongly expressed enolase in Arabidopsis (Andriotis *et al.*, 2010), and is also the main enolase that ensures cytosolic PEP provision, as ENO3/ENOc, the second cytosolic enolase of Arabidopsis, is strongly compromised in its catalytic function (Andriotis *et al.*, 2010). Four independent mutant lines were analysed in which enolase activity was reduced to different extents. The reduction in enolase activity correlated with the degree of phenotypes with milder defects in *los2-1* and *los2-2*, and severe defects in *los2-3* and *los2-4*, providing evidence that enolase deficiency accounts for major aspects of the constitutive growth defects revealed.

As the LOS2/ENO2 locus in addition to the enolase ENO2 also encodes the transcription factor AtMBP-1, it was important to dissect the effects conferred by the enolase function of the locus from those imposed by its transcription factor activity. To do so we tested whether the developmental defects of *los2/eno2* mutants could be restored by the expression of a LOS2/ENO2 version compromised in the transcription factor bifunctionality, and indeed a partial rescue that also correlated with partially recovered enolase activity was conferred. In addition, and even more importantly, the overexpression of *AtMBP-1* (in *35S:AtMBP-1-YFP* and *35S:LOS2-YFP* lines) produced phenotypes that strongly resemble those of the *los2/eno2* knock-out lines. These growth defects are correlated with a strong reduction in enolase activity, but not with a reduction in *AtMBP-1* transcript abundance, which on the contrary in these plants is increased (Kang *et al.*, 2013).

More evidence that a lack of enolase activity is a central reason for the constitutive developmental defects revealed comes from the fact that *los2/eno2* mutants and *AtMBP-1* overexpressing plants show a strong phenotypic resem-

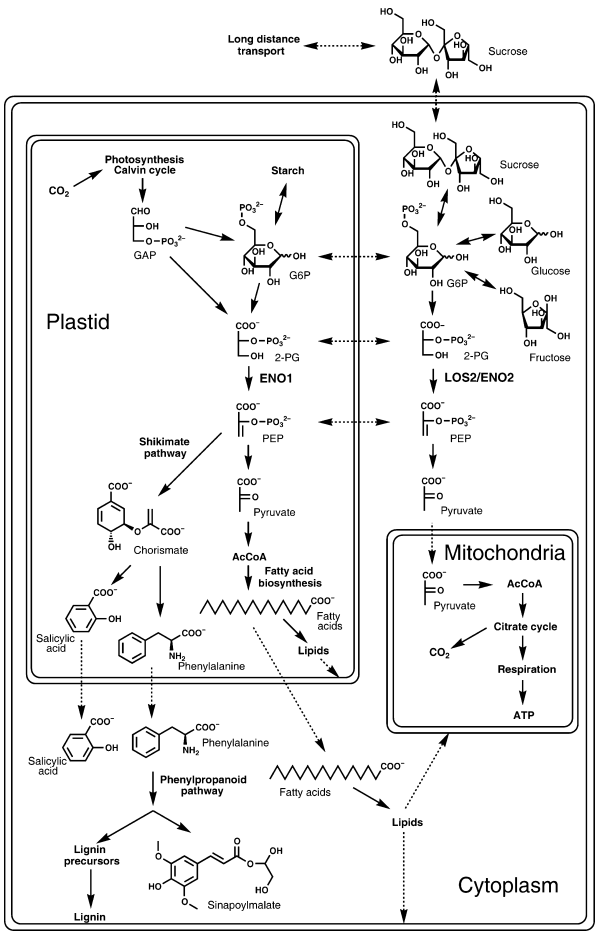


**Figure 8.** Model of ENO2 function in glycolysis.

blance with other mutants impaired in glycolysis, all of which display severe dwarfism, leave reticulation, are disturbed in flower development, and have reduced fertility or are sterile (Rius *et al.*, 2008; Lim *et al.*, 2009; Prabhakar *et al.*, 2010; Zhao and Assmann, 2011). These phenotypes only become pronounced 8–10 days after germination. Different, not mutually exclusive, reasons may account for this fact. Firstly, in young seedlings the carbon skeletons required for growth are mainly generated from the breakdown of storage oils (Vanholme *et al.*, 2010; Gallego-Giraldo *et al.*, 2011a), potentially limiting the consequences of defects in glycolysis. Secondly, lignin synthesis, which requires PEP provision, is essential in particular in later developmental stages as it enables secondary growth.

Lignin is a major structural component of secondary cell walls, and protects cell wall polysaccharides from degradation and impregnates vascular structures such as the xylem (Vanholme *et al.*, 2010). *los2/eno2* mutants are strongly compromised in lignin production and their phenotypes resemble other mutants defective in lignin formation, such as phenylpropanoid mutants, which are characterized by growth reduction and dwarfing (Li *et al.*, 2010; Gallego-Giraldo *et al.*, 2011a; Zhao *et al.*, 2013), as well as defective reproductive organ development (Prabhakar *et al.*, 2010; Zhao and Assmann, 2011). Accordingly, histological and biochemical analyses revealed that lignification in *los2/eno2* mutants is compromised. In addition, *los2/eno2* shows defects in secondary growth. Secondary growth results in an increased diameter of shoots and roots through the formation of secondary vascular structures (Sanchez *et al.*, 2012), and in the stems of *los2/eno2* knock-out plants the founding features of secondary stems are lost. *los2/eno2* is defective in the differentiation and spatial separation of xylem and phloem. Moreover, the position of vascular bundles is shifted towards the stem centre and the overall radial organization as well as the differentiation of tissues in the stele is compromised. As these phenotypes correlate in severity with a reduction in enolase activity in different *los2/eno2* mutant alleles, our work provides evidence that enolase activity is essential for the secondary, lateral growth of plant organs. Secondary growth defects in *los2/eno2* mutants coincide with a loss in the structural integrity of plant cell walls. Thus, there is evidence that LOS2/ENO2 activity is required for secondary cell wall assembly, and it is likely that this function is central to the role of LOS2/ENO2 in the secondary growth of plants.

In addition to reduced lignin contents, and in analogy to other mutants defective in monolignol production (Li *et al.*, 2010; Gallego-Giraldo *et al.*, 2011a), *los2/eno2* mutants over-accumulate SA. Two scenarios that may account for a correlation of reduced lignin levels with increased SA concentrations have been discussed: a back-up of flux from the monolignin pathway could increase SA production in monolignol mutants, or improperly lignified cell walls that collapse may release elicitors, which could trigger endogenous defence responses and thereby induce SA synthesis (Gallego-Giraldo *et al.*, 2011a,b). An altered flux through the pathways appears unlikely for *los2/eno2* mutants as ENO2 produces PEP, which is an upstream precursor for the production of both SA and lignin. Therefore, it appears more likely that, in analogy with other mutants (Jones *et al.*, 2001; Franke *et al.*, 2002), collapsed cell walls in *los2/eno2* mutant plants are causal for the activation of SA production. Alternatively, in *los2/eno2* mutants another aspect could account for increased SA levels: the fact that the 18:1 unsaturated fatty acid content is reduced, as 18:1 and its derivatives are required for the repression of SA biosynthesis and signalling (Kachroo *et al.*, 2003).

Elevated SA levels are often correlated with dwarf phenotypes in plants (Gallego-Giraldo *et al.*, 2011a; Rivas-San Vicente and Plasencia, 2011). How SA mediates its growth-repressing effects is not fully understood, although there are indications that SA impacts on the biosynthesis and signalling of growth-promoting hormones. Some mutants

with increased SA biosynthesis contain decreased auxin levels and show a decreased sensitivity to auxin (Wang *et al.*, 2007). Also, an impact of SA on gibberellin (GA) responsiveness and signalling has been revealed. Plants silenced in the expression of hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase, which over-accumulate SA because of decreased lignification (Li *et al.*, 2010; Gallego-Giraldo *et al.*, 2011a), show reduced GA biosynthetic gene expression and reduced responsiveness to GA (Gallego-Giraldo *et al.*, 2011a). Thus, increased SA levels caused by the lost structural integrity of cell walls could, in a feed-forward mode, further repress the growth of *los2/eno2* plants by reducing GA responses.

Enolase is a key enzyme in glycolysis (Plaxton, 1996) and the glycolytic pathways play a central role in plant growth and development. It is therefore perhaps not surprising that enolase activity must be controlled. Here, we provide evidence that AtMBP-1 represses *LOS2/ENO2* promoter activity and thereby controls ENO2 abundance. This feedback regulatory control could be used to maintain homeostasis of enolase activity, and would justify the evolution of bifunctionality of the locus. In humans, MBP-1 was first identified as a transcription factor that regulates the expression of cMyc, a bHLH/LZ transcription factor best known for its role in tumorigenesis (Feo *et al.*, 2000). MBP-1 represses the activity of cMyc (Feo *et al.*, 2000; Hsu *et al.*, 2009), which directly controls the expression of α-enolase *ENO1* as well as that of other glycolytic enzymes (Osthus *et al.*, 2000). We now provide evidence that glycolysis in plants is also subjected to homeostatic regulation. It will be important to determine whether AtMBP-1 directly binds to the *LOS2/ENO2* promoter to impose this control or whether additional proteins participate. Also, it will be interesting to investigate whether the known roles of AtMBP-1 in abiotic stress responses (Lee *et al.*, 2002; Barkla *et al.*, 2009; Kang *et al.*, 2013) are related to its central function in glycolysis.

In summary, we provide evidence that LOS2/ENO2 is essential for plant growth and development and uses an elegant regulatory mode to control its own activity: the alternative translation of the transcription factor AtMBP-1 that acts to repress *ENO2* transcription, and thereby governs glycolysis.

## EXPERIMENTAL PROCEDURES

### Plant material and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) was the wild type of the *los2-2*, *los2-3* and *los2-4* mutant alleles, and ecotype C24 was the wild type of the *los2-1* mutant line. *los2-2* (SALK_021737), *los2-3* (SALK_077784) and *los2-4* (SAIL_208_B09) were obtained from the NASC (http://arabidopsis.info). The position of the T-DNA insertions was determined by PCR using gene-specific and T-DNA border-specific primers (all primer sequences are given in Table S1), and sequencing the resulting PCR products.

For generation of the *los2-4* complementation lines a 1.7-kb fragment of the *LOS2/ENO2* promoter and the *LOS2/ENO2* coding sequence were amplified by PCR and cloned into the binary plant expression vector pGWR8 (Rozhon *et al.*, 2010). For generation of the nuclear export signal the NES-fw and NES-rv oligonucleotides were hybridized and treated with T4 polynucleotide kinase (Thermo Scientific, http://www.thermoscientific.com). The double-stranded NES fragment was cloned to the C-terminal part of the *LOS2p:LOS2* sequence. Heterozygous plants of the *los2-4* line were transformed with the *LOS2p:LOS2-NES* construct using the floral-dip method (Clough and Bent, 1998). For transient protoplast transformation, double-stranded NES and a YFP tag were cloned to the C-terminal part of the *LOS2p:LOS2* sequence.

For metabolite analysis (Appendix S1) and growth response assays, seeds were sterilized using the chlorine vapour method (Clough and Bent, 1998) and plated on half-strength MS medium (Sigma-Aldrich, https://www.sigmaaldrich.com). After stratification for 2 days at 4°C the plates were moved to growth chambers and were incubated at 21°C in long-day (LD) growth conditions of 16 h white light with a light intensity of 80 μmol $m^{-2}$ $sec^{-1}$. For all metabolite measurements plant material was collected 5 h into the light phase.

### *In vitro* pollen germination

The germination efficiency of pollen grains was assessed *in vitro* according to Fan *et al.* (2001). Pollen of fresh, anther-dehisced flowers was transferred to germination medium [5 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES), pH 5.8, 1 mM KCl, 10 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.5 mM boric acid, 1% (w/v) agar, 17% (w/v) sucrose] and was incubated for 20 h in a climate-controlled chamber at 21°C, 100% humidity and in low light (30 μmol $m^{-2}$ $sec^{-1}$). For each line 100 pollen grains were assessed on four plates and the germination efficiency was calculated. Photos of pollen grains were taken with an Olympus binocular microscope (Olympus, http://www.olympus-global.com).

### Histochemical analysis and light microscopy

Stem sections were made from primary inflorescence stems of 6-week-old plants 2–3 mm above the rosette. Tissue fixation and embedding was performed as described previously (Beeckman and Viane, 2000). Briefly: the stems were harvested and fixed in FAA solution (45% ethanol, 5% acetic acid and 5% formalin in water), the fixed plant material was dehydrated in a graded ethanol series (2 h each in 30, 50, 70 and 100% ethanol), infiltrated and embedded with Technovit 7100 embedding kit (Heraeus Kulzer, http://heraeus-kulzer.com). Sectioning was performed with a Leica RM 2065 microtome (Leica Mikrosysteme, http://www.leica.com). Sections were stained for 5 min in 0.02% aqueous Toluidine blue O (Sigma-Aldrich), rinsed with water and analysed with an Olympus BX-61 microscope.

### Scanning electron microscopy

For images of the adaxial leaf epidermis the second leaf pair of 4-week-old plants was shock-frozen in liquid nitrogen and visualized with a Hitachi TM3000 scanning electron microscope (Hitachi High-Tech, http://www.hitachi.com). The flowers and hypocotyls were fixed on a metal support rack and visualized without treatment.

### Quantitative RT-PCR analysis

Total RNA was isolated with the GeneJET RNA Purification Kit (Thermo Scientific, http://www.thermoscientific.com) and treated

with DNaseI to digest traces of DNA. First-strand cDNA was synthesized from 1 μg RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's instructions. qRT-PCRs were performed with the GoTaq qPCR Master Mix (Promega, http://www.promega.com) using a Mastercycler Realplex (Eppendorf, http://www.eppendorf.com) cycler. qRT-PCR experiments were performed from three independent biological samples that were each measured with four technical repeats.

### Measurement of total enolase activity

Enolase activity was determined spectrophotometrically as described previously (Van Der Straeten *et al*., 1991). Reactions were performed in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM $MgCl_2$ and 2.5 mM 2-phospho-D-glycerate. The samples were incubated at 25°C and the increase in absorbance at 230 nm was recorded for 20 min after adding the protein extract. The linear part of the graph was used for the calculation of enzymatic activity. PEP was used to establish a calibration curve. Values are presented as the mean of three independent biological replicates.

### Protoplast transformation

Protoplast generation and transformation was performed as described previously (Yoo *et al*., 2007).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Phenotypes of wild-type C24 and *los2-1* mutant plants.

**Figure S2.** Lignin, sinapoyl malate, soluble sugar and starch contents in different *los2/eno2* mutant alleles.

**Figure S3.** Phenotype of 4-week-old *35S:LOS2-YFP* and *35S:MBP1-YFP* mutant plants, as compared with the wild type.

**Figure S4.** Expression of a LOS2-NES-YFP construct in protoplasts and *los2-4* plants.

**Table S1.** List of primers used in this study.

**Methods S1.** Experimental procedures.

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