Functional analysis of orthologous transcription factors FLOWERING LOCUS C and PERPETUAL FLOWERING 1 in *A. thaliana* and *A. alpina*

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Abstract

Variation of transcription factor (TF) binding sites (BSs) is a major source of variation within and between species. In plants, evolution of TF BSs remains to be poorly studied. Here, we performed the first comparative ChIP-seq study in combination with gene expression analysis in knock-out mutants in two related plant species. We used the FLOWERING LOCUS C (FLC) TF in annual *A. thaliana* and the perennial sister species *A. alpina* as a model system. In *A. thaliana*, FLC represses flowering before vernalization. The *A. alpina* FLC ortholog PERPETUAL FLOWERING 1 (PEP1) not only represses floral induction prior to vernalization but also represses flowering in newly formed side shoots after vernalization to ensure that the flowering phase is followed by vegetative growth which is crucial for the perennial life-history of *A. alpina*.

We found that FLC and PEP1 BSs were highly divergent but both TFs bound identical CArG-box sequence motifs in the promoters of their target genes. Conserved BSs were associated with conserved CArG-boxes that often were extended by the 'TTT' trinucleotide. Species-specific BSs were correlated with the absence of a CArG-box in the other species. Although these correlations were highly significant, we found evidence that interactions with other TFs might affect binding as well. GO-term enrichment analysis of target genes revealed that conserved targets were mainly associated with the control of flowering time and flower development. Species-specific target genes of both species were associated with the responses to hormones and environmental stimuli, suggesting that convergent evolution resulted in similar roles of PEP1 and FLC. Both TFs bound a high number of cold-regulated (COR) genes and repressed their induction by intermittent cold, suggesting that PEP1 and FLC act in cold to negatively regulate the cold response. Intermittent cold causes growth retardation, thus this role of FLC and PEP1 might affect the trade-off between growth and the cold stress response to ensure growth under cold but non-freezing temperatures prior to vernalization.

In addition, PEP1 and FLC bound different sets of genes involved in GA metabolism and signaling. The *A. alpina pep1-1* mutant showed several phenotypes of GA-treated plants including elongated internodes. During vernalization of *A. alpina*, GA was involved in the promotion of floral induction and genes involved in GA metabolism and signaling were induced, which was counteracted by PEP1. Unexpectedly, GA levels were not induced but were reduced during vernalization independently of PEP1. In *pep1-1*, GA levels were elevated in young apices. In addition, we found locally enriched GA signaling in *pep1-1*. Taken together,

these findings suggest that PEP1 negatively regulates GA levels causing reduced elongation of vegetative branches, possibly to increase plant stability. Furthermore, PEP1 might act during vernalization to repress GA signaling to suppress floral induction during vernalization. This interaction of two flowering pathways that respond to GA and vernalization represents a species-specific interaction of conserved pathways and might act to prevent flowering after short periods of vernalization before the end of the alpine winter. *A. thaliana* FLC also regulated gene expression within the GA network but no GA-related phenotypes could be identified. Expression of GA-related genes was furthermore induced by intermittent cold. In *A. thaliana*, FLC did not affect this cold response. In *A. alpina*, intermittent cold caused induction of genes encoding GA metabolic enzymes and GA signaling components and PEP1 had a buffering effect on this, possibly to maintain plant growth under cold but non-freezing temperatures.

In conclusion, we used comparative ChIP-seq to identify a conserved core function of PEP1 and FLC in the regulation of flowering as well as species-specific functions based on novel interactions between conserved developmental and environmental response pathways. Thus, the evolution of new TF BSs provides a mechanism to connect gene networks possibly to allow plants to adapt their developmental cycle to specific environments.

Zusammenfassung

Variation von Transkriptionsfaktorbindungsstellen ist eine Hauptursache der Artenvielfalt. Die Evolution von Transkriptionsfaktorbindungsstellen in Pflanzen bleibt weitgehend unerforscht. Hier führten wir die erste vergleichende ChIP-seq Studie in Kombination mit Genexpressionsanalysen in Gen-Knockout-Mutanten in zwei verwandten Pflanzenarten durch. Wir nutzen den FLOWERING LOCUS C (FLC) Transkriptionsfaktor in der einjährigen Pflanze *A. thaliana* und ihrer mehrjährigen Schwesterart *A. alpina* als Modelsystem. In *A. thaliana* reprimiert FLC die Blühinduktion vor der Vernalisierung. Das FLC Ortholog PERPETUAL FLOWERING 1 (PEP1) in *A. alpina* reprimiert die Blühinduktion nicht nur vor der Vernalisierung, sondern auch in Seitensprossen nach der Vernalisierung, sodass die Blühphase von einer vegetativen Wachstumsphase unterbrochen wird, was wesentlich für die mehrjährige Lebensweise von *A. alpina* ist.

Wir stellten fest, dass sich FLC- und PEP1-Bindungsstellen stark unterscheiden aber beide Transkriptionsfaktoren identische CarG-Box Sequenzmotive in den Promotoren ihrer Zielgene binden. Konservierte Bindungsstellen hingen mit konservierten CArG-Boxen zusammen, welche oft um das ‚TTT'-Trinukleotid erweitert waren. Artenspezifische Bindungsstellen hingen mit der Abwesenheit einer CArG-Box in der anderen Art zusammen. Obwohl diese Zusammenhänge hochsignifikant waren, fanden wir Hinweise, dass auch andere Transkriptionsfaktoren das Binden von DNA beeinflussen. Funktionale Analyse der Zielgene zeigte, dass konservierte Zielgene hauptsächlich in die Blühinduktion und Blütenentwicklung involviert waren. Artenspezifische Zielgene von beiden Transkriptionsfaktoren waren in Hormon- und Umweltantworten involviert, was darauf hindeutet, dass FLC und PEP1 durch konvergente Evolution ähnliche Rollen evolviert haben.

Beide Transkriptionsfaktoren banden zahlreiche Kälte-regulierte Gene und reprimierten ihre Induktion durch vorrübergehende Kälte. Dies deutet darauf hin, dass PEP1 und FLC in Kälte agieren und die Kälteantwort unterdrücken. Zeitweilige Kälte führt zu einer Wachstumsretardierung. Somit könnte diese Rolle von FLC und PEP1 einen Kompromiss representieren, der das Wachstum bei vorrübergehender Kälte vor der Vernalisierung aufrecht erhält.

Desweiteren banden PEP1 und FLC beide unterschiedliche Gene mit Funktionen im GA Metabolismus und in der GA Signaltransduktion. Die *A. alpina pep1-1* Mutante zeigte verschiedene Phänotypen GA behandelter Pflanzen, wie zum Beispiel verlängerte Internodien. GA förderte die Blühinduktion während der Vernalisierung in *A. alpina* und Gene mit Funktionen im GA Metabolismus und in der GA Signaltransduktion wurden induziert und PEP1 wirkte entgegen diese Induktion. Die GA Konzentration war während der Vernalisierung jedoch verringert und nicht erhöht und PEP1 beeinflusste dies nicht. In *pep1-1* war die GA Konzentration lediglich in jungen Sprossspitzen leicht erhöht. Zudem zeigte *pep1-1* eine verstärkte GA Signaltransduktion. Zusammengefasst suggerieren diese Ergebnisse, dass PEP1 ein negativer Regulator der GA Konzentration ist, was zu verminderter Elongation von vegetativen

Sprossen führt und womöglich zu einer erhöhten Pflanzenstabilität beiträgt. Zudem könnte PEP1 während der Vernalisierung die GA Signaltransduktion inhibieren um die Blühinduktion zu unterdrücken. Diese Interaktion zweier Signalwege der Blühinduktion welche auf GA und Vernalisierung antworten representiert eine artenspezifische Interaktion von zwei konservierten Signalwegen und könnte bewirken, dass die Blühinduktion nach kurzer Vernalisierungszeit, bevor der alpine Winter vorrüber ist, verhindert wird. Auch FLC in *A. thalinana* regulierte die Expression von Genen im GA Netzwerk, allerdings konnten keine GA-Phänotypen identifiziert werden. Auch vorrübergehende Kälte führte zu einer Induktion der Expression von GA-Genen. In *A. thaliana* war dies nicht von FLC beeinflusst. In *A. alpina* führte vorrübergehende Kälte zur Induktion von Genen mit Funktionen im GA Metabolismus und in der GA Signaltransduktion und PEP1 wirkte als Puffer auf diesen Effekt, möglicherweise um das Wachstum bei vorrübergehend kalten Temperaturen über dem Gefrierpunkt aufrecht zu erhalten.

In dieser Studie nutzten wir vergleichende ChIP-seq Experimente um eine konservierte Funktion von PEP1 und FLC in der Blühzeitpunktkontrolle sowie artenspezifische Funktionen, basierend auf neuen Interaktionen zwischen konservierten Entwicklungs- und Umweltantwortsignalwegen, zu identifizieren. Somit stellt die Evolution neuer Transkriptionfaktorbindungsstellen einen Mechanismus zur Interaktion von bestehenden Gennetzwerken dar, welche Pflanzen eine Anpassung des Entwicklungszyklus and spezielle Umweltbedingungen erlaubt.

Table of contents

1 Introduction

1.1 Phenotypic variation and the role of transcriptional regulation

Natural phenotypic variation within individuals of one species is the basis for local adaptation to different habitats. Separation of subpopulations of one species due to local adaptation to different habitats is thought to result in the evolution of new species. Already in 1975, it was proposed that differential gene regulation, rather than differences in gene content, makes a major contribution to phenotypic variation between species (King & Wilson, 1975). More recent genome-wide studies in primates and other model organisms confirmed that gene regulation is a major aspect defining phenotypic variation (Romero *et al*, 2012). In plants, transcriptional networks that control plant development are well described (Kaufmann *et al*, 2010a) and examples demonstrate the important role of differential gene expression in interspecific variation of floral organ identity (Kanno *et al*, 2003; Di Stilio *et al*, 2005; Sather *et al*, 2010). Gene expression is regulated by transcription factors (TFs) that bind to DNA and positively or negatively regulate recruitment to genes of RNA Polymerase II, which catalyzes transcription. Differential gene regulation can be either due to changed TF activity (*trans*-effects) or to changes in the DNA sequence that affects TF binding (*cis*-effects). Studies using interspecies hybrids of yeasts, plants and fruit flies suggested that differential gene regulation between species was mainly due to *cis*-effects which likely caused variation in TF binding, while *trans*-effects played a minor role (He *et al*, 2012; Tirosh *et al*, 2009; Wittkopp *et al*, 2008).

1.2 Variation of transcription factor binding sites

Modern sequencing-based technologies allow the identification of genome-wide binding profiles for TFs. Numerous studies focused on conservation of TF binding sites (BSs) in different vertebrate species (Villar *et al*, 2014), but BS conservation of plant TFs remains largely uninvestigated (Muiño *et al*, 2016). Among vertebrates, conservation of BSs for developmental TFs is rather low and generally, the rate of conservation decreases exponentially with increasing evolutionary distance (Villar *et al*, 2014). For example, conservation of the CEBPα TF, a TF involved in liver cell specification, decreases exponentially with evolutionary distance in seven vertebrate species and six rodent species (Schmidt *et al*, 2010; Stefflova *et al*, 2013; Ballester *et al*, 2014). Conservation of CEBPα BSs between human and macaque, that have an evolutionary distance of about 30 million years, is less than 30 % (Ballester *et al*, 2014). Also the analysis of binding profiles of various TFs in different tissues of human and mouse showed varying but low degrees of conservation (Denas et al, 2015). Low conservation of TF

binding profiles was also observed in yeast. For instance, a ChIP-chip study comparing BSs for the MADS-box TF MCM1, a TF controlling mating and cell-cycle regulation, revealed low conservation in three yeast species (Tuch *et al*, 2008). Also BS conservation of pseudohyphal regulators Ste12 and Tec1 in three yeast species was limited (Borneman *et al*, 2007). Conservation of TF BSs in *Drosophila* species, however, is rather high and the rate of conservation decreases linearly, not exponentially with evolutionary distance (Villar et al, 2014). BSs of the embryo development TF Twist were highly conserved in six *Drosophila* species and more than 60 % of Twist BSs were conserved between two *Drosophila* species with an evolutionary distance of 30 million years (He et al, 2011). Similar results were obtained for six TFs that regulate segmentation in two *Drosophila* species (Bradley et al, 2010). The higher level of BS conservation in *Drosophila* species compared to vertebrates might be explained by their smaller genome size (Villar *et al*, 2014). Compared for example to the human genome, the genomes of *Drosophila* species are much smaller and include less intergenic space. This, together with the larger population size, leads to a decreased tolerance of random mutations (González & Petrov, 2012), which might have resulted in the evolution of new TF BSs.

Despite high variation of BSs between species, TFs generally bind identical DNA sequence motifs in different species and the majority of species-specific TF BSs could be correlated with the presence of a DNA-binding motif in that species that was absent in the other species. Various studies in vertebrates, yeast and *Drosphila* found the same DNA motif enriched in BSs of orthologous TFs in different species and for species-specific BSs, this DNA motif was absent in the other species (Borneman *et al*, 2007; Odom *et al*, 2007; Tuch *et al*, 2008; Wilson *et al*, 2008; Bradley *et al*, 2010; Schmidt *et al*, 2010; He *et al*, 2012; Ballester *et al*, 2014). A suggested mechanism for evolution of short DNA-binding motifs on a short time scale is local base-pair substitutions (Stone & Wray, 2001). Indeed, numerous studies showed that species-specific DNA motifs mainly evolved by small sequence changes (Schmidt *et al*, 2010; Bradley *et al*, 2010; He *et al*, 2011). Furthermore, in some cases, species-specific DNA motifs were found to be associated with the insertion of transposable elements (TEs) (Kunarso *et al*, 2010; Schmidt *et al*, 2012). Evolution of species-specific binding can also be more complex. Denas *et al*., described repurposing of TF BSs in mouse and human, where binding to a DNA motif occurs at different times, in different tissues or by different TFs (Denas et al, 2015). In other studies, for example in the case of the yeast TF Ste12, most BSs do not have a consensus DNA-motif (Borneman et al, 2007). A recent review on comparisons between human individuals of TF BSs even describes a paradigm shift, claiming that most changes in TF binding are not associated with changes in the short DNA-motif they recognize (Deplancke et al, 2016). One

possible explanation could be that TF binding is influenced by interacting TFs that bind DNA-motifs in the proximity, as described in several publications (Bradley et al, 2010; He et al, 2011; Heinz et al, 2013; Stefflova et al, 2013). Ballester *et al*. found for instance that in vertebrates, BSs that are clustered with BSs of other TFs are generally more conserved and more strongly bound and absence of one TF affects binding of the others (Ballester et al, 2014). Also in plants, TFs were found to affect binding of their interacting TFs. For example, binding of the plant MADS-box TF FLOWERING LOCUS C (FLC) can depend on the presence of SHORT VEGETATIVE PHASE (SVP) and *vice versa* (Mateos et al, 2015). Moreover, binding of SVP is affected by the presence of its interactor FLOWERING LOCUS M (FLM) (Posé et al, 2013a). TFs can also be recruited to DNA indirectly by binding to a DNA-bound interactor. The important role of indirect binding of plant TFs was emphasized by the finding that indirect and direct TF-DNA interactions equally affect gene expression (Heyndrickx et al, 2014). In addition, other factors that could influence the conservation of binding are regions flanking the core motif that might affect the DNA structure (Muiño et al, 2014), chromatin accessibility (Degner et al, 2012; Shibata et al, 2012) or DNA methylation (Domcke et al, 2015).

Even though conservation of BSs is generally rather low, the core function of developmental transcription factors is often quite conserved. This functional conservation was explained by higher conservation of binding to genes related to the core function of the TF (Tuch et al, 2008; Odom et al, 2007; Schmidt et al, 2010; Muiño et al, 2016; Ballester et al, 2014; He et al, 2011). In many other cases, the core function of a TF was maintained by compensatory binding to a different site associated with the same gene in the other species (Odom et al, 2007; Kunarso et al, 2010; Schmidt et al, 2010; Denas et al, 2015; Heinz et al, 2013). BSs associated with species-specific target genes, on the other hand, were suggested to be an adaptation to different environmental conditions in yeast (Borneman et al, 2007; Tuch et al, 2008), whereas in animals, species-specific BSs are often considered to be non-functional (MacArthur et al, 2009; Schmidt et al, 2010; He et al, 2011).

To date, only one comparative ChIP-seq study has been performed in plants. BSs of the MADS-box TF SEPALATA 3 (SEP3), which is a key factor in flower development, were compared between *A. thaliana* and *Arabidopsis lyrata* and around 21 % of the BSs were conserved (Muiño *et al*, 2016). *A. thaliana* and *A. lyrata* have an evolutionary distance of about 10 million years (Clauss & Koch, 2006), suggesting that divergence of SEP3 BSs rather resembles the exponential decrease with evolutionary distance observed in vertebrates than the higher conservation that was found in *Drosophila* species. Species-specific BSs were associated with

the species-specific presence of *cis*-elements that evolved by small scale sequence changes and by TE insertion in the case of *A. lyrata*. Common target genes were associated with flower development, the core function of SEP3. Since no *sep3* mutant was available for *A. lyrata*, binding events could not be correlated with gene expression in this study. To obtain a broader picture of the evolution of TF BSs in plants and their influence on plant development, it will be crucial to extend the comparative analysis of plant TF BSs to additional TFs and additional species and to include the analysis of knock-out mutants. In addition, such analyses will reveal how the huge variation in genome size and TE content of closely related plant species (Michael, 2014) affects the evolution of TF BSs and thereby phenotypic variation in plants.

1.3 Variation in flowering time in *A. thaliana* and *A. alpina*

Flowering time is a highly adaptive trait and tight regulation of the timing of flowering ensures that reproduction occurs under optimal conditions to maximize seed production and thereby reproductive success. In the annual model species *A. thaliana*, flowering is controlled by environmental and internal signals and the different flowering pathways are interconnected, resulting in a complex regulatory network.

A. thaliana is a facultative long day plant. Under long photoperiods, as they occur in spring, flowering is promoted by the photoperiod pathway via transcriptional activation of the florigen-encoding gene *FLOWERING LOCUS T (FT)* (Turck *et al*, 2008). Under short photoperiods, as they occur in autumn and winter, the photoperiod pathway is inactive and flowering is delayed. In winter annual accessions of *A. thaliana*, flowering is actively repressed before winter by the floral repressor FLOWERING LOCUS C (FLC). Prolonged exposure to cold winter temperatures (vernalization) accelerates flowering by silencing *FLC* expression (Kim *et al*, 2009). In rapid cycling summer annual accessions, which complete their life cycle within one growing season, the vernalization pathway is not active. Mutations in the vernalization pathway account for a major proportion of the flowering time variation in *A. thaliana* (Johanson *et al*, 2000; Michaels *et al*, 2003). In addition, flowering time of *A. thaliana* is affected by ambient temperatures. Warm ambient temperatures promote flowering via the thermosensory pathway (Capovilla *et al*, 2015), while intermittent cold (short periods of cold temperature) that might occur in autumn, delays flowering by increasing expression levels of floral repressor *FLC* (Kim *et al*, 2004; Seo *et al*, 2009; Jung *et al*, 2012, 2013). Moreover, flowering time of *A. thaliana* is controlled by several endogenous pathways. Plant age regulates flowering through the two microRNAs miR156 and miR172 that have complementary expression patterns (Huijser & Schmid, 2011). The miR156 declines in abundance with plant age and in younger plants

represses flowering by repressing accumulation and translation of mRNAs encoding members of the family of SQUAMOSA PROMOTER BINDING-LIKE (SPL) TFs. SPL TFs induce transcription of floral promoting *MIR172b* and other targets (Wu *et al*, 2009; Hyun *et al*, 2016). The Gibberellin (GA) pathway promotes flowering in response to the plant hormone GA (Mutasa-Göttgens & Hedden, 2009) and finally, the autonomous pathway promotes flowering under non-inductive conditions by reducing the *FLC* transcript levels (Simpson, 2004). Signals of the different flowering pathways are integrated by floral integrator genes, including *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) at the shoot apical meristem (SAM) (Samach *et al*, 2000; Moon *et al*, 2003; Hepworth *et al*, 2002; Helliwell *et al*, 2006; Searle *et al*, 2006; Wang *et al*, 2009a) and transcriptional reprogramming is initiated causing the meristem identity to change into a floral meristem (Schmid *et al*, 2003). Analysis of natural variation in flowering time in 1135 *A. thaliana* accessions proposed components of the vernalization and photoperiod pathway as well as meristem regulators as main factors causing differences in flowering time (Alonso-Blanco *et al*, 2016). The effects of other flowering pathways and interactions among them on natural variation in flowering time is just beginning to be uncovered (reviewed in Koornneef *et al*, 2004). For instance, recently natural variation within the ambient temperature pathway was discovered (Lutz *et al*, 2015).

The alpine perennial plant *Arabis alpina* is a close relative of *A. thaliana* and became a model for the perennial life cycle within the Brassicaceae family. *A. alpina* is a polycarpic perennial plant, and thus flowers repeatedly during its life span. Floral transition of several meristems occurs in every growing season but the flowering period is restricted and is followed by a period of vegetative growth (Wang et al, 2009b). Flowering time of *A. alpina* is regulated by similar pathways as in *A. thaliana* but several regulatory differences were associated with its perennial flowering behavior. Flowering in *A. alpina* is repressed before winter and vernalization is obligatory for many accessions of *A. alpina* to flower. As in *A. thaliana*, vernalization causes silencing of a floral repressor, but silencing of *FLC* ortholog *PERPETUAL FLOWERING 1* (*PEP1*) in *A. alpina*, is not stable. In consequence, PEP1 can be active after floral induction when it causes restriction of the flowering period and represses flowering in some of the side shoots (Wang *et al*, 2009b). Thereby PEP1 ensures that the flowering phase is followed by a period of vegetative growth, which allows the plant to survive flowering and grow vegetatively until the next winter, a crucial aspect of perennialism. Mutations in the vernalization pathway in *A. alpina* were associated with natural variation in flowering time and in the duration of the flowering period (Albani *et al*, 2012). Compared to *A. thaliana*, *A. alpina* also has a prolonged juvenile phase when flowering is repressed by the age pathway and the plant is not competent

to respond to floral-promoting stimuli (Wang *et al*, 2011; Bergonzi *et al*, 2013). In *A. alpina*, the miR156 pathway appears not to be mechanistically coupled to the miR172 pathway. The two pathways act in parallel and plants need both activating stimuli, adult plant age and vernalization, to be competent to flower (Bergonzi *et al*, 2013). In addition, AaTFL1, the ortholog of *A. thaliana* floral repressor TERMINAL FLOWER 1 (TFL1), prevents flowering of juvenile plants after vernalization by blocking induction of floral meristem identity genes (Wang *et al*, 2011).

The *A. thaliana* and *A. alpina* species pair has proven to be an instructive study system to investigate interspecific differences in the regulation of flowering pathways and their contribution to variation in the flowering behavior that is associated with different life histories of these species (Wang *et al*, 2009b; Albani & Coupland, 2010; Andrés & Coupland, 2012; Bergonzi *et al*, 2013).

1.4 The FLC transcription factor and its role in the regulation of flowering

The FLC TF is a floral repressor that inhibits flowering of *A. thaliana* prior to vernalization (Koornneef *et al*, 1994; Lee *et al*, 1994; Michaels & Amasino, 1999; Sheldon *et al*, 1999). The *A. alpina* FLC ortholog PEP1 also represses flowering before vernalization, however PEP1 has additional functions that are crucial for the perennial flowering behavior of *A. alpina* (Wang *et al*, 2009b). In addition to repressing flowering before vernalization, PEP1 also represses flowering in some of the axillary shoots and restricts the flowering phase to ensure succession of vegetative and reproductive phases in the life cycle of *A. alpina* (Wang *et al*, 2009b).

1.4.1 Regulation of *FLC*

FLC encodes a MADS-box TF and its transcription is repressed by vernalization (Michaels & Amasino, 1999; Sheldon *et al*, 1999). Before vernalization, *FLC* transcription is promoted by co-transcriptional activator FRIGIDA (FRI) (Michaels & Amasino, 1999; Sheldon *et al*, 1999; Johanson *et al*, 2000; Geraldo *et al*, 2009). Silencing of *FLC* by vernalization is associated with the accumulation of the histone mark H3K27me3 at the locus, which is generally linked to repression of gene expression (Bastow *et al*, 2004; Sung & Amasino, 2004). H3K27me3 of *FLC* is mediated by POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) and associated PHD-finger proteins, including VERNALIZATION INSENSITIVE 3 (VIN3) (Sung & Amasino, 2004). *FLC* is stably repressed during vernalization (Sheldon *et al*, 2000), so that after vernalization flowering is induced in all meristems and the plant can produce a maximum number of seeds before senescence. During embryo development, *FLC* transcription is reset and FLC is active to repress flowering in the progeny (Sheldon *et al*, 2008). In *A. alpina*, *PEP1*

transcription is also silenced during vernalization, which is also associated with the accumulation of H3K27me3, but *PEP1* silencing is not stable, allowing it to repress flowering repeatedly in subsequent growing seasons (Wang *et al*, 2009b).

1.4.2 The function of FLC in development of *A. thaliana*

FLC acts in the leaves to repress flowering by directly repressing transcription of *FT*, a key gene in the photoperiod pathway (Samach *et al*, 2000; Helliwell *et al*, 2006; Searle *et al*, 2006). In addition, FLC functions in the SAM to directly repress transcription of floral integrator *SOC1* (Hepworth *et al*, 2002; Helliwell *et al*, 2006; Searle *et al*, 2006) and of *FLOWERING LOCUS D* (*FD*) (Searle *et al*, 2006), which encodes an FT-interacting protein in the photoperiod pathway (Abe *et al*, 2005; Wigge *et al*, 2005). FLC is part of a multimeric complex (Helliwell *et al*, 2006) and physically interacts with the related MADS-box TF SVP (Li *et al*, 2008). Like FLC, SVP directly represses transcription of *FT* and *SOC1* (Li *et al*, 2008). Moreover, SVP is involved in the regulation of flowering in response to ambient temperatures (Lee *et al*, 2007) and acts to repress flowering via the GA pathway by indirectly repressing transcription of GA biosynthesis gene *GA20OX2* (Andrés *et al*, 2014). Comparative analysis of the effects of FLC and SVP on the transcriptome and of their genome-wide DNA binding profiles in the presence and absence of the other protein showed that FLC and SVP have mutually dependent, independent and redundant roles in regulating target gene expression (Mateos *et al*, 2015). This study also revealed a complex-dependent role of SVP and FLC in the GA-mediated control of flowering time (Mateos *et al*, 2015).

In addition, FLC interacts with several other flowering pathways. FLC directly represses expression of *SPL15* (Deng *et al*, 2011), which promotes flowering by positively regulating transcription of, for instance, floral integrator gene *FRUITFUL (FUL)* (Hyun *et al*, 2016). *SPL15* itself is negatively regulated by the age pathway in juvenile plants through miR156 and its protein activity is positively affected by the GA pathway (Schwarz *et al*, 2008; Hyun *et al*, 2016). Finally, *FLC* integrates ambient temperature signaling as well as signals from the autonomous pathway. *FLC* transcription decreases in response to warm ambient temperatures consistent with accelerated flowering (Blázquez *et al*, 2003; Lee *et al*, 2013), whereas intermittent cold induces *FLC* transcription (Seo *et al*, 2009). The latter involves key players in the cold-stress response CRT/DRE BINDING FACTOR 1 (CBF1) and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1) and causes delayed flowering in response to cold stress (Seo *et al*, 2009; Jung *et al*, 2013). Genes in the autonomous pathway,

as for example *FCA* or *FVE*, negatively regulate *FLC* transcript levels and thereby induce flowering in the absence of other activating stimuli (Simpson, 2004).

FLC has several hundreds of target genes in the *A. thaliana* genome (Deng *et al*, 2011; Mateos *et al*, 2015) and is expressed throughout the whole plant (Sheldon *et al*, 1999). Consistently, various functions besides the repression of flowering have been reported for FLC. In the vegetative stage, FLC promotes cold-induced seed germination (Chiang *et al*, 2009) and delays the progression of the juvenile to adult transition (Deng *et al*, 2011; Willmann & Poethig, 2011). In the reproductive phase, FLC was associated with a positive effect on shoot branching (Huang *et al*, 2013) and was found to be involved in the regulation of flower development (Deng *et al*, 2011). Furthermore, FLC was shown to contribute to the high-temperature compensation mechanism of the circadian clock (Edwards *et al*, 2006). Finally, GO-term enrichment analyses in the genome-wide studies suggested a role of FLC in the response to environmental stresses caused by light and temperature and the response to the phytohormones ABA, JA and GA (Deng *et al*, 2011; Mateos *et al*, 2015).

1.4.3 The family of MADS-box transcription factors

FLC belongs to the family of MADS-box TFs. MADS-box TFs regulate key developmental processes in yeast, animals and plants (Shore & Sharrocks, 1995). All TFs in the family contain a conserved DNA-binding domain, the MADS-box domain, which was named after four founding members of the protein family; the yeast MINICHROMOSOME MAINTENANCE 1 TF, the *A. thaliana* flower development TF AGAMOUS (AG), the *Antirrhinum majus* flower development TF DEFICIENS A and the human SERUM RESPONSE FACTOR (Schwarz-Sommer *et al*, 1990). The MADS-box domain binds the DNA at a CArG-box motif with the consensus sequence $CC[A/G]_6GG$ (de Folter & Angenent, 2006). Plant MADS-box TFs form heterodimers (Folter *et al*, 2005; Smaczniak *et al*, 2012b) and those form higher order complexes and interact with other types of TFs and chromatin remodelers (Egea-Cortines *et al*, 1999; Honma & Goto, 2001; Smaczniak *et al*, 2012b). A dimer of MADS-box TFs binds one CArG-box, each TF occupying one half-site of the motif (Schwarz-Sommer *et al*, 1992; Pellegrini *et al*, 1995). According to the quartet model for floral development, tetramers of MADS-box TFs bind two CArG-boxes and different tetramers specify the different floral organs (Theissen, 2001; Theissen & Saedler, 2001). How DNA binding specificity for different MADS-box TFs is determined is only starting to become clear. Different MADS-box TFs preferentially bind distinct CArG-box sequences and cause different degrees of DNA bending due to sequence changes in their MADS-box domain (Nurrish & Treisman, 1995; Riechmann

et al, 1996). DNA binding affinity is determined by the energy that is required to bend the DNA, and recently it has been shown that different MADS-box TFs favor so-called A-tracts (a stretch of A_mT_n with a minimum length of 4 bp) of different length in the CArG-box and in the flanking regions (Muiño *et al*, 2014). In addition, different MADS-box TFs bind different sites at different developmental stages, which might be due to the presence of different interaction partners (Pajoro *et al*, 2014) and might influence whether they activate or repress transcription (Kaufmann *et al*, 2010b; Wuest *et al*, 2012).

1.4.4 Evolutionary conservation of FLC

Repression of flowering prior to vernalization is not specific to *A. thaliana* and other members of the Brassicaceae but can be found throughout the plant kingdom. In monocotyledonous species, however, the vernalization pathway involves other genes than *FLC*. In wheat, vernalization causes induction of the MADS-box TF *VERNALIZATION 1 (VRN1)*, which is a homolog of the *A. thaliana* floral meristem identity genes *AP1/FUL* (Yan *et al*, 2003). VRN1 then represses the floral repressor *VERNALIZATION 2* (*VRN2*), which encodes a CCT-domain protein (Yan *et al*, 2004). A recent study, however, identified *FLC*-like genes in monocots and *FLC* in *Brachypodium* was found to be regulated by vernalization, suggesting it might contribute to the vernalization response (Ruelens *et al*, 2013). A function of *FLC* in the vernalization response was confirmed in dicotyledonous species outside of the Brassicaceae family, for instance in *Beta vulgaris* (Reeves *et al*, 2007).

Within the Brassicaceae family, *FLC* has been identified and associated with the vernalization response in various winter annual species like *Raphanus sativus* and three different *Brassica* species *B. napus*, *B. rapa* and *B. oleracea* (Tadege *et al*, 2001; Kim *et al*, 2007; Ridge *et al*, 2014; Li *et al*, 2016). Furthermore, as described above for *A. alpina*, *FLC* expression was found to cycle with the seasons in several perennial Brassicaceae species including *Arabidopsis halleri*, *A. lyrata* and *Cardamine flexuosa* (Wang *et al*, 2009b; Aikawa *et al*, 2010; Kemi *et al*, 2013; Zhou *et al*, 2013a). In these perennial species, FLC was shown to repress flowering repeatedly during the plant life cycle, corresponding to the seasonal flowering pattern. Thus, *FLC* orthologs have a similar function in the background of different life histories in *A. thaliana* and perennial relatives, however, conservation of the molecular function or target gene conservation between species has not been investigated.

1.5 The Role of GA in the regulation of flowering

Genome-wide analyses of FLC direct target genes suggested a role of FLC in the regulation of GA-mediated signaling (Deng *et al*, 2011; Mateos *et al*, 2015). GAs are plant hormones that

promote flowering in *A. thaliana*. GA is necessary for flowering under non-inductive SD conditions (Wilson *et al*, 1992) and has a weaker effect under LDs (Griffiths *et al*, 2006), when the photoperiod pathway is dominant (Reeves & Coupland, 2001). GA levels strongly increase at the SAM of SD grown plants prior to floral induction (Eriksson *et al*, 2006). This increase was concluded to be a result of GA transport, since it does not correlate with increased expression of genes that encode GA biosynthetic enzymes (Eriksson *et al*, 2006). The form of GA that is transported to the SAM might be GA12, a precursor of active GAs, which was suggested to be the major mobile GA in *A. thaliana* (Regnault *et al*, 2015). However, GA metabolism might also play a role in floral induction since mutations in GA-biosynthesis genes *GA3-OXIDASE 1* (*GA3OX1*) and *GA3OX2*, as well as mutations in genes encoding GA2 oxidases affect flowering time (Mitchum *et al*, 2006; Rieu *et al*, 2008a). The GA metabolic pathway is summarized in Figure 1A. Under SD conditions, GA acts in the apex to induce transcription of the floral integrator *SOC1* (Moon *et al*, 2003) and the floral meristem identity gene *LEAFY (LFY)* (Blázquez *et al*, 1998). Under LD conditions, GA promotes flowering via the photoperiod and the age pathway as GA is required to induce *FT* expression in the leaves and the expression of *SPL* genes in leaves and apices (Hisamatsu & King, 2008; Galvão *et al*, 2012; Porri *et al*, 2012).

GA is bound by GIBBERELLIN INSENSITIVE DWARF 1 (GID1) GA receptors, which, in the presence of GA, interact with DELLA repressor proteins (Figure 1B). This interaction promotes the interaction of DELLAs with an E3 ubiquitin ligase complex that targets DELLA proteins for degradation by the 26S proteasome (Griffiths *et al*, 2006; Nakajima *et al*, 2006) (Figure 1B). In the absence of GA, DELLA repressor proteins affect the flowering-promoting activity of several transcription factors. DELLA proteins interact with SPL9 and affect its function (Yu *et al*, 2012; Yamaguchi *et al*, 2014). SPL9 induces transcription of *AP1* and this is positively affected by the interaction with a DELLA protein (Yamaguchi *et al*, 2014) whereas the induction of *MIR172b* and *SOC1* by SPL9 is negatively affected by DELLAs (Wang *et al*, 2009a; Yu *et al*, 2012). Thus GA positively affects floral induction but negatively affects flower development via SPL9. DELLAs also interact with SPL15, a closely related paralog of SPL9 (Hyun *et al*, 2016). SPL15 promotes flowering under non-inductive SD conditions by inducing transcription of its target genes *FRUITFUL* (*FUL*) and MI*R172b* (Hyun *et al*, 2016, 2017). DELLA proteins bind SPL15 at the promoter of its target genes at the SAM to repress its activity, thus GA promotes flowering under SD conditions. *SPL15* transcription is regulated by the age pathway via miR156 (Schwab *et al*, 2005) and by the vernalization pathway via FLC (Deng *et al*, 2011). In consequence, signals from the age, vernalization and GA flowering

pathway are integrated at the level of SPL15. In addition, DELLA degradation causes increased activity of the GAMYB33 TF that induces *LFY* expression (Gocal *et al*, 2001; Achard *et al*, 2004). DELLAs also repress activity of the PHYTOCHROME INTERACTING FACTOR (PIF) TFs (Feng *et al*, 2008; de Lucas *et al*, 2008). PIF3 induces flowering by directly repressing transcription of flowering regulators *GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED* (*GNC*) and *GNC-LIKE* (*GNL*) (Richter *et al*, 2010), and PIF3 as well as PIF4 promote flowering in the ambient temperature pathway (Kumar *et al*, 2012; Galvão *et al*, 2015).

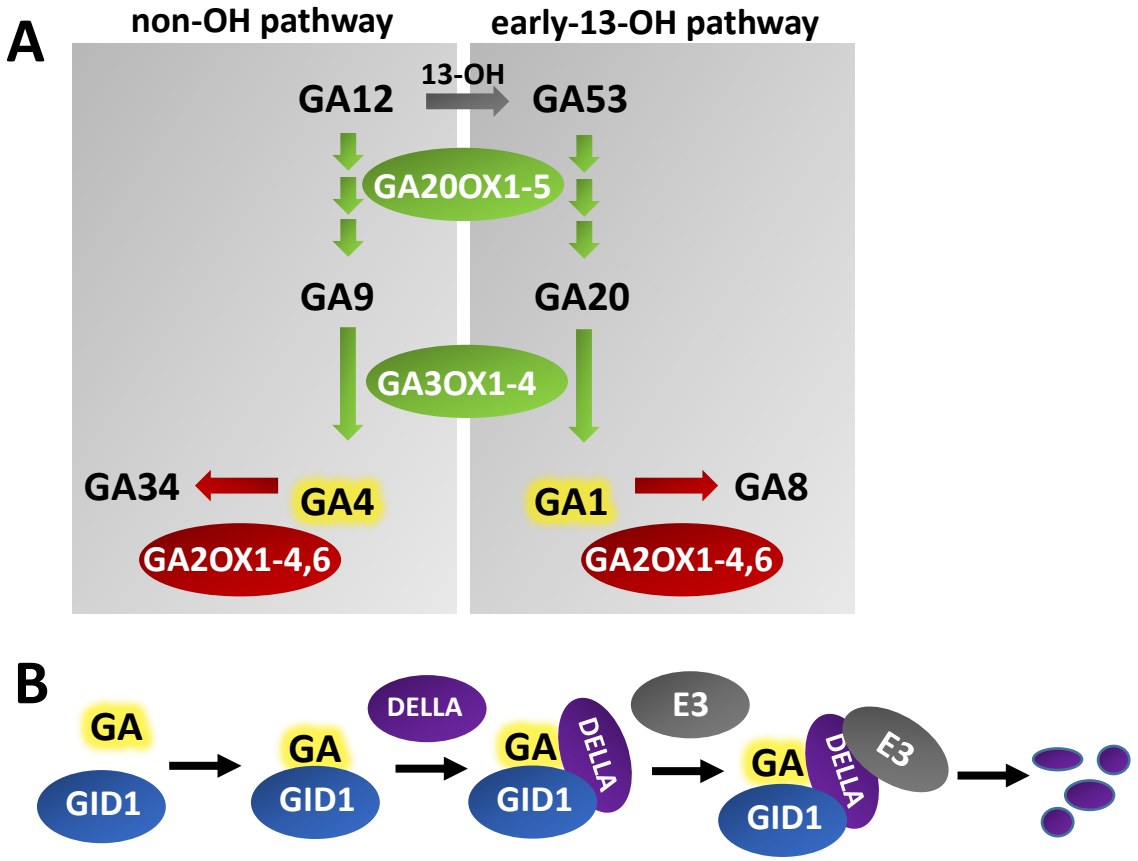


Figure 1 Model of GA metabolism and signaling.

(A) Model of GA metabolism in *A. thaliana*. GA12 is the common precursor of active GAs and can be hydroxylated to GA53 and processed in the early-13-OH pathway or the non-OH pathway by the same enzymes. GA12 and GA53 are oxidized by GA20OX1-5 in three steps leading to GA9 and GA20 (Phillips 1995). GA3OX1-4, then transform GA9 and GA20 into active GA4 and GA1 (Williams 1998, Yamaguchi 1998, Yamaguchi 2008, Hedden 2002). Which form of active GA is playing the major role varies between species (kobaiashi 1998, Polle 1995, Jordan 1995, Eriksson, Talon 1990, Lange 2005, Smith 1991, Metzger 1990). Active GA4 and GA1 as well as precursors GA9 and GA20 are degraded by GA2OX1-4, 6 (Rieu 2008). A different type of GA2OXes, GA2OX7-8, degrade early intermediates of the GA pathway including GA12 and GA53 (Schomburg 2003). Color code: Green: GA biosynthesis; Red: GA degradation; Yellow: active GA **(B)** Model of GA signaling in *A. thaliana*. Active GA binds GA receptor GID1 which then allows DELLAS and GID1-GA complex to interact. This interaction triggers E3 ubiquitin ligases to interact with DELLAs which targets them for degradation (Griffiths *et al*, 2006; Nakajima *et al*, 2006).

Besides promoting floral induction at the SAM and through regulation of different flowering pathways, GA is also required for other flowering-related traits, including bolting, flower development and determinacy of the floral meristem (Koornneef & van der Veen, 1980;

Griffiths *et al*, 2006; Rieu *et al*, 2008a; Achard *et al*, 2004; Hay *et al*, 2002; Jasinski *et al*, 2005). Furthermore, analysis of multiple mutants or overexpressors of genes encoding GA metabolism enzymes and GA receptors revealed that GA also affects various phenotypes at the vegetative stage. GA promotes germination, cell elongation which affects elongation of hypocotyl, stem and root as well as leaf expansion and morphology, trichome formation and branching and GA negatively regulates chlorophyll content (Koornneef & van der Veen, 1980; Schomburg *et al*, 2003; Griffiths *et al*, 2006; Mitchum *et al*, 2006; Rieu *et al*, 2008b, 2008a; Porri *et al*, 2012).

FLC was found to regulate several GA-related genes in *A. thaliana* (Deng *et al*, 2011; Mateos *et al*, 2015), however so far, FLC has not been implicated in the GA response on the phenotypic level and genetic analysis did not reveal a role in the vernalization response (Chandler *et al*, 2000). In summer annual *A. thaliana*, GA levels were found to increase prior to floral induction under non-inductive SD conditions (Eriksson *et al*, 2006) and increased GA levels can overcome the requirement for inductive LDs for flowering (Lang, 1957; Koornneef & van der Veen, 1980; Griffiths *et al*, 2006). In other species, like *Silene armeria* and *Spinacia oleracea*, GA levels increase in response to inductive LD conditions (Talon & Zeevaart, 1990; Zeevaart *et al*, 1993). Vernalization is not known to alter GA levels in *A. thaliana*, however, *A. thaliana* as well as *Eustoma grandiflorum* were found to be more responsive to exogenous GA after vernalization (Oka *et al*, 2001). It remains to be tested whether this phenomenon is due to direct repression of GA biosynthesis or of the GA signaling pathway by FLC or due to repressive effects of FLC on genes downstream of the GA pathway.

In contrast to vernalization, short cold treatments do affect GA levels in *A. thaliana*. Intermittent cold caused a reduction of GA levels by increasing transcription of genes encoding catabolic GA2OX enzymes, which leads to growth retardation under cold stress (Achard *et al*, 2008). During seed germination, cold has the opposite effect and causes an increase of GA levels by inducing transcription of *GA3OX1* and *GA20OX2/3*, which encode biosynthetic enzymes (Yamauchi *et al*, 2004). These data indicate that the effects of environmental stimuli on GA levels in *A. thaliana* are complex and dependent on developmental, tissue-specific factors.

In species other than *A. thaliana*, vernalization promotes induction of GA levels. In and *Thlaspi arvense*, another Brassicaceae species, and in *E. grandiflorum*, levels of precursors of active GAs increase during vernalization, whereas winter canola contains higher levels of active GA after vernalization (Hazebroek *et al*, 1993; Zanewich & Rood, 1995; Hisamatsu *et al*, 2004). Moreover, as shown in early studies, application of exogenous GA could overcome the vernalization requirement in *Raphanus sativus* and *T. arvense* (Suge & Rappaport, 1968;

Metzger, 1985). To summarize, GA plays different roles in the induction of flowering in different plant species in and outside of the Brassicaceae family. Interestingly, in trees, GA even has the opposite effect and inhibits flowering (Wilkie *et al*, 2008). Whether the vernalization and GA pathways interact in *A. thaliana* and whether this is conserved in the perennial sister species *A. alpina* remains to be tested.

1.6 Aims of this thesis

Diversification of TF BSs causing differential gene regulation is a major source of phenotypic variation between species. Here, the orthologous TFs *A. thaliana* FLC and *A. alpina* PEP1 were used as a model to investigate evolution of TF BSs in plants. To address this major aim, we performed the first study coupling ChIP-seq with expression analysis in knockout mutants for two related plant species. The resulting genome-wide data sets were first used to determine the rate of conservation and diversification of BSs in the two plant species with considerably different genome sizes. In a next step, the mechanism of diversification was investigated on DNA sequence level. Finally, functional diversification of the TFs was assessed by correlating BSs with gene expression data and furthermore, by analyzing gene ontology terms enriched among conserved and species-specific target genes.

FLC and PEP1 repress flowering in annual *A. thaliana* and perennial *A. alpina*, respectively and PEP1 defines perennial flowering traits in *A. alpina*. A second major aim of this work was to test whether differences in BSs and regulatory functions of FLC and PEP1 contribute to the distinct flowering behaviors of *A. thaliana* and *A. alpina* that are associated with their different life histories. Previously, FLC was suggested to regulate the GA response in *A. thaliana*. Here we aimed to investigate whether FLC and PEP1 regulate the GA flowering pathway as part of the vernalization response. Finally, we addressed whether a putative interaction between the vernalization and the GA flowering pathway is conserved between species or could contribute to the distinct life histories of *A. thaliana* and *A. alpina*.

2 Evolution of PEP1 direct targets

2.1 Introduction

Changes in gene regulation caused by variation in TF BSs are a major source of phenotypic variation between species (Romero *et al*, 2012). In plants, conservation of TF BSs in related species has not been widely studied (Muiño *et al*, 2016). This study aimed to extend the knowledge on conservation of TF BSs in plants by comparing BSs of PEP1 and FLC, two

orthologous MADS-box TFs that repress flowering in *A. alpina* and *A. thaliana*, respectively (Koornneef *et al*, 1994; Lee *et al*, 1994; Wang *et al*, 2009b).

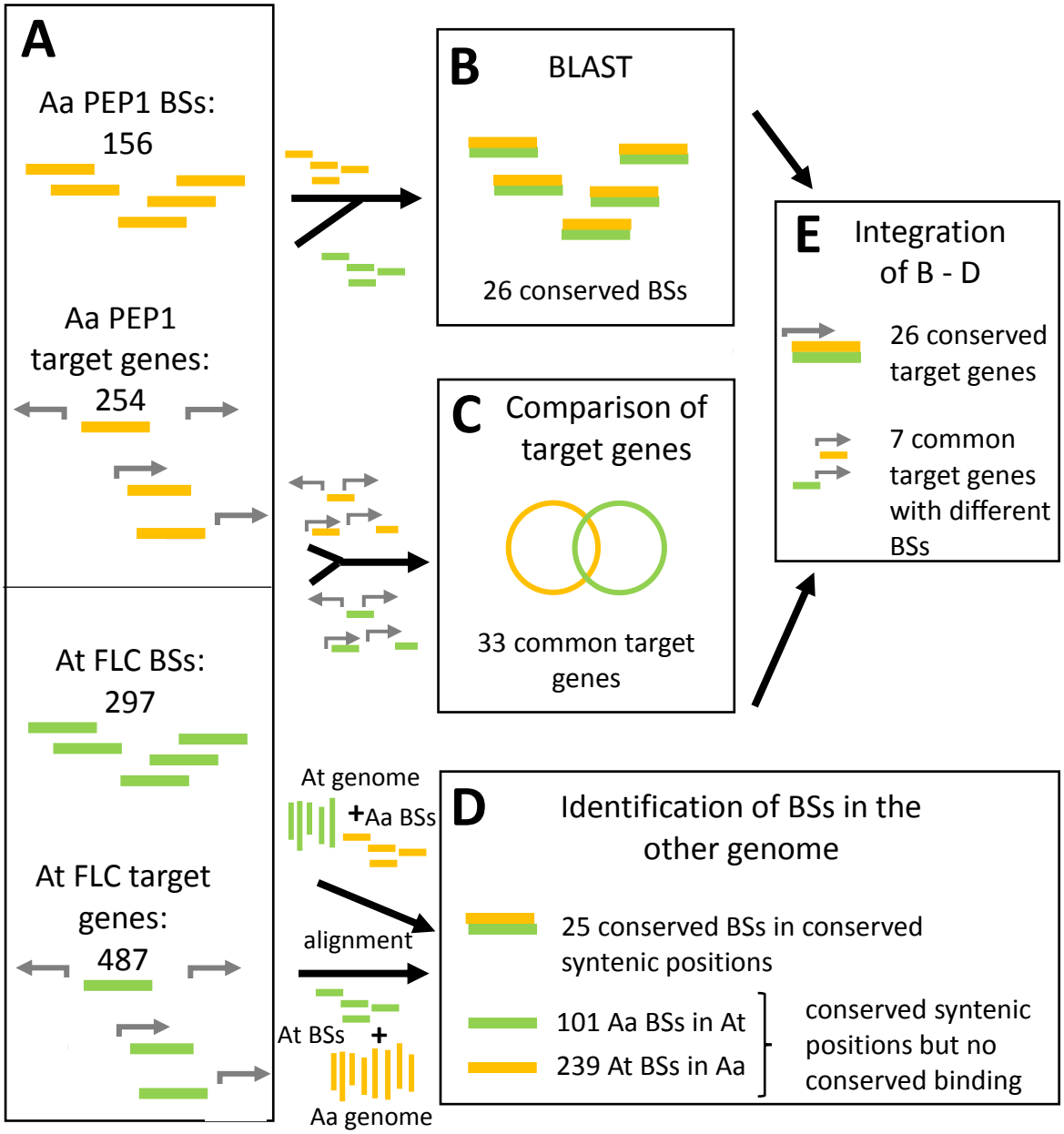


Figure 2 Comparative analysis of PEP1 and FLC BSs and target genes.

Flow diagram representing the different steps of data analysis. **(A)** Top: PEP1 BSs and associated neighboring genes (PEP1 direct target genes). Bottom: FLC BSs and associated neighboring genes (FLC direct target genes). **(B)** BLAST was used to compare PEP1 BSs with FLC BSs to identify conserved BSs. **(C)** PEP1 and FLC direct target genes were compared to identify common target genes. **(D)** PEP1 BSs were identified in the *A. thaliana* genome and FLC BSs were identified in the *A. alpina* genome. Conservation of synteny was tested based on associated genes, in consequence only BSs with associated genes were included in the analysis. **(E)** Results of B - D were integrated to identify conserved target genes with conserved BSs in contrast to common target genes with diverging BSs. PEP1 BSs are represented in orange, FLC BSs in green. Target genes are represented by grey arrows. Vertical bars represent *A. alpina* (orange) and *A. thaliana* (green) chromosomes.

Chromatin-immunoprecipitation followed by high-throughput sequencing (ChIP-seq) was performed with PEP1 antiserum to identify PEP1 BSs in the *A. alpina* genome. Wild-type plants

grown for 2 weeks under LD conditions were compared to the *pep1-1* mutant as negative control in three biological replicates. This analysis identified 156 BSs present in at least two replicates, which were associated with 254 neighboring genes, considered as PEP1 direct target genes (Figure 2A, Table A1, data were kindly provided by J. Mateos and P. Madrigal). To have a comparable dataset for FLC, ChIP-seq was performed under the same conditions in two replicates of *A. thaliana* wild-type and *flc-3* using novel FLC antiserum. 297 BSs, which were associated with 487 FLC direct target genes, were identified (Figure 2A, Table A2, data were kindly provided by J. Mateos, R. Richter and P. Madrigal). Despite different experimental conditions, this experiment showed high overlap (50-60 %) with previously published ChIP-seq studies for FLC (Deng *et al*, 2011; Mateos *et al*, 2015). Comparison of PEP1 BSs with FLC BSs by BLAST analysis revealed that only 26 of the BSs (17 % of PEP1 BSs) were conserved between species (Figure 2B, data provided by J. Mateos). Comparison of target genes, independently of BSs, identified 33 genes that were commonly bound by PEP1 and FLC (Figure 2C, Table A1, Table A2, data provided by J. Mateos). To rule out that the lower number of BSs in *A. alpina* and the limited overlap with *A. thaliana* is due to lower genome coverage in the ChIPseq experiment, the PEP1 ChIPseq experiment was repeated with and a higher number of reads was obtained. 2 replicates of the previous experiment were re-sequenced together with one new replicate and 204 PEP1 BSs, which were associated to 331 target genes were identified (Table A3). 84 % of previously identified target genes are also in the new dataset and the overlap with FLC target genes is 11 % compared to previously 13 %. Thus the new experiment confirms that PEP1 has less BSs than FLC and the overlap between species is low. All analyses presented here were performed with the first dataset.

In addition, RNAseq was performed comparing apices and leaves of wild-type and *pep1-1* mutant plants to identify genes that were regulated by PEP1 (Figure 3A, Table A4, data provided by J. Mateos). Between the genotypes, 96 genes were differentially expressed in apices and 325 genes in leaves (Figure 3A, Table A4). Most of the genes differentially regulated in *pep1-1* were up-regulated in the mutant, suggesting that PEP1 acts almost exclusively as a transcriptional repressor, in agreement with what was previously shown for FLC (Mateos *et al*, 2015). In addition, a relatively low proportion of genes differentially expressed in *pep1-1* were direct targets of PEP1, but all of those were up-regulated in *pep1-1* (Figure 3A, Table A1Table A1, Table A4), suggesting that all direct effects of PEP1 are repressive. The transcriptomic data for *pep1-1* were compared to published results for FLC-regulated genes in *A. thaliana* (Mateos *et al*, 2015). Only a low number of genes were commonly regulated by PEP1 and FLC in leaves or apices, similar to what was described above for the ChIP-seq data

(Figure 3B). Interestingly, PEP1 regulated a higher number of genes in apices than in leaves whereas FLC regulated a higher number of genes in leaves than in apices.

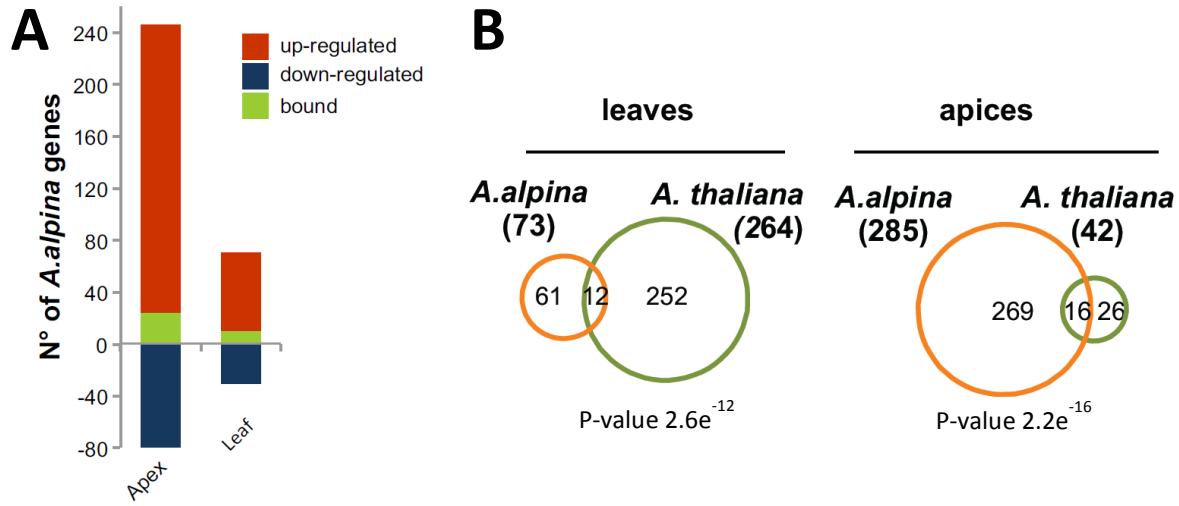


Figure 3 Comparison of genes that were regulated by PEP1 and FLC.

(A) Genes that were up-regulated or down-regulated in apices or leaves of *pep1-1* and the proportion of those that were bound by PEP1. **(B)** Venn diagrams comparing DEG in leaves and apices of the *A. thaliana flc-3* mutant and the *A. alpina pep1-1* mutant. P-value indicates significance of overlap, tested by hypergeometric test.

These datasets were used in the present study to investigate the rate of divergence of PEP1 and FLC BSs, the molecular mechanism that might have caused this divergence and the consequences of BS divergence on FLC and PEP1 functions.

2.2 BSs of PEP1 and FLC are largely divergent

Comparison of sets of PEP1 and FLC BSs, direct target genes and genes that are regulated by these TFs indicated low levels of conservation between species (Figure 2B, C; Figure 3B). To pave the way for understanding the evolution of PEP1 and FLC BSs, in this first section, I analyzed and compared BSs and direct or indirect target genes of PEP1 and FLC in more detail.

First, binding of PEP1 to BSs associated with selected target genes was validated by ChIP-qPCR. Significant enrichment of PEP1 binding was detected for all sites tested, whereas no significant enrichment could be detected in the negative control regions that were 1-2 kb distant from the BSs (Figure 4). Thus, this experiment confirms that our dataset contains high confidence PEP1 BSs. These validated BSs include some sites that were in common with FLC that were previously validated (Deng *et al*, 2011).

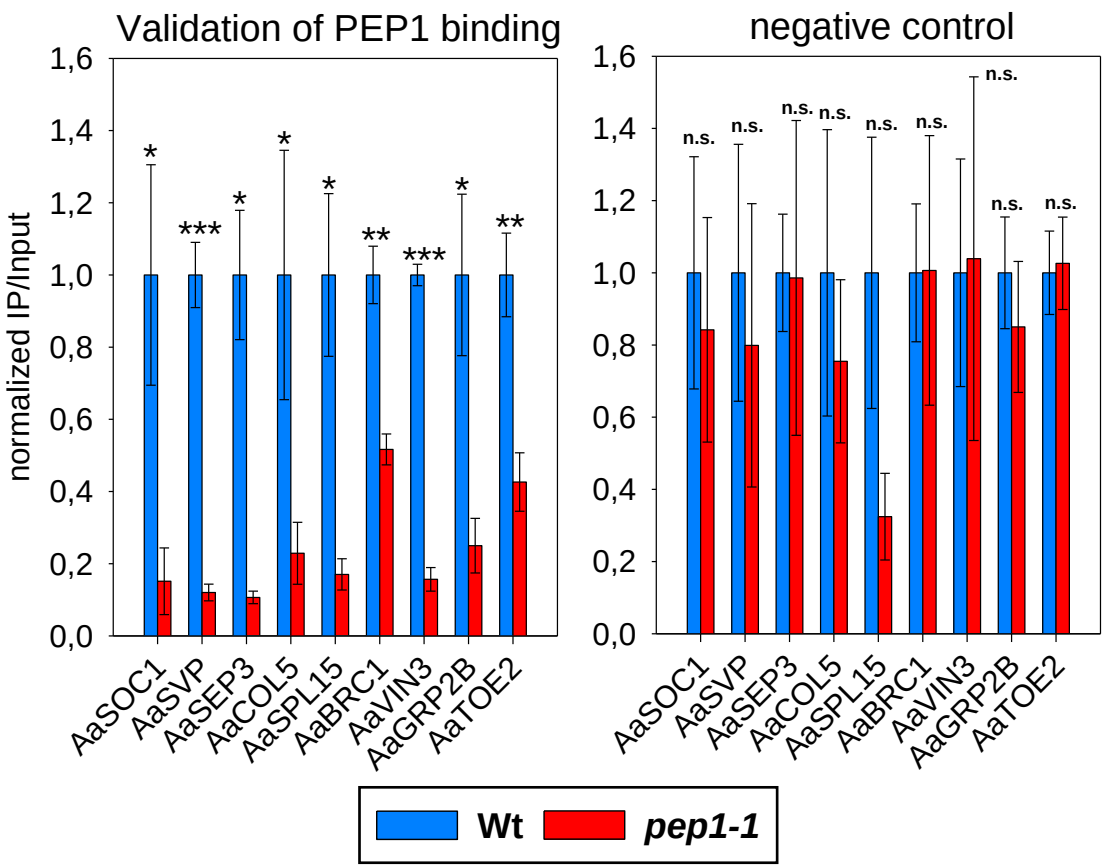
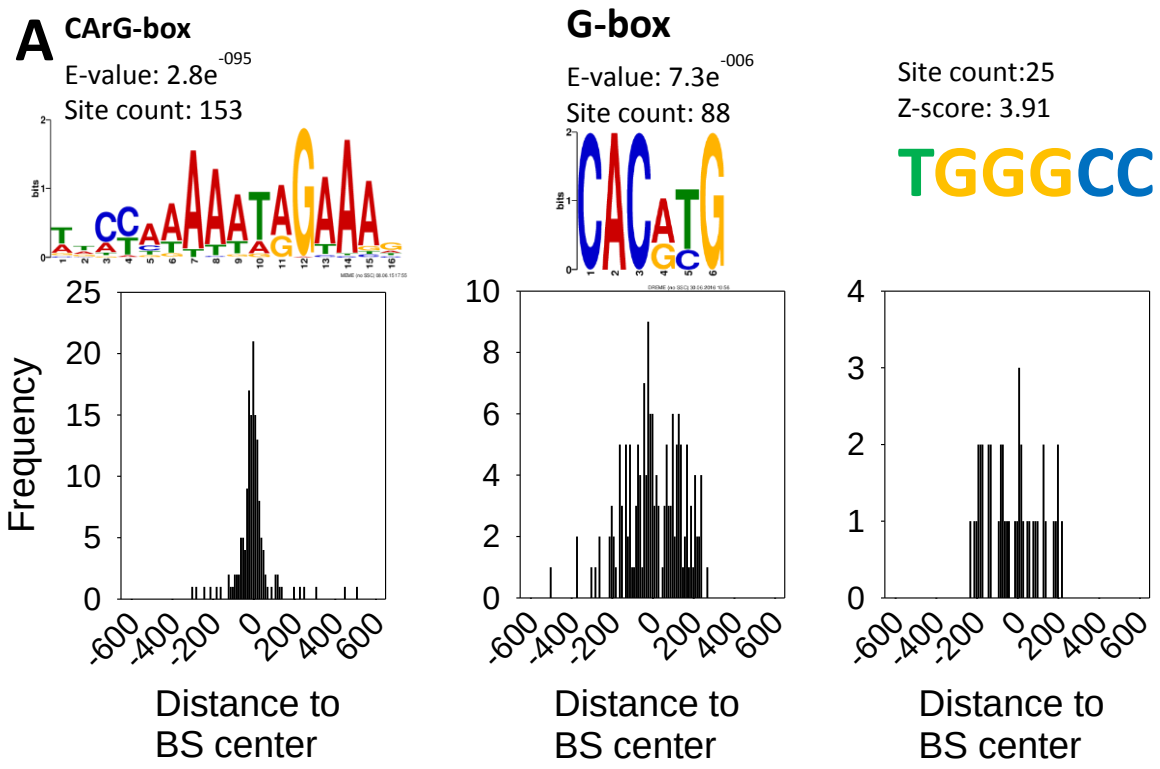


Figure 4 Validation of PEP1 binding to selected target genes.

Validation of binding detected by ChIP-seq for selected PEP1 BSs using ChIP-qPCR. For each target, fold-enrichment relative to its input is shown. Negative controls were performed with primers not flanking predicted BSs (1-2 kb distance to BS). Data are shown as mean ± SEM (n = 3 biological replicates). Primers are listed in the appendix. Asterisks indicate significant enrichment in wild-type compared to *pep1-1* (n.s. not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; Student's t-test).

PEP1 BSs were analyzed for enriched DNA-motifs using the MEME software (Bailey & Elkan, 1994; Bailey, 2011). The most significantly enriched motif was a CArG-box. CArG-boxes were present in almost all of the BSs and showed enrichment in the center of the BSs, supporting the idea that PEP1 directly binds this motif (Figure 5A). This finding further suggests that the detected PEP1 BSs were high confidence BSs, because the canonical CArG-box with the sequence $CC[A/T]_6G$ is the known binding motif for MADS-box TFs (de Folter & Angenent, 2006) and was also identified in published FLC BSs (Deng *et al*, 2011; Mateos *et al*, 2015) as well as in the newly identified set of FLC BSs presented here (Figure 5B). In the present study, not all FLC BSs contained a CArG-box motif but enrichment in the center of the peak was detected, supporting the idea that FLC binds this motif (Figure 5B). The second most enriched motif in PEP1 BSs was a G-box with the canonical sequence CACGTG, which is the known binding motif of bHLH and bZIP TFs (Menkens *et al*, 1995). G-boxes in PEP1 BSs were not enriched in the center of the BSs (Figure 5A), suggesting that these motifs are probably not

bound by PEP1 but by interacting TFs. G-boxes, at a certain distance from the central CArG-box, were also identified in a previous FLC study (Mateos *et al*, 2015). In addition, a third motif with the sequence TGGGCC was previously identified to be enriched in FLC BSs (Deng *et al*, 2011). This motif was present in 25 out of 156 PEP1 BSs and enrichment was significant compared to the genomic background in *A. alpina*, as indicated by a Z-score >3 (Figure 5A). Similar to the G-box, the TGGGCC-motif is most likely not bound by PEP1 since it is not enriched in the center of the BSs (Figure 5A). G-boxes and TGGGCC-motifs were also found to be significantly enriched in the new set of FLC BSs (Figure 5B), suggesting that PEP1 and FLC directly bind to identical CArG-box motifs and that they might both interact with other TFs that bind G-boxes and TGGGCC-motifs.



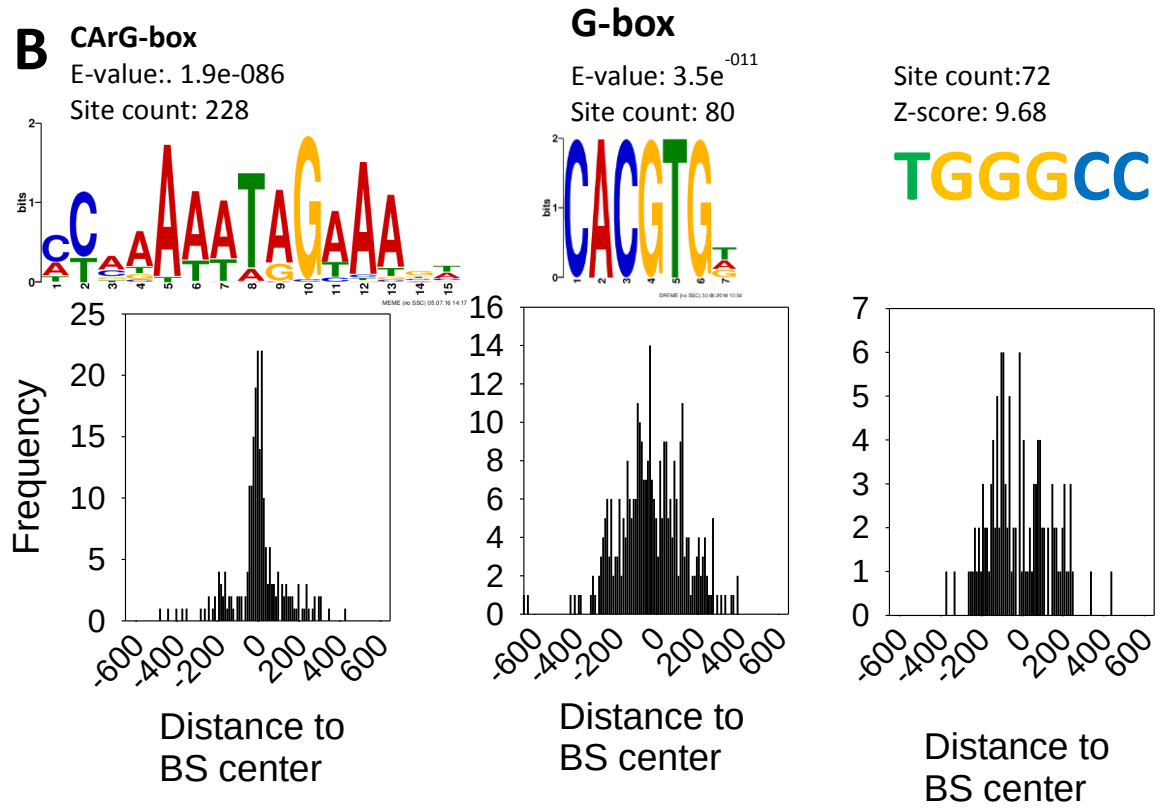


Figure 5 Enriched DNA-motifs in PEP1 and FLC BSs.

CArG-box and G-box were identified by MEME software and TGGGCC-motif was screened for manually. Distance of the closest motif to the center of the BS is represented in a histogram below the motif. Numbers of BSs that contain a motif and significance (P-value for MEME-results or Z-score for TGGGCC) are given above the position-weight matrices. **(A)** PEP1 BSs. **(B)** FLC BSs.

To test whether conserved BSs, as defined by BLAST (Figure 2B), are located in conserved syntenic regions and to test if species-specific BSs are present in the other genome, orthologous regions of PEP1 BSs in the *A. thaliana* genome and orthologous regions of FLC BSs in the *A. alpina* genome were identified (Figure 2D, see Methods). This analysis revealed that all BSs that were identified as conserved BSs by BLAST (Figure 2B) and had associated genes (23 PEP1 BSs and 25 FLC BSs) were present in conserved syntenic positions. Furthermore, most species-specific BSs (79 % of PEP1 BSs and 82 % of FLC BSs) were present in the other genome and sequence identity between species was not substantially lower compared to conserved BSs (Figure 6), indicating that those sites are present in both genomes but not bound by both TFs. Visual inspection of read density in the peak regions of a large number of those species-specific BSs confirmed that they are truly not bound in one species rather than not called due to a high significance threshold. Given that conserved BSs are located in conserved syntenic positions, it was tested whether the genes that were bound in both species (common target genes, Figure 2C) were bound at conserved BSs (Figure 2B, D). 26 target genes were associated with the conserved BSs (Figure 2E) and therefore represent conserved target genes

that were bound at conserved BSs in both species (From here on referred to as ‘conserved target genes’ as a subgroup of all common target genes. For example see Figure 7A). The remaining 7 genes that were common targets were bound at different BSs. BSs can be different if (1) PEP1 and FLC bind to similar genomic positions but these positions show very low homology (Figure 7A) or if (2) synteny around the genes is not conserved or if (3) PEP1 and FLC bind different genomic positions within the locus (Figure 7C).This analysis suggests that 7 of the common target genes might have evolved independently in the two species because they were bound at different sites in *A. alpina* and *A. thaliana*. By contrast 26 conserved target genes (only 10 % of PEP1 target genes) likely evolved in a common ancestor of both species. Common target genes with and without conserved BSs are listed in Table A5.

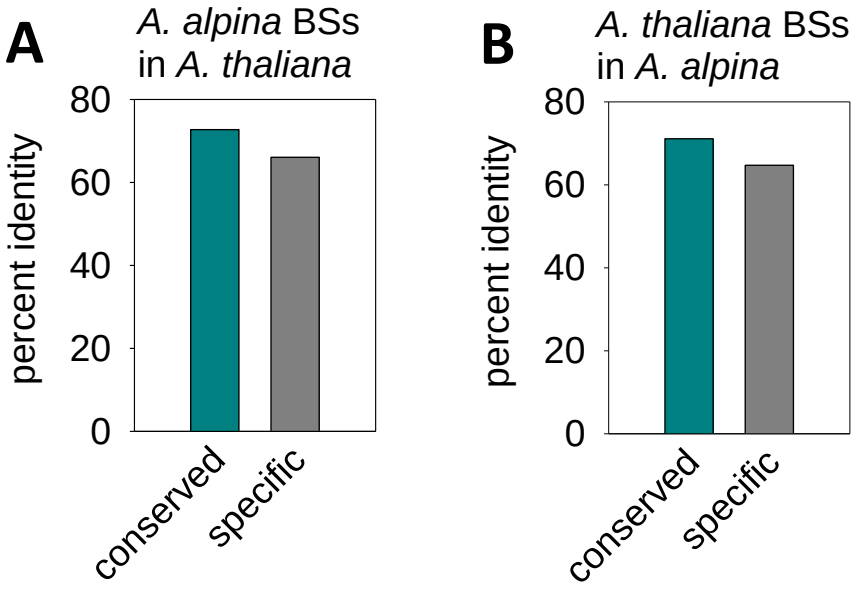
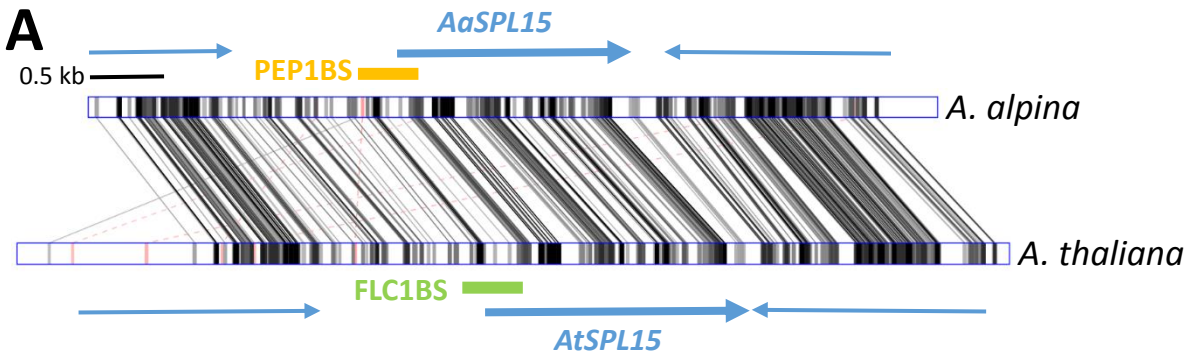


Figure 6 Sequence conservation of BSs with orthologous sequence in the other species.

Average percent identity between conserved or species-specific BSs and orthologous regions in the other species (Results of analysis in Fig. 1D). **(A)** *A. alpina* BSs in *A. thaliana* genome. Analysis includes BSs with associated genes that have orthologs in *A. thaliana*, (23 conserved and 101 PEP1-specific BSs). **(B)** *A. thaliana* BSs in *A. alpina* genome. Analysis includes BSs with associated genes that have orthologs in *A. alpina* (25 conserved and 239 FLC-specific BSs). Note that the number of conserved BSs in *A. alpina* and *A. thaliana* is different due to the distance of the BS to associated genes.



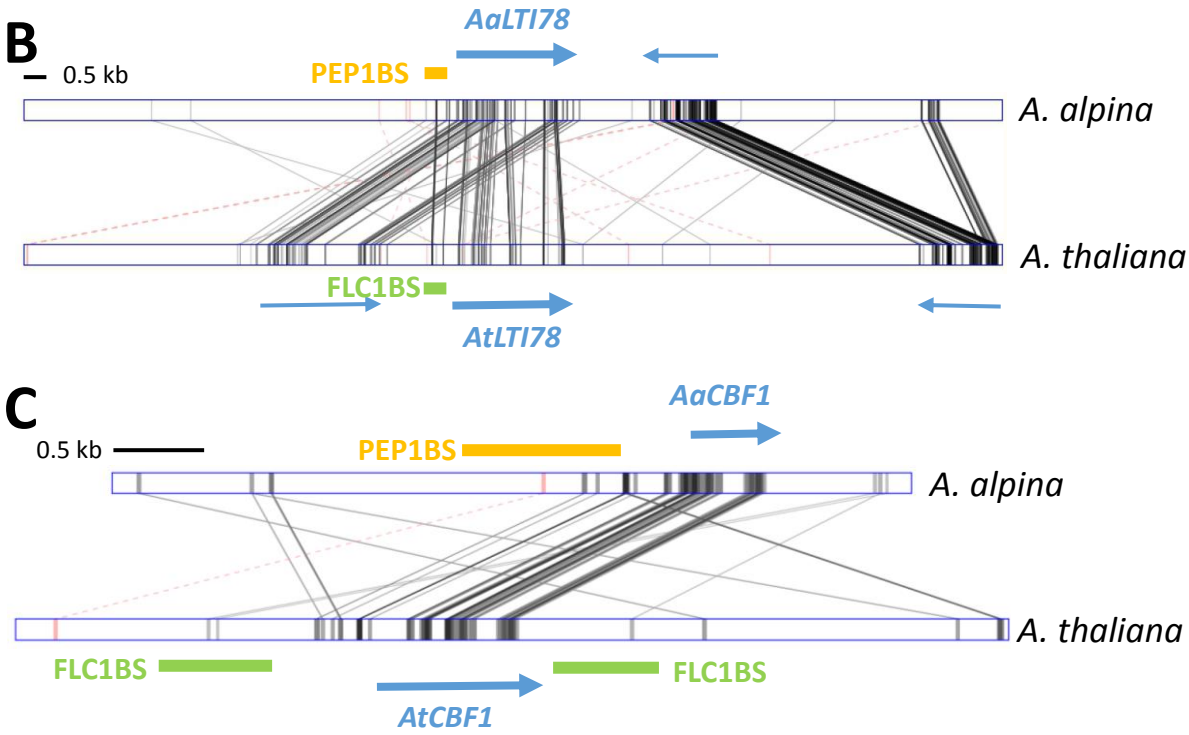


Figure 7 Illustration of BS conservation for common target genes.

GATA-alignments illustrating homology between orthologous sequences for loci with **(A)** conserved BS, example: *SPL15;* **(B)** FLC and PEP1 BSs at the same position that has low sequence conservation, example *LTI78;* **(C)** FLC and PEP1 BSs at different positions within the same locus, example *CBF1*. Black lines indicate homology. Red lines indicate inversions. Intensity of the color represents degree of homology. PEP1 BSs are indicated in orange, FLC BSs in green. Genes are marked by blue arrows.

Expression of selected validated PEP1 direct target genes (Figure 4) was tested by qPCR using the same conditions as were used for the RNA-seq experiment. This experiment confirmed that PEP1 directly regulates *SOC1*, *SEP3* and *TOE2* as suggested by the ChIP-seq and RNA-seq experiments (Figure 8). In addition, qPCR analysis detected differential expression of *VIN3* and *BRC1* (Figure 8), two genes that were not found to be regulated by PEP1 in the RNA-seq experiment. *BRC1* was only differentially expressed in leaf tissue, that might include axillary meristems, where *BRC1* is expressed in *A. thaliana* (Aguilar-Martínez *et al*, 2007). *SVP*, *COL5* and *GRP2B* seem to be bound but not regulated by PEP1 under the conditions tested (Figure 8). *SOC1*, *SEP3*, *SPL15* and *BRC1* are examples of conserved targets that were differentially regulated in *flc-3* and *pep1-1* (Figure 4, Figure 8 and Deng *et al*, 2011). Generally, the percentage of conserved target genes that was differentially regulated in *pep1-1* was significantly higher compared to target genes with PEP1-specific BSs (Figure 9, middle bars), indicating that, at least under the conditions tested, conserved target genes were more likely to be regulated by PEP1 than species-specific target genes. This effect was slightly weaker, but still significant, for common targets without conserved BSs than for those with conserved BSs (Figure 9, left bars). This observation suggests that also the target genes that appear to have

evolved independently are functionally important under the conditions tested. Interestingly, *VIN3* was an example of a gene that was only bound by PEP1 in *A. alpina* but showed the same expression changes in *pep1-1* and *flc-3* mutants in both species (Figure 8), suggesting that different mechanisms resulted in the same molecular phenotype.

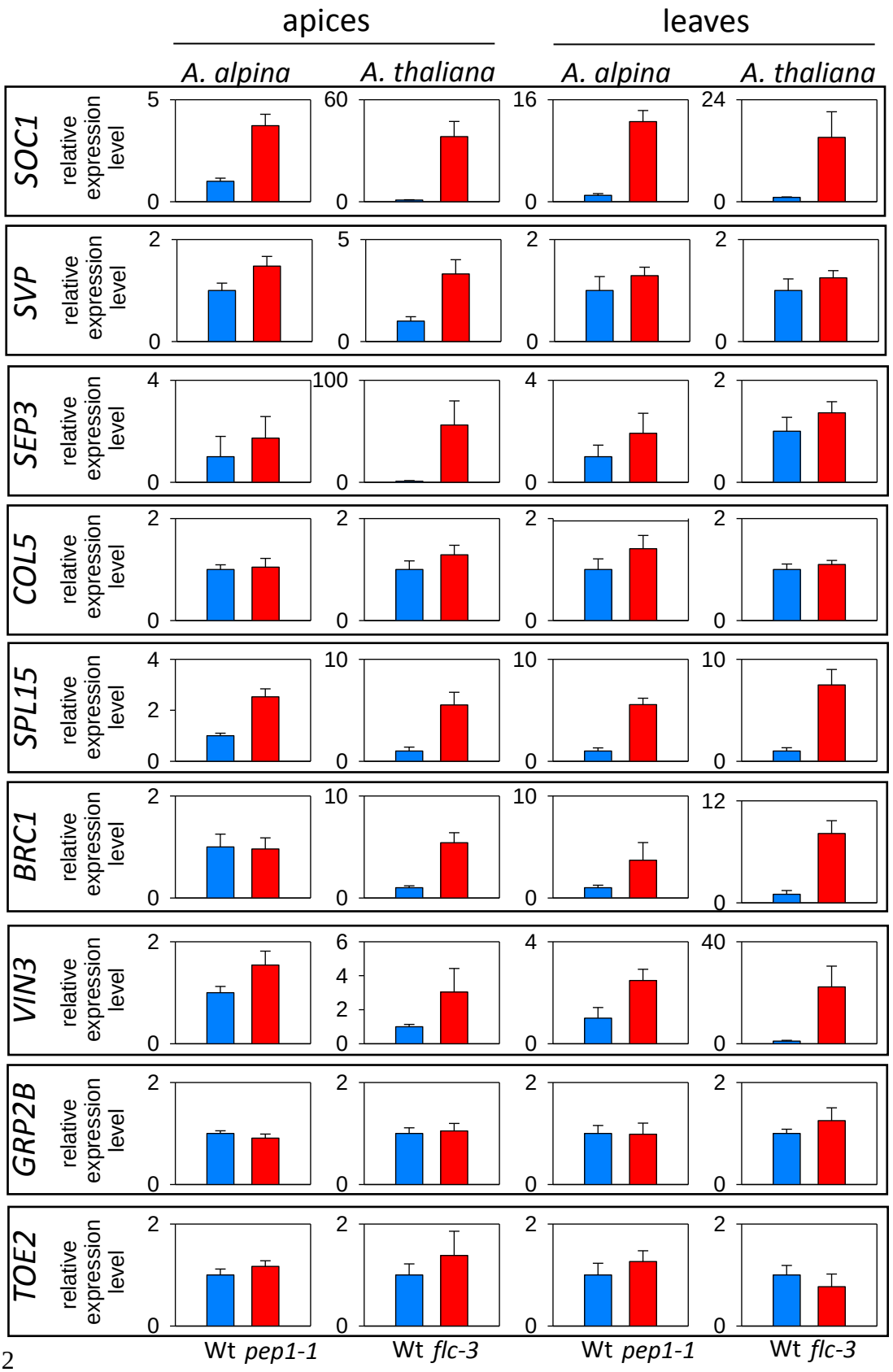


Figure 8 Expression analysis of genes regulated by PEP1 and FLC by qPCR.

Validation of RNA-seq results for selected PEP1 direct target genes using qPCR. Data are shown as mean ± SEM (n = 4 biological replicates). Expression was analyzed in leaves and apices of Wt and mutant in both species and all genes were normalized to *PP2A*. Asterisks indicate significant enrichment in wild-type compared to the mutant (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; Student's t-test). Note that in some cases, differences between genotypes were highly reproducible but not statistically significant because low expression levels in one genotype caused high variation between replicates.

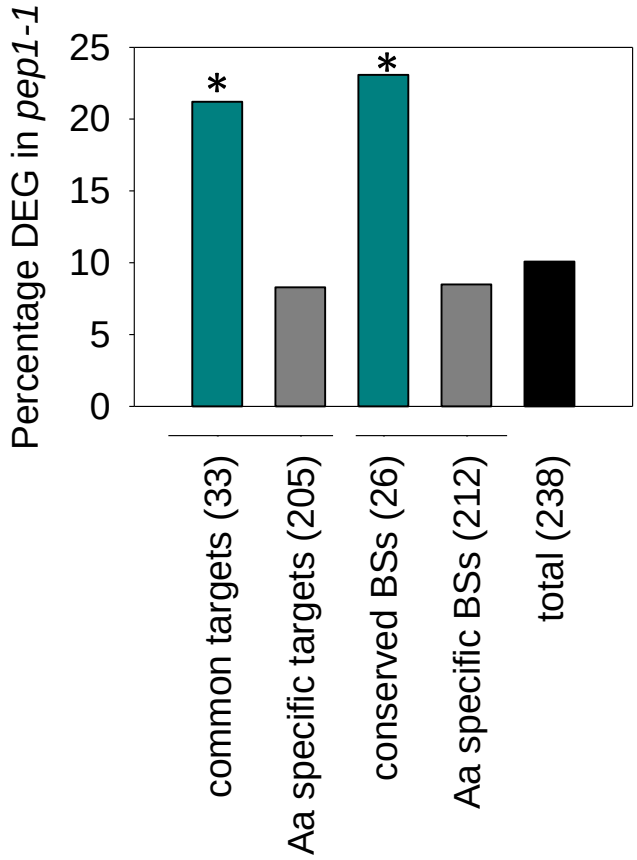


Figure 9 Differential expression of conserved and species-specific target genes in the *pep1-1* mutant.

Percentage of target genes that were differentially expressed in *pep1-1*. Left set of bars: common target genes irrespective of BS conservation vs. species-specific target genes. Right set of bars: Common target genes with conserved BSs vs. species-specific BSs. Asterisks indicate significant difference compared to the total set of PEP1 target genes tested by hypergeometric test ($P \leq 0.05$).

In summary, high confidence sets of PEP1 and FLC BSs revealed that PEP1 and FLC bind identical DNA-motifs but bind to largely different sets of target genes although these included a common set of core target genes. Common target genes were bound at conserved or different sites and were more likely to change in expression in the respective mutants under the conditions tested.

2.3 Species-specific binding usually correlates with a species-specific CArG-box

Despite the high similarity of PEP1 and FLC protein sequences, their binding landscapes in the genomes of *A. alpina* and *A. thaliana*, respectively, were highly different. As shown in Figure 6, overall sequence conservation at conserved and species-specific BSs was similar (Figure 6), indicating that the divergence of binding is not due to large re-arrangements of DNA-sequence. This result differs from previous findings of Muiño *et al*., showing that SEP3 BSs that are conserved in *A. thaliana* and *A. lyrata* have higher conservation scores than species-specific BSs (Muiño *et al*, 2016). To test whether PEP1 and FLC always bind DNA-motifs with identical consensus sequences, CArG-boxes in the sequence of conserved and species-specific subsets of BSs were compared. CArG-boxes were significantly enriched in all subsets of BSs and the motifs were highly similar between species (Figure 10), suggesting that PEP1 and FLC bind identical DNA-motifs. Comparing CArG-boxes enriched in conserved BSs with those in species-specific BSs, however, revealed slight differences. The 'CC' dinucleotide at the 5'end of the core-motif was more abundant in the conserved BSs. In addition, conserved BSs showed significant enrichment (Z-score > 3) of the 'TTT' trinucleotide at the 5' end of the CArG-box, whereas this was not found in either subset of species-specific BSs (Figure 10). Since CArG-boxes can be considered as palindromic sequences, the 'TTT' extension of the first half-side of the motif is a functional equivalent of the 'AAA' extension of the second half-side that was identified in this study and was previously described (Deng *et al*, 2011). Both of these extensions might be important for the binding of FLC/PEP1 and their interactors to the conserved set of target sites.

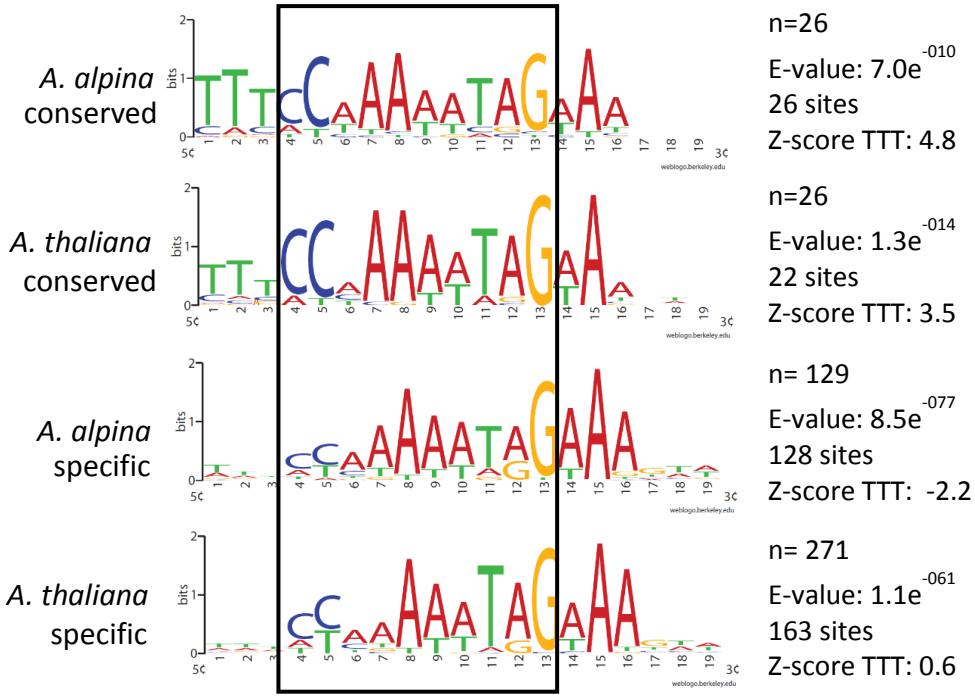


Figure 10 Enriched CArG-box variants in conserved and species-specific BSs.

CArG-boxes enriched in conserved and species-specific BSs in *A. alpina* and *A. thaliana* identified by MEME (consensus sequences are boxed). The number of BSs bearing the motifs and E-value in each subset of BSs is indicated to the right of each motif. Z-scores indicate significance of TTT enrichment at positions 1-3.

Since the same characteristics of CArG-boxes were identified in both species, they do not explain species-specific binding. Comparing BSs of PEP1 and FLC to orthologous regions in *A. thaliana* and *A. alpina*, respectively, revealed a strong correlation between the conservation of binding and the presence of a CArG-box motif at the orthologous sequence. More than 80 % of conserved PEP1 BSs contained a CArG-box at the orthologous site in *A. thaliana*, which is bound by FLC. By contrast less than 30 % of PEP1-specific BSs contained a CArG-box at the orthologous site, which is not bound by FLC (Figure 11A). Enrichment of CArG-boxes at orthologous sites of conserved BSs was statistically significant as indicated by a Z-score > 3 whereas no significant enrichment at orthologous sites of PEP1-specific BSs was detected. Similar results were obtained for FLC BSs (Figure 11B). These results suggest that the conservation of binding correlates with the presence of a CArG-box in both species. Also *A. thaliana*- and *A. lyrata*-specific SEP3 BSs were explained by species-specific CArG-boxes and *A. lyrata*-specific CArG-boxes were associated with TE insertions (Muiño *et al*, 2016). To test if TEs could explain species-specific CArG-boxes in PEP1/FLC BSs in *A. thaliana* or *A. alpina*, it was tested if any subset of BSs or set of orthologous sequences in the other species had an altered content of TEs compared to the genomic background. A higher TE content in species-specific BSs might indicate that the TEs introduced new CArG-boxes, while a higher TE content in orthologous non-bound sites might indicate that TE insertions disrupted existing CArG-boxes. *A. alpina* regions contained more TEs compared to *A. thaliana* regions (Figure 12A) corresponding to the higher TE content in the *A. alpina* genome (Willing *et al*, 2015). No significant enrichment (which would be enrichment ratio >1, see methods), however, was detected in any subset of BSs compared to the genomic background. The low enrichment ratios represent rather selection against TEs within the selected regions compared to the whole genome. Since the enrichment of a specific type of TE might be masked by the high number of various different types of TEs in the genomes, enrichment of different TE-types was investigated separately (Figure 12B). As listed in Figure 12B, several TEs were significantly enriched in *A. alpina*-specific BSs in both genomes and in *A. thaliana*-specific BSs in the *A. alpina* genome. Most of them, however, appeared only in a very small fraction of BSs and would explain less than 4 % of the binding events. The only exception is the enrichment of Helitron_Confused TEs in orthologous sites of *A. alpina*-specific BSs in the *A. thaliana* genome

(Figure 12B). Almost 10 % of these BSs (10 BSs) contained at least one Helitron_Confused TE in the non-bound region in *A. thaliana*. Of these, 7 BSs contained the TE in the central 100 bp of the BSs (data not shown) and therefore, in these cases TE insertion could have disrupted the CArG-box. Taken together, species-specific CArG-boxes that probably conferred species-specific binding of FLC/PEP1 were not associated with TE insertions or dramatic sequence changes in general, since nucleotide conservation of species-specific BSs is similar to conserved ones (Figure 6). In accordance with these results, small sequence changes appear to be responsible for species-specific CArG-boxes at least in some cases (Figure 13). In these three cases, the CArG-box was present in *A. alpina* but modified in *A. thaliana*. We included orthologous sequences of other related Brassicaceae species and *T. hassleriana* as outgroup from the sister family Cleomaceae in the alignment to determine whether binding was rather gained in *A. alpina* or lost in *A. thaliana*. These alignments showed that the CArG-box motifs were also absent in *A. arabicum* which is a basal Brassicaceae species (Figure 13), suggesting that the CArG-box (and thereby PEP1 binding) evolved in the *A. alpina* lineage rather than being lost in the *A. thaliana* lineage.

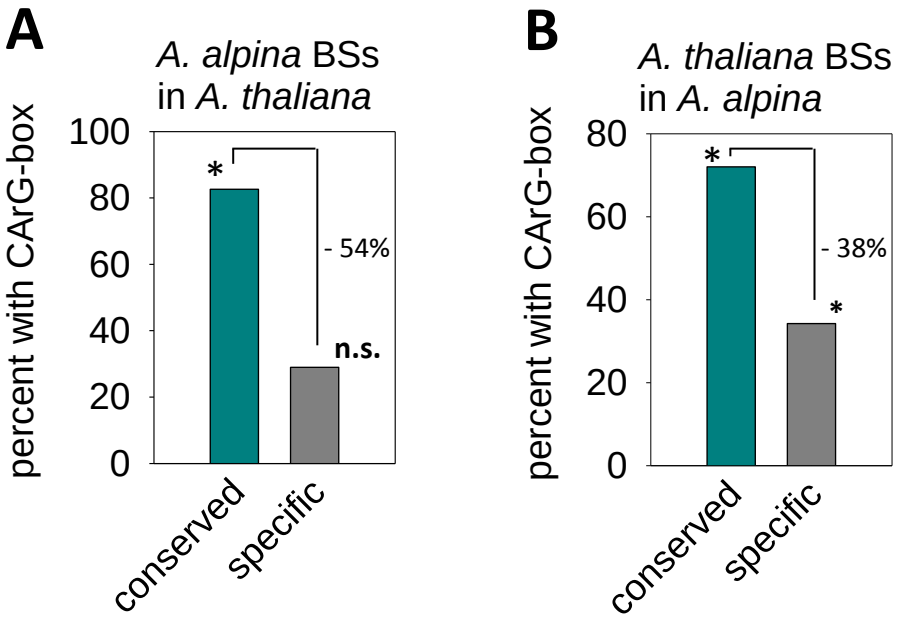
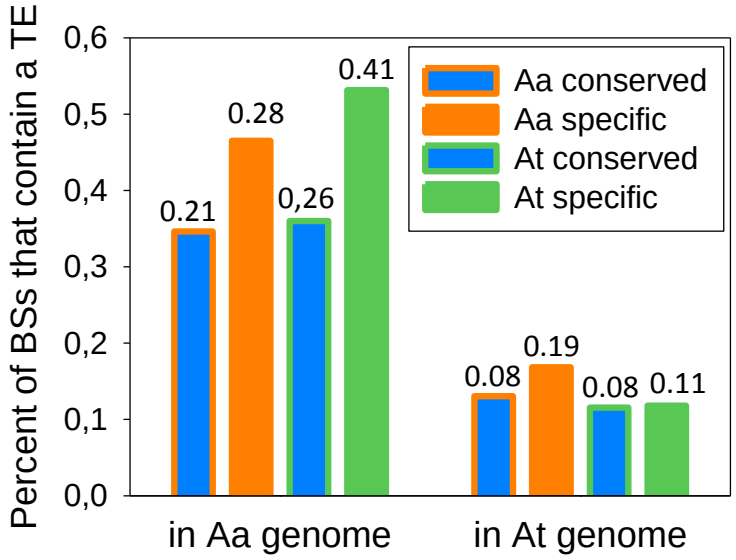


Figure 11 Conservation of the presence of a CArG-box in conserved and species-specific BSs.

Presence of CArG-box motifs in orthologous regions of **(A)** *A. alpina* and **(B)** *A. thaliana* BSs. Orthologous regions as defined in Fig. 1D. CArG-boxes were defined as MYHWAWWWRGWWW which is closest to the position weight matrix identified by MEME without allowing too much variation and detection of random sequences. Note that the method used to find CArG-boxes differed from previous figures because MEME software can only be used to identify significantly enriched motifs. Asterisks indicate significant enrichment of CArG-boxes as defined by Z-score ≥3. Percentage is percent difference of sequences that contain a CArG-box.

A



B

subset of BSs	genome	enriched TEs	enrichment ratio	no. of BSs with TE	% of BSs
Aa specific	*A. alpina*	Harbinger	2.33*	1	0.78
		hAT_Confused	8.11*	2	1.55
		putative_nonLTR_RT	1.13*	3	2.32
		LINE_Confused_withOTHER	20.79*	1	0.78
		LINE_Confused	1.51*	4	3.10
		SINE	2.87*	2	1.55
At specific	*A. alpina*	putNAclassII	1.83*	3	1.26
		Mariner_Confused	2.51*	2	0.84
		Copia_Confused	1.47*	7	2.93
		Tase	1.48*	1	0.42
		putNA_hAT	3.71*	1	0.42
		Helitron_Confused_withOTHER	2.02*	2	0.84
		Confused	3.02*	3	1.26
		LINE_Confused	1.62*	9	3.77
Aa specific	*A. thaliana*	Copia_Confused	2.05*	1	0.99
		Helitron_Confused	1.15*	10	9.90
At specific	*A. thaliana*	no enriched TEs			

Figure 12 Occurrence of TEs in PEP1 and FLC BSs.

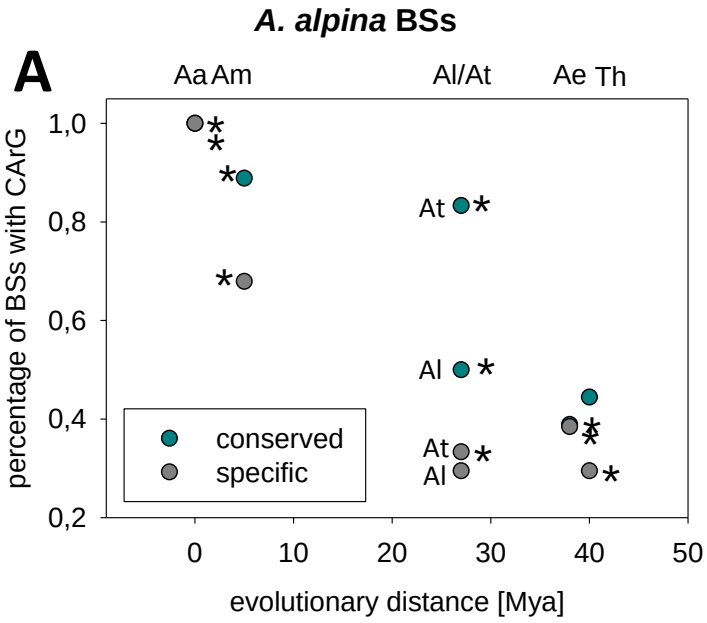
(A) Percentage of BSs and orthologous sequences in the other genome that contained at least one TE. Left: BSs of *A. alpina* PEP1 and orthologous sites of *A. thaliana* FLC BSs in the *A. alpina* genome. Right: BSs of *A. thaliana* FLC and orthologous sites of *A. alpina* PEP1 BSs in the *A. thaliana* genome. Numbers above the bars are ratio of enrichment of TEs (bp that correspond to a TE/bp that are not TE) in BSs vs. rest of the genome. Ratios >1 indicate enrichment of TEs in the BSs. **(B)** List of all types of TEs that were significantly enriched in a subset of BSs. Asterisks behind enrichment ratio indicate significant enrichment (P-value ≥ 0.05 as defined by χ^2 -test).

Figure 13 Examples showing how species-specific CArG-boxes arose by small sequence changes.

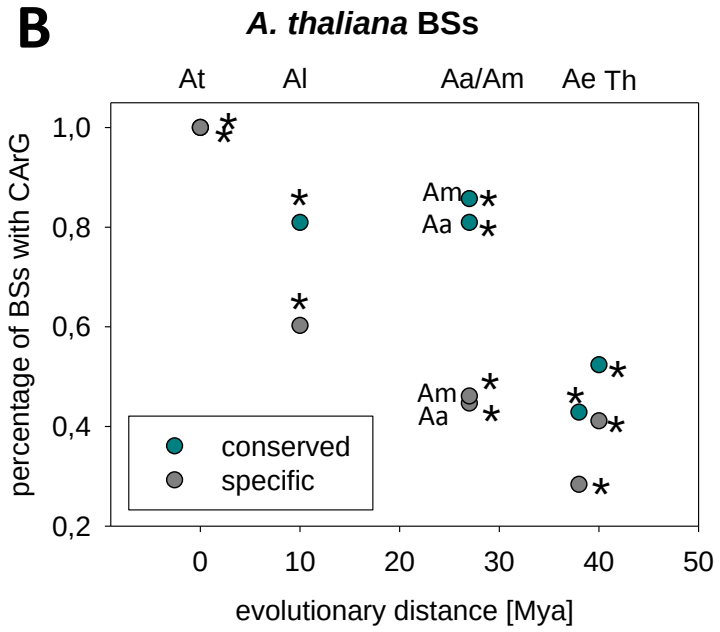
Three examples of *A. alpina*-specific BSs that contained a CArG-box in *A. alpina* which was lost in other Brassicaceae. *A. alpina* sequence around CArG-box motif was aligned to orthologous regions of other Brassicaceae species (At: *A. thaliana*, Am: *A. montbretiana*, Al: *A. lyrata*, Ae: *A. arabicum*) and *T. hassleriana* (abbreviated Th). *A. alpina* CArG-box is marked in red. Sequence changes relative to the consensus motif in *A. thaliana* are highlighted by a green box. Alignments were performed using mvista.

In vertebrates, conservation of BSs of developmental TFs decreases exponentially with the evolutionary distance between species (Schmidt *et al*, 2010; Stefflova *et al*, 2013; Ballester *et al*, 2014). Also conservation of BSs between *A. thaliana* SEP3 and *A. lyrata* SEP3 is low (Muiño *et al*, 2016). In *Drosophila* species, however, conservation of BSs is higher and decreases only linearly with the evolutionary distance (Bradley *et al*, 2010; He *et al*, 2011). Conservation of CArG-boxes in related species was plotted for conserved PEP1 and FLC BSs as well as for species-specific BSs (Figure 14). For both datasets, linear and exponential regression curves represented the decrease of conservation with evolutionary distance equally well as indicated by similar R^2-value (Figure 14). This might indicate that the species are too closely related, to show a difference between linear and exponential curves (all data points were located in the linear range of the exponential curve) and analysis with additional species might clarify which curve fits better. The linear regression curves for conserved and species-specific BSs had similar slopes of around -0.01 percent of BSs with CArG per million years (Figure 14), suggesting that the rate of binding loss with evolutionary distance is similar but the conserved BSs start at a higher conservation level. Both sets of *A. alpina* PEP1 BSs were also

analyzed without the *A. thaliana* data, to test whether conservation is generally higher (in all species) for BSs that are conserved between *A. thaliana* and *A. alpina* or whether conservation is rather limited to *A. thaliana*. This analysis resulted in higher R^2 of linear and exponential fit for conserved but not for the species-specific BSs (Figure 14A and Figure 14B shows similar results for *A. thaliana* BSs), suggesting that at least some conserved BSs are conserved only between *A. thaliana* and *A. alpina* but not deeply conserved in all Brassicaceae species. Taking this into account, removal of these not-deeply conserved BSs from the datasets would result in a linear regression curve with a softer slope, indicating that the decrease of conservation for the deeply conserved BSs is actually lower than that of the species-specific BSs. Similar effects could be observed for the *A. thaliana* BSs (Figure 14B). However, here the slope for the conserved BSs was indeed slightly softer compared to species-specific ones, indicating that the decrease of conservation with the evolutionary distance is lower for the conserved BSs. Furthermore, the increase of R^2 for the conserved BSs without the *A. alpina* data is much weaker (Figure 14B) compared to the *A. alpina* data (Figure 14A). This weaker effect might be due to similarly high conservation of *A. thaliana* BSs with *A. alpina* and *A. monbretiana*, whereas for the *A. alpina* BSs, conservation is much higher in *A. thaliana* compared to *A. lyrata*. These findings suggest that the divergence of BSs between *A. thaliana* and *A. lyrata* is higher compared to the divergence between *A. alpina* and *A. montbretiana*, which would be in line with their longer evolutionary distance (Willing *et al*, 2015).



Subset of BSs	Linear fit	Exponential fit
conserved	y = -0.014x + 0.99 R^2 = 0.80	$y = 1.03e^{-0.02x}$ R^2 = 0.76
conserved w/o At	y = -0.015x + 0.97 R^2 = 0.97	$y = 0.98e^{-0.023x}$ R^2 = 0.97
Specific	y = -0.015x + 0.85 R^2 = 0.80	$y = 0.83e^{-0.027x}$ R^2 = 0.81
Specific w/o At	y = -0.015x + 0.86 R^2 = 0.82	$y = 0.84e^{-0.027x}$ R^2 = 0.83



Subset of BSs	Linear fit	Exponential fit
conserved	$y = -0.012x + 1.01$ $R^2 = 0.72$	$y = 1.06e^{-0.017x}$ $R^2 = 0.67$
conserved w/o Aa	$y = -0.012x + 1.0$ $R^2 = 0.78$	$y = 1.03e^{-0.018x}$ $R^2 = 0.74$
Specific	$y = -0.015x + 0.88$ $R^2 = 0.84$	$y = 0.89e^{-0.025x}$ $R^2 = 0.86$
Specific w/o Aa	$y = -0.015x + 0.88$ $R^2 = 0.84$	$y = 0.89e^{-0.025x}$ $R^2 = 0.86$

Figure 14 Presence of a CArG-box in sites orthologous to BSs in related species.

Percentage of orthologous sites that contain a CArG-box was plotted vs. evolutionary distance. **(A)** *A. alpina* PEP1 BSs. **(B)** *A. thaliana* FLC BSs. Asterisks indicate significant enrichment of CArG-boxes defined by Z-score ≥ 3. Table shows linear and exponential regression curves and R^2 for conserved and specific BSs and for the same subsets without *A. thaliana* (A) or *A. alpina* (B), respectively. Species: Aa: *A. alpina*, At: *A. thaliana*, Am: *A. montbretiana*, Al: *A. lyrata*, Ae: *A. arabicum*, Th: *T. hassleriana*.

In addition it should be noted that conservation of CArG-boxes does not always reflect conservation of binding as indicated by the fact that around 30 % of species-specific BSs have a CArG-box in the species where no binding was detected (Figure 11). To investigate why in these cases the CArG-box is conserved although binding is not, it was tested whether the presence or absence of G-boxes or TGGGCC-motifs can explain conservation of binding. The presence of a binding motif for an interacting TF might be required for PEP1/FLC binding or binding of another TF might prevent binding of PEP1/FLC. G-boxes were more enriched in conserved BSs compared to species-specific BSs but they were not lost with binding in the orthologous sequences (Figure 15A), suggesting that G-boxes are functionally important for the regulation of conserved target genes but do not explain conservation of CArG-boxes in species-specific BSs. TGGGCC-motifs were present at similar frequencies in the different subsets of BSs and only for *A. alpina*-specific BSs, their absence correlated with the absence of binding in *A. thaliana*, however enrichment of the motif was not significant compared to the background (Z-scores < 3) (Figure 15B).

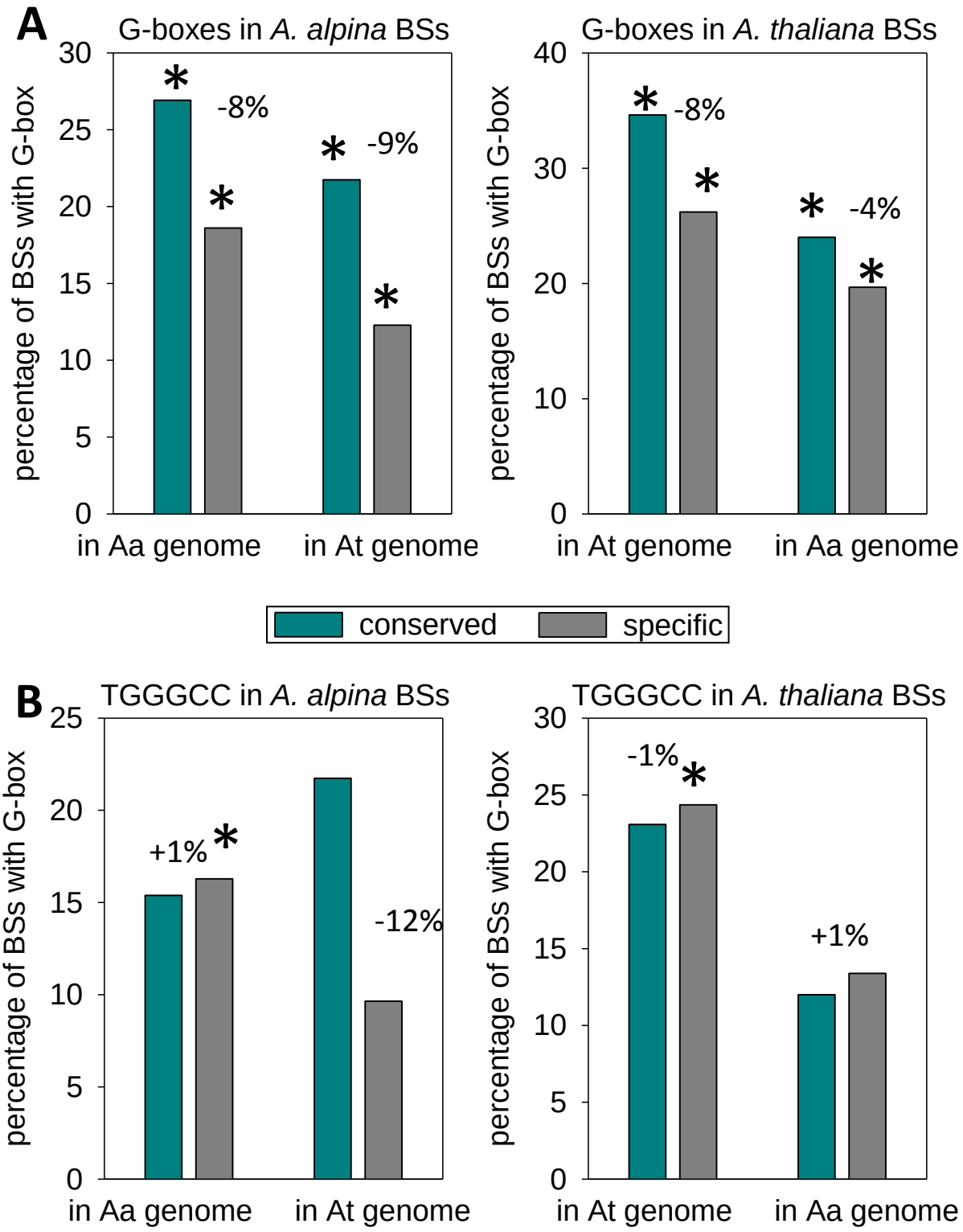


Figure 15 Enrichment of other motifs in conserved and species-specific BSs and orthologous regions in the other species.

Presence of G-box motifs **(A)** or TGGGCC-motifs **(B)** in *A. alpina* BSs and orthologous regions in *A. thaliana* (left) and in *A. thaliana* BSs and orthologous regions in *A. alpina* (right). Asterisks indicate significant enrichment as defined by Z-score ≥ 3. Percentage is percent difference of sequences that contained a motif.

In a next step, *A. thaliana*-specific BSs that contained a CArG-box in *A. thaliana* and in the *A. alpina* orthologous sequence (although binding was not conserved) were screened for additional

G-boxes and TGGGCC-motifs (Figure 16). An additional motif present in both species might indicate that the region is part of a conserved regulatory module that is bound by various types of TFs, possibly including other MADS-box TFs than FLC/PEP1 that might bind the CArG-box. The presence of an additional motif only in *A. thaliana* would suggest that this motif might be required for FLC binding and might therefore explain the absence of binding in *A. alpina*. Presence of an additional motif only in *A. alpina* suggests that another TF might bind this motif in *A. alpina* and that this prevents PEP1 binding. As shown in Figure 16, all of the three different possibilities were identified. In total, 41 % of *A. thaliana*-specific BSs with conserved CArG-box contained at least one other motif in *A. thaliana*, *A. alpina* or in both species. Thus, interactions of PEP1/FLC with other TFs might affect binding and cause differences between species despite conservation of the CArG-box.

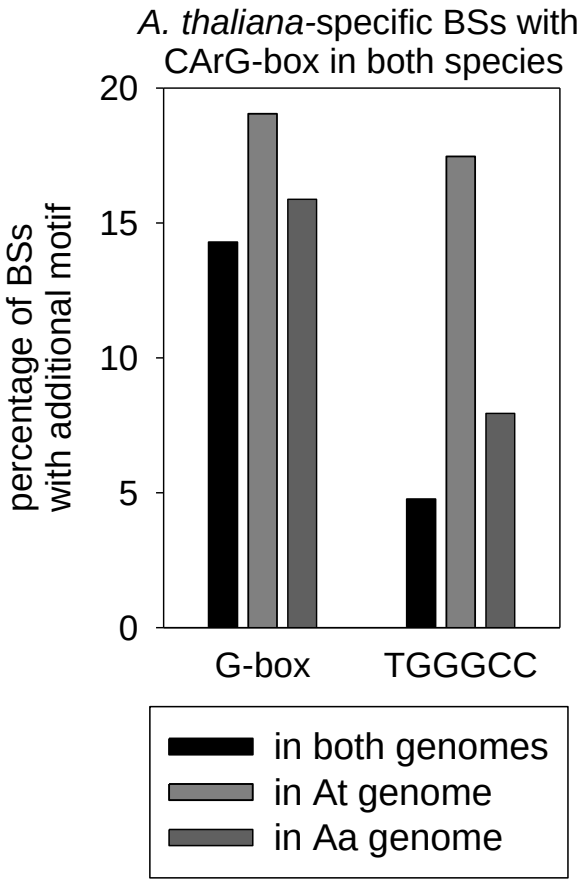


Figure 16 Presence of a second motif in A. thaliana-specific BSs with conserved CArG-box.

Percentage of *A. thaliana*-specific BSs that have a CArG-box in *A. alpina* and contain an additional G-box or TGGGCC motif (besides the CArG-box that is present in both species) in both genomes (black), only in the *A. thaliana* genome (light grey) or only in *A. alpina* genome (dark grey). The total proportion of BSs with at least one additional motif in one species or both is 41 %.

CArG-boxes in *A. alpina*-specific BSs that were conserved in *A. thaliana*, were also more conserved in other species, compared to CArG-boxes that were absent in the non-bound regions in *A. thaliana* (Figure 17A). This suggests that there is general selection pressure to keep these motifs, implying that these motifs might be necessary for other processes while they are not sufficient to determine binding of FLC/PEP1. Similar results were also obtained for *A. thaliana*-specific BSs with conserved CArG-boxes in *A. alpina* (Figure 17B). One explanation for conservation of CArG-boxes despite divergence of FLC/PEP1 binding could be binding of other MADS-box TFs to these motifs. Such repurposing of DNA-binding motifs was previously described in human and mouse (Denas *et al*, 2015). Genome-wide BSs of several MADS-box TFs with functions in flowering time control or flower development were described in *A. thaliana* (Deng *et al*, 2011; Gregis *et al*, 2013; Immink *et al*, 2012; Kaufmann *et al*, 2009, 2010b; Mateos *et al*, 2015; Pajoro *et al*, 2014; Posé *et al*, 2013b; Wuest *et al*, 2012). Comparison of these BSs with FLC BSs revealed that approximately 80 % of *A. thaliana* FLC-specific BSs were bound by at least one other MADS-box TF. Furthermore, for approximately 40 % of *A. alpina* PEP1-specific BSs the orthologous region in *A. thaliana* was found to be bound by at least one other MADS-box TF (Figure 18). Overlap with other MADS-box TFs was higher for *A. thaliana* FLC-specific BSs that contained a conserved CArG-box compared to all FLC-specific BSs (Figure 18A, light vs. dark grey bars). This suggests that there is selection pressure on these CArG-boxes due to binding of other MADS-box TFs. In addition, binding of other MADS-box TFs might have effects on binding of FLC/PEP1 and those might vary depending on external and internal conditions. Interestingly, BSs that were conserved between *A. thaliana* and *A. alpina* showed a higher frequency of overlap with BSs of other MADS-box TFs than the species-specific BSs (Figure 18, dark grey vs. green bars), suggesting that the conserved function of FLC and PEP1 involves interaction with these other MADS-box TFs or conserved BSs tend to be recognized by multiple TFs more frequently. CArG-boxes at these sites might be more readily repurposed for binding of different TFs at different times, or they might be more likely to be in areas of open chromatin.

In contrast to PEP1 BSs, not all *A. thaliana* FLC BSs contained a CArG-box (Figure 10). Screening for G-boxes and TGGGCC-motifs in these sites, revealed that 48 % of *A. thaliana* FLC BSs without a CArG-box contained at least one other motif (Figure 19) suggesting that FLC binding to these sites might occur indirectly via interaction of FLC with a TF that binds the G-box or TGGGCC-motif, respectively.

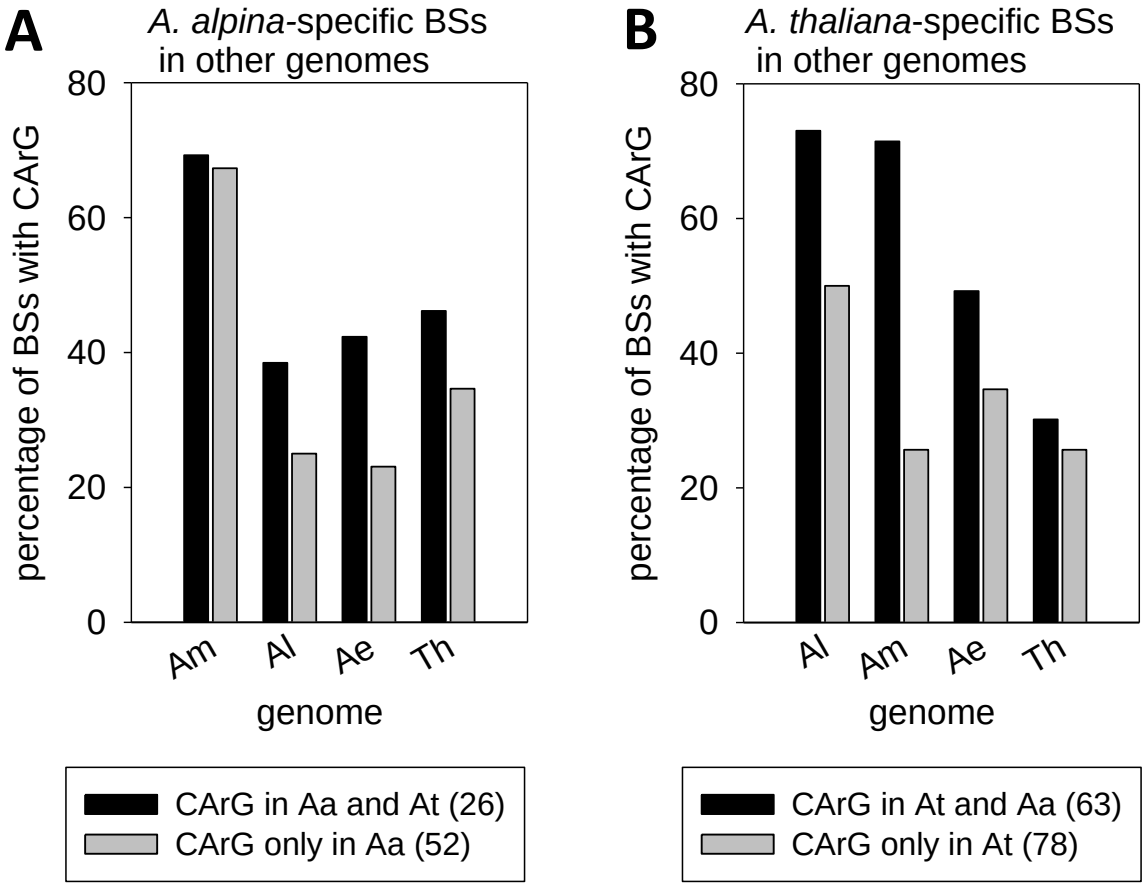
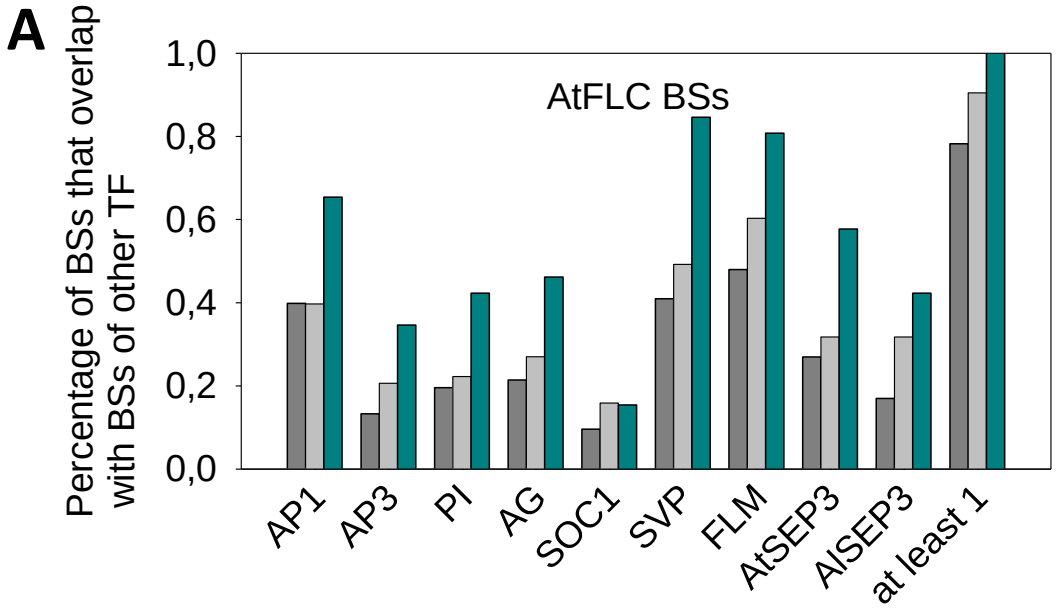


Figure 17 Conservation of conserved vs. species-specific CArG-boxes in species-specific BSs in related species.

Conservation of CArG-boxes that were conserved in *A. alpina* and *A. thaliana* although binding was not (black) and of CArG-boxes that were absent in the species where binding did not occur (grey) in related species. **(A)** *A. alpina* BSs. **(B)** *A. thaliana* BSs. Species: Am: *A. montbretiana*, Al: *A. lyrata*, Ae: *A. arabicum*, Th: *T. hassleriana.*



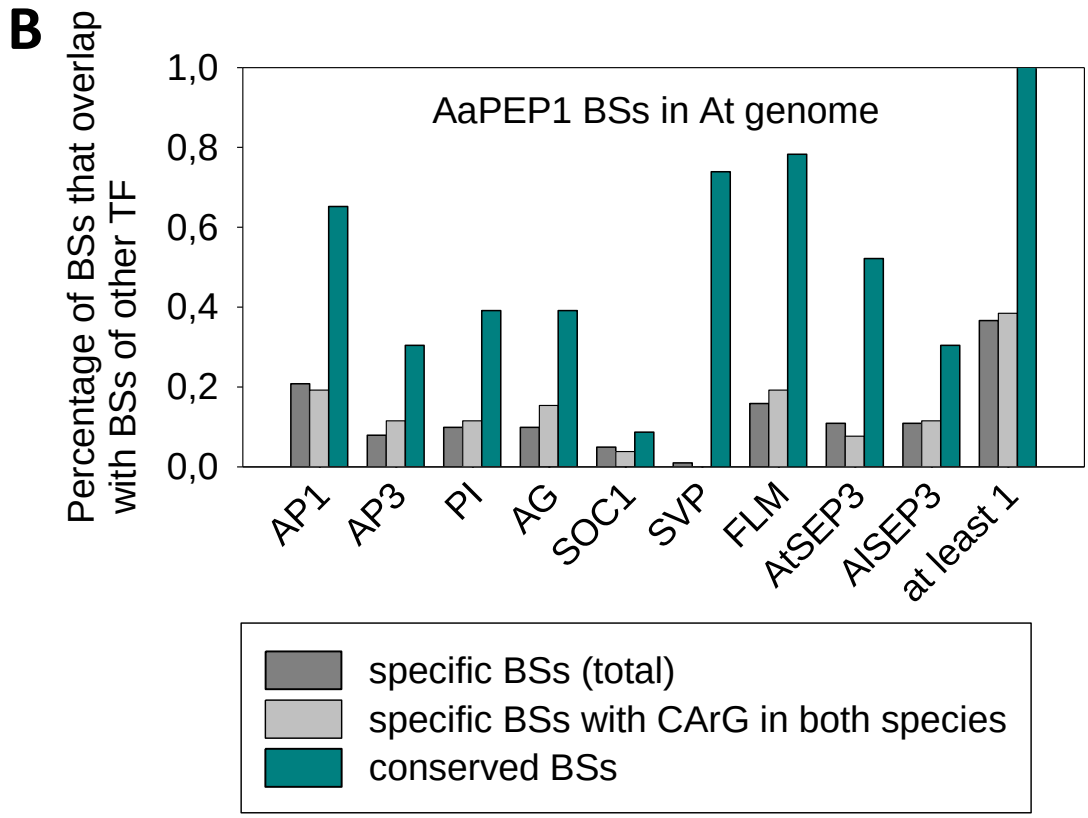


Figure 18 Overlap of PEP1/FLC BSs with BSs of other MADS-box TFs.

Percentage of different subsets of FLC or PEP1 BSs that overlapped with BSs described for other MADS-box TFs in *A. thaliana*. Subsets of BSs for each species are: Specific (dark grey), specific with conserved CArG-box (light grey) and conserved BSs (green). **(A)** *A. thaliana* FLC BSs. **(B)** *A. alpina* PEP1 BSs. Note that orthologous regions of *A. alpina* peaks in the *A. thaliana* genome were used for the analysis.

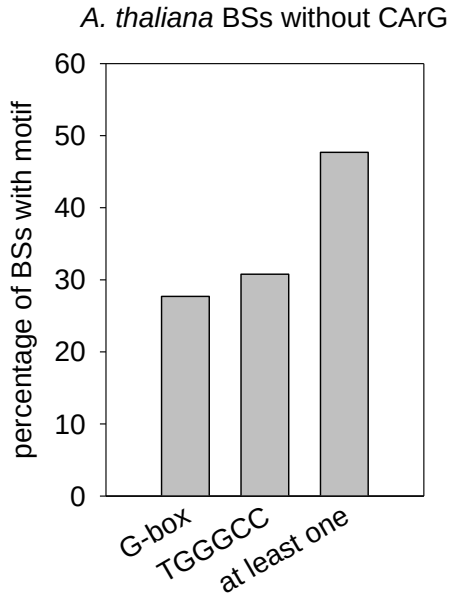


Figure 19 Presence of other motifs in *A. thaliana* FLC BSs without CArG-box.

Percentage of *A. thaliana* FLC BSs that contained no CArG-box but a G-box, a TGGGCC-motif or at least one of the two motifs.

In summary, conservation of binding correlates with the conserved presence of a CArG-box. The absence of a CArG-box in one species, correlating with species-specific BSs, seems to be mainly due to small sequence changes rather than large scale sequence re-arrangements. Although the correlation between conservation of CArG-boxes and conserved binding is highly significant, it does not allow accurate prediction of binding in other species, since FLC seems to act in a complex network consisting of various types of interacting TFs that influence each other's activities.

2.4 PEP1 and FLC are involved in similar biological processes and flowering genes are evolutionary conserved target genes

PEP1 and FLC regulated largely different sets of target genes, suggesting that they might be involved in different biological functions. To test this hypothesis, GO-term enrichment analysis for PEP1 and FLC target genes was performed (Figure 20). PEP1 and FLC were both found to be involved in the control of flowering time and flower development, in the regulation of transcription, in the response to hormone stimuli, in particular to GA, and in the response to abiotic stimuli, in particular to cold temperatures (Figure 20). Fold enrichment of some categories varied between species. For example, genes involved in GA metabolism, which is not a classical GO-category but a group including all genes involved in GA biosynthesis and degradation, were highly over-represented among PEP1 target genes but only weakly enriched among FLC target genes. In addition, both TFs regulated genes involved in the response to GA, indicating that both TFs do regulate GA-related processes (Figure 20). Interestingly, no GO category associated with a truly species-specific function was found to be enriched in either of the datasets, suggesting that PEP1 and FLC, despite the high divergence of their target genes, regulate similar biological processes.

GO-term analysis was repeated specifically for the common target genes of PEP1 and FLC to test which functions are associated with these target genes. Common target genes showed a very strong enrichment for genes in flowering-related GO-categories (Figure 20). Genes involved in cold- and GA-related processes were also overrepresented among common target genes but the enrichment was much weaker, indicating that the regulation of flowering is the main function conferred by common target genes (Figure 20). To assess which functions of PEP1 and FLC could be conferred by conserved binding events, GO-term enrichment analysis was also performed for target genes with conserved BSs. Conserved target genes were mainly associated with flowering-related processes, whereas no enrichment for genes encoding GA metabolic enzymes was detected (Figure 20). Furthermore, categories related to GA and cold responses were less strongly enriched in conserved targets compared to all common target genes

(Figure 20), suggesting that these functions are conferred by non-conserved binding events and therefore arose by convergent evolution.

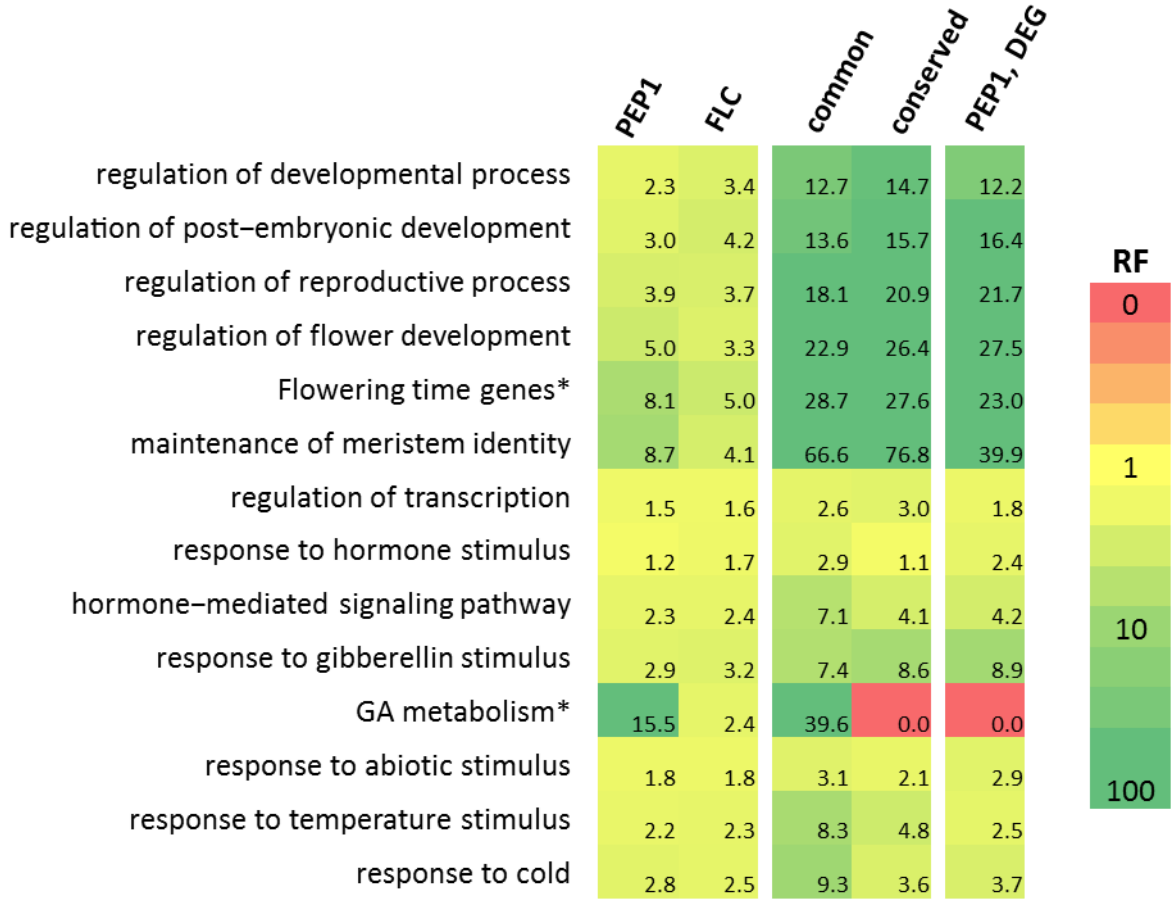


Figure 20 GO-term enrichment analysis for different subsets of PEP1 and FLC target genes.

Table shows selected GO-terms that were significantly ($P \leq 0.05$) enriched in at least one of the gene lists. Functional categories of our interest that are not standard GO categories were labelled with *. The representation factor (RF) is given in each box and symbolized by the color in the heat map. RF is the observed frequency of genes in the GO-term category within the subset of target genes divided by the expected frequency. RF >1 represents higher frequency than expected (green color) and RF >1 represents lower frequency than expected (red color). The different sets of target genes are indicated above the chart. (Left side: all PEP1 and FLC direct target genes, middle: common and conserved target genes, right: PEP1 direct target genes that were DEG in the RNAseq study).

GO-term analysis was also performed for all *A. alpina* PEP1 target genes that were found to be differentially regulated in the *pep1-1* mutant to identify processes regulated by PEP1 under our experimental conditions. Similar to conserved target genes, this analysis revealed a very strong enrichment for genes in flowering-related categories and much weaker enrichment for other functions (Figure 20). This suggests that the regulation of flowering is the main function of PEP1 under LD conditions. Other processes, like the response to cold temperatures or hormone responses, might be more relevant under different environmental conditions or developmental stages (For more details see section 2.5 and part 3).

Taken together, the GO-term enrichment analysis suggests that the regulation of flowering is the main function of PEP1 under LD conditions and this function is conserved between *A. alpina* and *A. thaliana*. These findings are in accordance with previous studies showing that *pep1-1* and *flc-3* mutants have strong flowering phenotypes (Wang *et al*, 2009b; Michaels & Amasino, 1999; Sheldon *et al*, 1999). In total, 6 out of 26 (almost 25 %) of conserved target genes were involved in flowering-related processes. Conserved targets that are involved in flowering included the floral integrators *SPL15*, *SOC1* and *SVP* (Samach *et al*, 2000; Moon *et al*, 2003; Schwab *et al*, 2005; Searle *et al*, 2006; Lee *et al*, 2007; Li *et al*, 2008; Hyun *et al*, 2016), suggesting that the regulation of flowering at the SAM is highly conserved between species, as well as *SEP3*, a master regulator of floral development (Pelaz *et al*, 2000; Honma & Goto, 2001) (Table A6). In addition, PEP1 and FLC regulated distinct genes involved in different flowering pathways (Table A6). PEP1-specific target genes were for example involved in the photoperiod pathway, for instance *COL5* and *TOE2*, and in the GA pathway, for instance *GA2OX2*, *GA3OX2* and *GID1B* (Table A6). Examples for FLC-specific target genes involved in the same two pathways are *FT*, *SPA2*, *CIR1* (photoperiod) and *GID1C* (GA) (Table A6). In conclusion, PEP1 and FLC regulated a conserved set of flowering-related core targets involved in the regulation of flowering at the meristem and additional species-specific target genes involved in different flowering pathways.

The conservation of BSs and CArG-box motifs within the BSs for three flowering-related genes *SOC1*, *SPL15* and *SEP3* is shown in Figure 21. To further explore conservation of PEP1/FLC targets involved in the regulation of flowering, sequence conservation was also analyzed in related Brassicaceae species and *T. hassleriana* (Figure 21B-C). The PEP1/FLC BSs showed high conservation in most of the species and at least one CArG-box motif in each BS was conserved in *A. arabicum*, a member of the basal Brassicaceae lineage, or *T. hassleriana* which belongs to the sister family of the Brassicaceae, the Cleomaceae (Figure 21B-C). This deep conservation of binding motifs suggests that binding to these target genes and thus the regulation of flowering, is deeply conserved within the Brassicaceae family and in some cases involves BSs that predate the Brassicaceae. The hypothesis that binding events that are associated with a conserved core function are also conserved in other species prompted us to identify these deeply conserved binding events among the BSs that were conserved between *A. alpina* and *A. thaliana*. Only a small set of BSs contained a CArG-box in all species tested (Figure 21D), suggesting that binding to these motifs is deeply conserved and associated with conserved core functions. Besides the already mentioned flowering-related genes, such deeply conserved target genes included for example *bZIP44*, which is involved in germination

(Iglesias-Fernández *et al*, 2013), and several other so far not well characterized target genes (Figure 21D). Conservation of binding can be verified experimentally to identify and confirm additional conserved core functions of FLC/PEP1.

In summary, PEP1 and FLC were found to be involved in similar biological functions despite the low number of conserved target genes. The regulation of flowering seems to be the most conserved core function while other functions that are related to environmental and hormonal responses, were mainly conferred by species-specific target genes and might have arisen by convergent evolution.

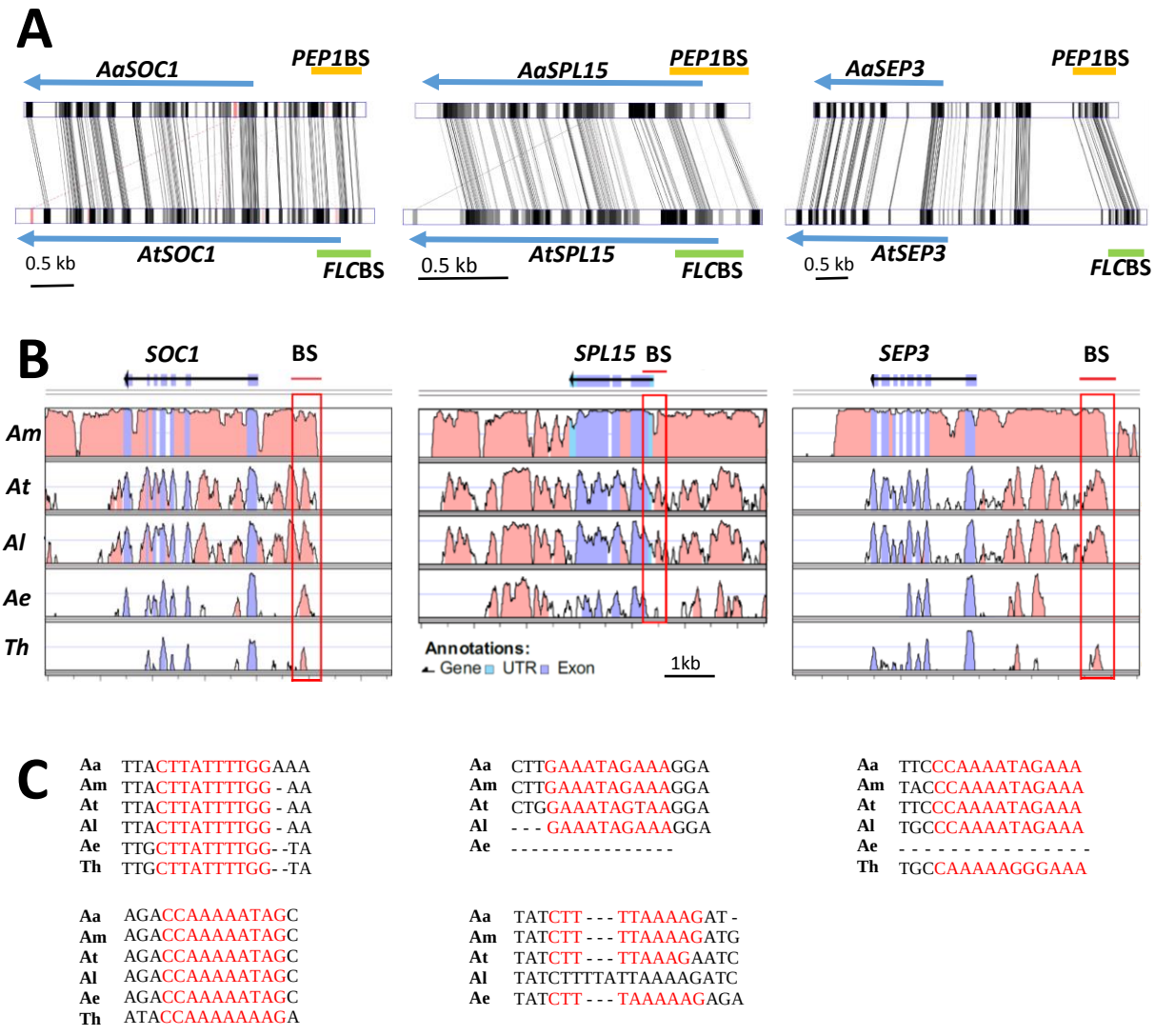


Figure 21 Conservation of BS and CArG-box for selected genes involved in flowering.

(A) Conservation of BSs at *SOC1*, *SPL15* and *SEP3* in *A. alpina* and *A. thaliana*. Homology between orthologous sequences is illustrated by GATA-alignments. *A. alpina* sequence is shown on the top, *A. thaliana* sequence on the bottom, respectively. Black lines indicate homology. Red lines indicate inversions. Intensity of the color represents degree of homology. PEP1 BSs are indicated in orange, FLC BSs in green. Genes are marked by blue arrows. **(B)** Conservation of BSs at *SOC1*, *SPL15* and *SEP3* in related species. Approximately 6 kb regions of *A.*

alpina were aligned to orthologous loci. Homology is illustrated by VISTA plots. Range of homology between 50 % and 100 % over a 100 bp sliding window is shown for each species, regions shown in red are at least 70 % conserved. PEP1 BSs are marked by red boxes. **(C)** Conservation of CArG-boxes in PEP1 and FLC BSs and in related species. Sequences around CArG-box motifs within PEP1 BSs were extracted from alignments shown in (B). All sequences corresponding to a consensus CArG-box are colored in red. **(D)** CArG-box conservation for conserved BSs in *A. alpina* and *A. thaliana* and related species. Heatmap indicates if at least one CArG-box in the PEP1 BS was conserved in the orthologous locus. Conservation with variation indicates that presence of CArG-box was conserved but sequence was slightly modified. *A. thaliana* orthologs of genes associated to BSs are listed on the right. Gene identifiers are given for genes with unknown function. If several genes were associated to one BS but only one gene contained the BSs in its promoter and therefore is probably the targeted gene, that gene is marked in bold. Alignments were performed using mVISTA. Species: Aa: *A. alpina*, At: *A. thaliana*, Am: *A. montbretiana*, Al: *A. lyrata*, Ae: *A. arabicum*, Th: *T. hassleriana*..Note that analysis of conservation of the *SPL15* locus in *T. hassleriana* did not give any result in our analysis because the reciprocal BLAST analysis did not reveal an unambiguous ortholog. More thorough analysis, however, showed that *T. hassleriana* has two putative orthologous loci and the first motif is conserved in one and the second motif in both loci (Y. Hyun, personal communication).

2.5 PEP1 and FLC regulate the response to short-term cold exposure through different target genes

Transcription of *PEP1* and *FLC* is repressed during prolonged cold treatment to allow flowering to proceed in response to vernalization (Michaels & Amasino, 1999; Wang *et al*, 2009b). GO-term enrichment analysis of PEP1 and FLC target genes revealed that these TFs regulated genes belonging to the GO-categories ‘response to cold’ and ‘response to temperature stimulus’ (Figure 20) suggesting a function for FLC and PEP1 in the response to cold stress. As represented in Figure 22, both PEP1 and FLC bound (Figure 22A) and regulated (Figure 22B) many genes described as cold regulated (COR) genes (list of robust COR genes defined by Park *et al*, 2015). However the overlap in identity of cold regulated PEP1 and FLC targets was very limited (Figure 22, Table A7). The only four cold regulated PEP1 direct target genes that were also targeted by FLC were *SOC1*, *CBF1*, *LTI78* and *COL1* (Figure 22A). Among these, *SOC1* has a major function in flowering and represses COR genes to counteract a delay in flowering (Seo *et al*, 2009). *CBF1* and *LTI78* were bound by both FLC and PEP1, but their BSs were not conserved (Figure 7B, C), leaving *COL1* as the only conserved cold regulated target gene of PEP1 and FLC. *COL1*, however was not among the conserved target genes that had a deeply conserved CArG-box motif (Figure 21D). Taken together, the function of PEP1 and FLC in the cold response does not seem to be evolutionarily conserved. Binding of PEP1 to selected COR genes was validated by ChIP-qPCR (Figure 23A) and RNA-seq results were validated by qPCR (Figure 23B).

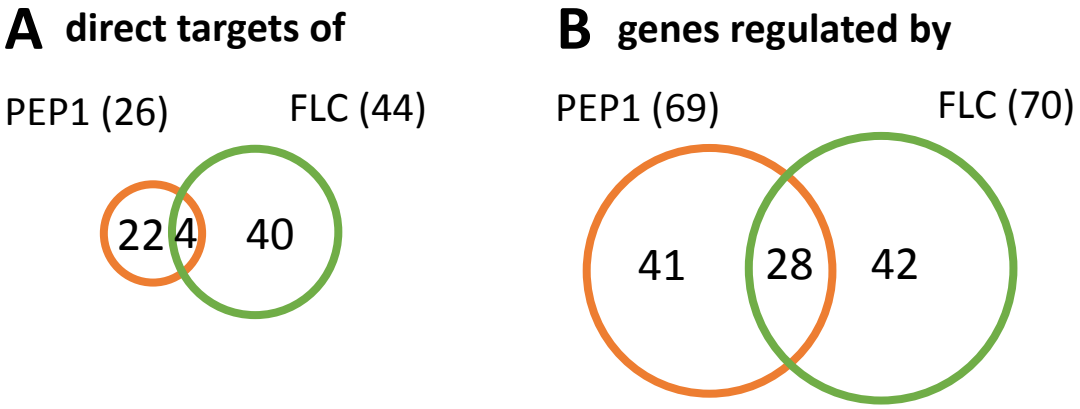
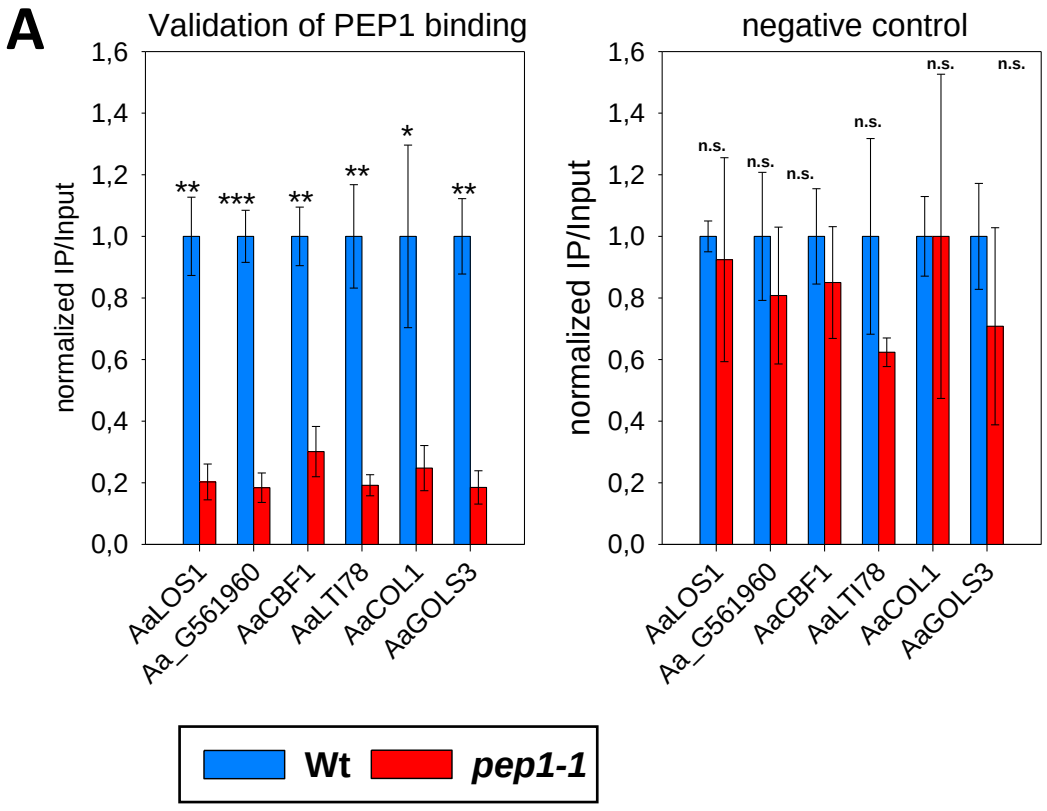


Figure 22 Comparison of COR genes that were bound and regulated by PEP1 and FLC.

Illustration showing overlap of the 1279 COR genes as defined in (Park *et al*, 2015) that were **(A)** PEP1 and FLC direct target genes and **(B)** genes regulated by these TFs.



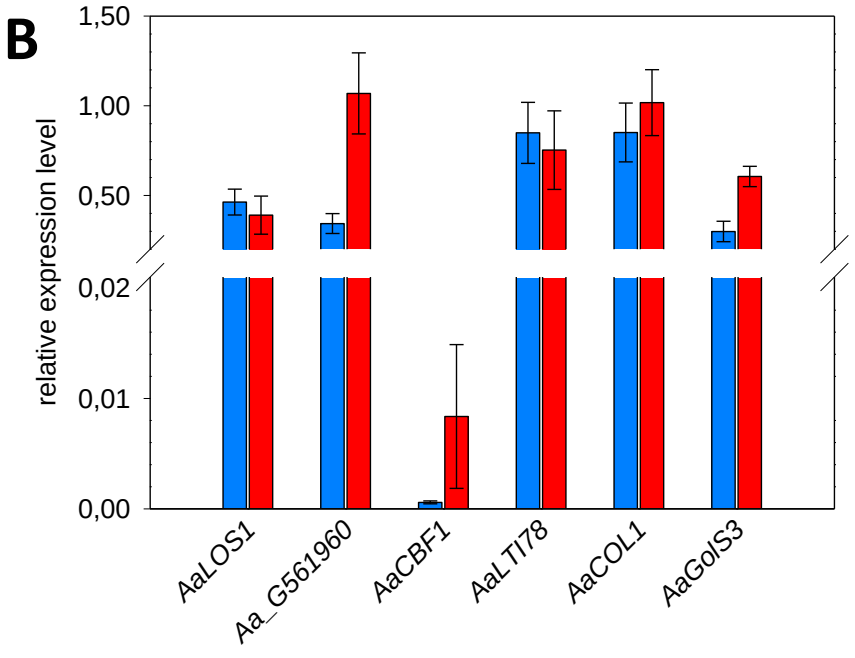
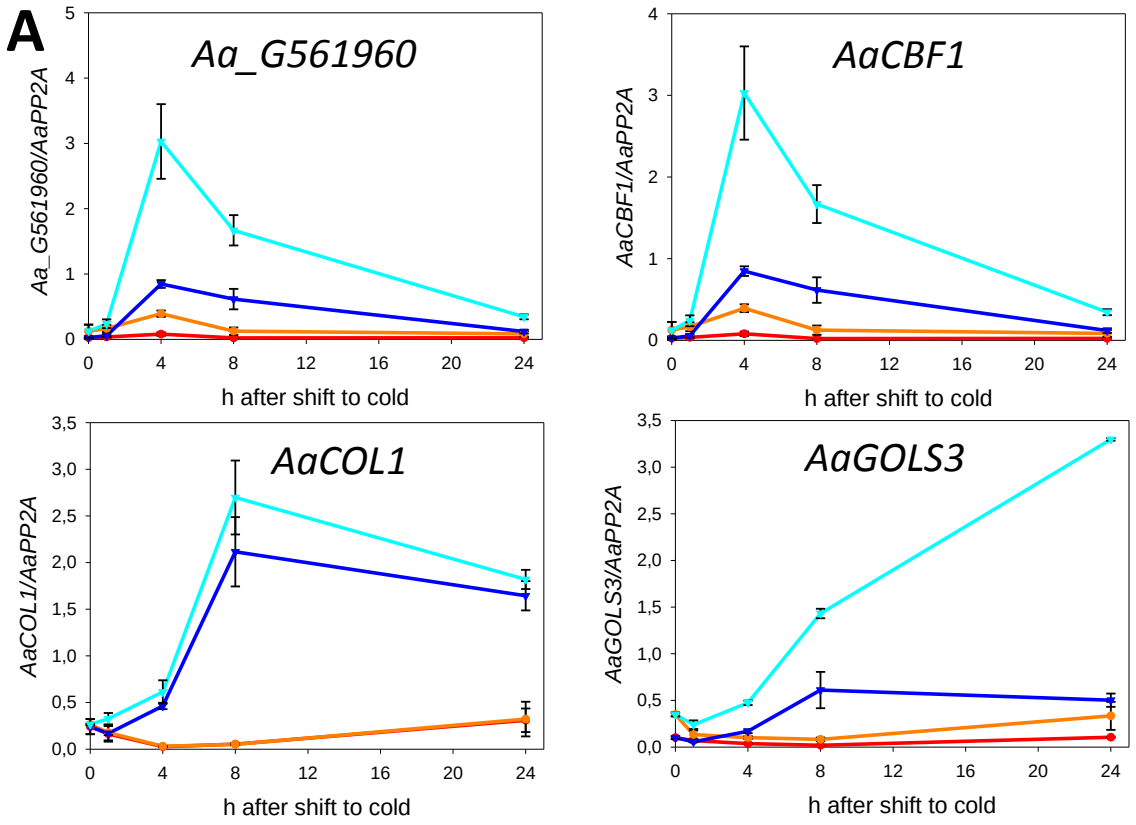


Figure 23 PEP1 binding and expression analysis for selected target genes involved in the cold response.

(A) Validation of PEP1 binding to selected COR genes by ChIP-qPCR. For each target, fold-enrichment relative to its input is shown. Negative controls were performed with primers not flanking predicted BSs (1-2 kb distance to BS). Data are shown as mean ± SEM (n = 3 biological replicates). Asterisks indicate significant enrichment in wild-type compared to *pep1-1* (n.s. not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; Student's t-test). Primers are listed in the appendix. **(B)** Validation of RNA-seq results for selected PEP1 direct target genes using qPCR. Data are shown as mean ± SEM (n = 4 biological replicates). Expression was analyzed in leaves of Wt and *pep1-1* mutant and all genes were normalized to *AaPP2A*.

Most of the COR genes analyzed by qPCR did not show expression differences in *pep1-1* compared to the wild-type, which was consistent with COR genes not being strongly enriched among PEP1 targets that are DEG in *pep1-1* (Figure 20 B, right column). Analysis of COR target gene expression after up to 24 h of cold exposure showed that these genes were induced by cold in *A. alpina* (Figure 24A), as was described for their orthologs *A. thaliana* (Park *et al*, 2015). Expression levels reached considerably higher levels in the cold-treated *pep1-1* mutant compared to cold-treated wild-type (Figure 24A), suggesting that PEP1 functions after short exposures to cold to repress the induction of COR genes. The same experiment was performed in *A. thaliana* and interestingly, also in the *flc-3* mutant, COR genes were induced to higher levels in response to cold compared to wild-type (Figure 24B). These findings suggest that FLC also acts to repress cold induction of COR genes although only *COL1*, *CBF1* and *LTI78* but not the other two genes tested were direct targets of FLC. Thus, PEP1 and FLC seem to have a similar function in the response to intermittent cold stress but the molecular mechanisms are not conserved.



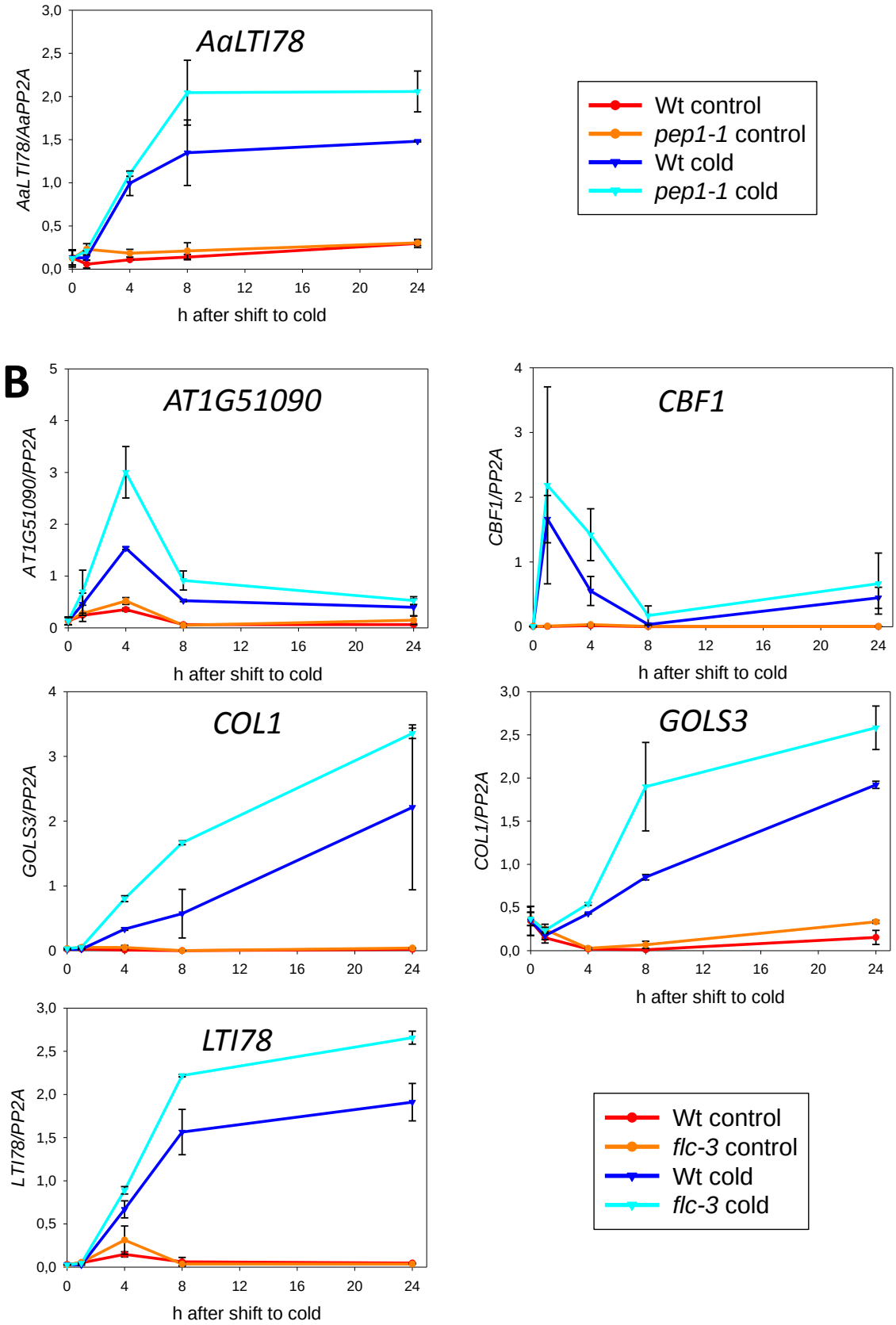


Figure 24 Effect of PEP1 and short cold treatment on COR gene expression and conservation in *A. thaliana*.

Effect of mutations in *PEP1/FLC* on expression of selected cold-regulated PEP1 target genes after transferring plants to 4°C for 24 h compared to control conditions (21°C). Data are shown as mean ± SEM (n = 2 biological replicates). Each experiment was normalized to the average expression across the time course of the mutant in cold. **(A)** *A. alpina*. **(B)** *A. thaliana*. Note that only *COL1* is a conserved target gene.

A role of PEP1 and FLC in regulating the stress response to cold temperatures was not described before. Previously, these TFs were described to function before vernalization to repress flowering and to be silenced during vernalization (Michaels & Amasino, 1999; Wang *et al*, 2009b). In *A. thaliana*, intermittent cold causes rapid induction of COR genes which permits the acclimation to cold temperatures leading to a higher tolerance to freezing temperatures (Thomashow, 1999). COR genes are strongly and transiently induced by intermittent cold but their expression levels are still elevated after prolonged cold treatment compared to control conditions (Zarka *et al*, 2003). To test whether PEP1 also affects COR gene expression after prolonged cold exposure in *A. alpina*, COR gene expression was analyzed during vernalization. All genes tested were still up-regulated after prolonged exposure to cold compared to control conditions. *COL1*, *LTI78*, *Aa_G561960* and *GolS3* showed higher levels of cold induction in *pep1-1* compared to wild-type after several weeks of vernalization (Figure 25), indicating that PEP1 still functions during vernalization to repress induction of COR genes. Plant exposure to cold temperatures causes a reduction of growth (Atkin *et al*, 2006) and in *A. thaliana*, overexpression of the COR gene *CBF1* causes severe growth reduction (Kasuga *et al*, 1999) suggesting that active repression of growth is part of the response to cold temperatures. To test whether PEP1 has an effect on the cold response at the phenotypic level, growth of *pep1-1* was analyzed during cold exposure. 2w old wild-type and *pep1-1* mutant seedlings were shifted to cold and plant diameter was measured. During cold exposure, wild-type plants had a greater diameter compared to *pep1-1*, whereas before or after cold treatment, and under control conditions (after 3w in warm temperature) this effect was not observed (Figure 26). These findings suggest that growth reduction in response to cold is stronger in *pep1-1*, indicating that PEP1 acts to modulate and lower the cold response, which is a previously unknown function of PEP1. Taken together, PEP1 and FLC seem to function during cold exposure to repress the cold induction of COR genes, which in *A. alpina* was shown to correlate with increased growth at cold temperatures. PEP1 and FLC bind highly divergent sets of COR genes, suggesting that their role in the regulation of the cold response evolved convergently.

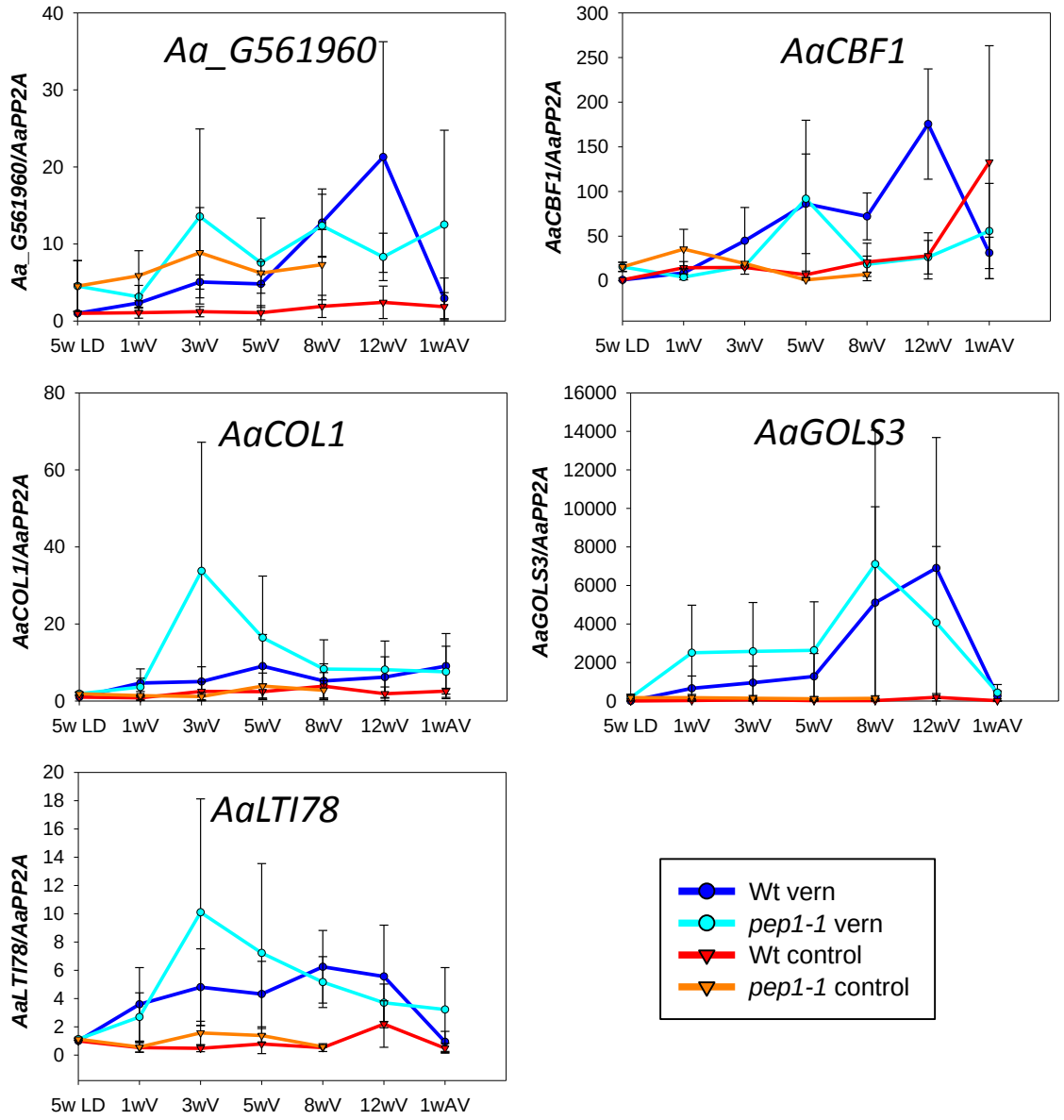


Figure 25 Effect of PEP1 and vernalization on COR gene expression.

Effect of PEP1 and vernalization treatment on expression of selected cold-regulated PEP1 target genes. Plants were grown for 5.5 w in LD and then transferred to 4°C or kept under control conditions (21°C) for 12 w and then shifted back to LD. Data are shown as mean ± SEM (n = 2 biological replicates). Each experiment was normalized to expression in Wt at the start of the experiment.

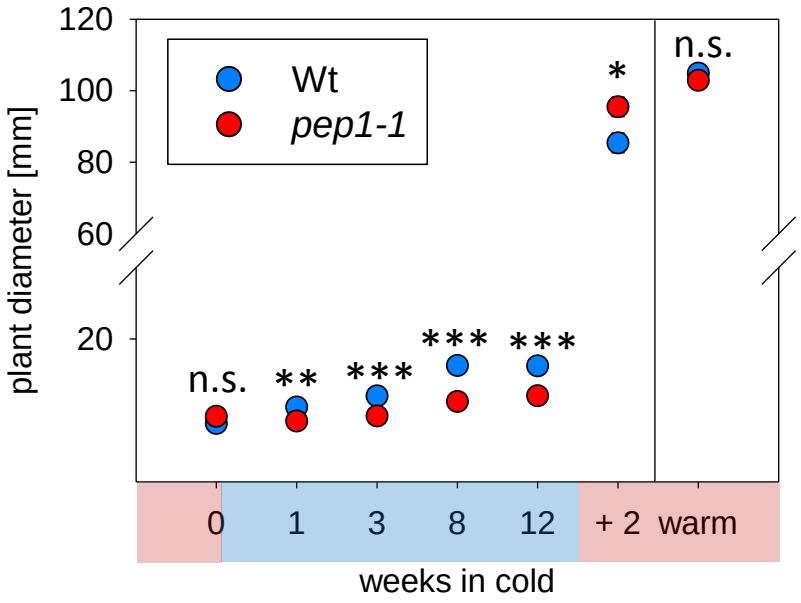


Figure 26 Growth phenotype of *pep1-1* in cold.

Growth phenotype of *pep1-1* in cold. Plant diameter during cold treatment is shown as mean ± SEM, 2 independent biological replicates (total number of replicates after combining all biological replicates: n ≥ 37). Student's t-test between wild-type and *pep1-1* was performed (n.s. not significant; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001). Controls (warm) represent plants that were grown at 21°C for 3 weeks.

2.6 Summary

In summary, a small set of PEP1 and FLC BSs were conserved. Most of these BSs contained a conserved CArG-box in both species that often contained the TTT extension preceding the core motif. Conserved target genes were mainly involved in the regulation of flowering and flower development, which seems to be the core function of PEP1 and FLC under LD conditions (Figure 27). On the other hand, species-specific BSs were correlated with the absence of a CArG-box in the species where binding was not detected. Despite the high number of species-specific target genes, PEP1 and FLC were involved in largely similar biological processes, which mainly involved the response to environmental stimuli and the phytohormone GA. PEP1 and FLC regulated different sets of COR genes, which was associated with a reduction in the cold response in both species. This function probably represents a similar biological role of PEP1 and FLC that arose by convergent evolution (Figure 27).

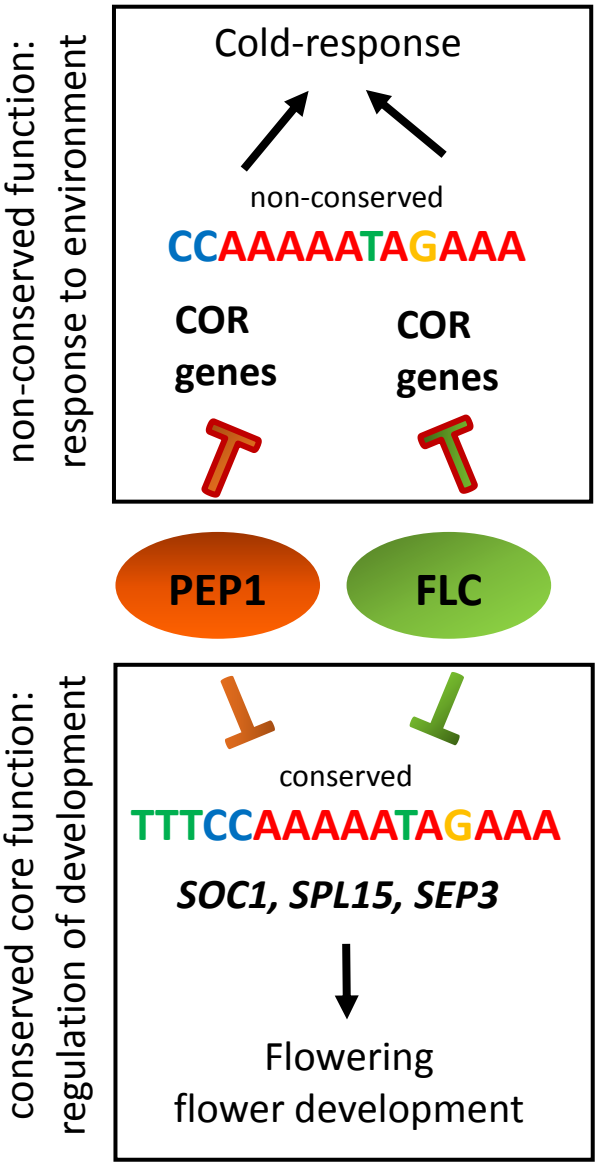


Figure 27 Model summarizing conservation and divergence of PEP1 and FLC in the regulation of flowering and the cold response.

PEP1 and FLC regulate a small set of conserved core target genes that is involved in the regulation of flowering by binding conserved CArG-box motifs that contain the TTT extension at the 5‘ end. In addition, PEP1 and FLC regulate a high number of species-specific target genes that contain a CArG-box only in the species where they are bound. Regulation of different sets of COR genes by FLC and PEP1 causing a repression of the response to cold is a representative example of species-specific target genes involved in similar biological processes. Arrows represent positive regulation, bars represent negative regulation. Red frames indicate non-conserved functionality.

2.7 Discussion

2.7.1 Conservation of FLC and PEP1 binding sites

In this study, we performed one of the first comparative ChIP-seq studies in two related plant species and found high divergence of BSs for the FLC TF in *A. thaliana* and its ortholog PEP1 in *A. alpina*. Studies investigating conservation of BSs of developmental TFs in vertebrates generally find a rapid turnover of BSs (summarized in Villar *et al*, 2014). For example, less

than 30 % of BSs of liver development TF CEBPα are shared between human and macaque, that diverged around 30 million years ago (Ballester *et al*, 2014). *A. thaliana* and *A. alpina* have a comparable evolutionary distance and only 17 % of PEP1 BSs were conserved between the two species. This indicates that conservation of FLC BSs in Brassicaceae is similar, even slightly lower, than that of CEBPα BSs in vertebrates, which is in line with what was previously found for the plant MADS-box TF SEP3 in *A. thaliana* and *A. lyrata* (Muiño *et al*, 2016). Taken together, these results suggest that BSs of developmental TFs in plants as in vertebrates evolve rapidly, which is in contrast to the higher conservation of BSs in *Drosophila* species (summarized in Villar *et al*, 2014).

Conservation of FLC and PEP1 BSs was associated with the conserved presence of a CArG-box motif in both species. For species-specific BSs, this motif was usually absent in the species where binding was not detected due to local sequence changes while general sequence conservation was maintained. Rapid evolution of TF BSs by point mutations that introduce new *cis*-elements was predicted more than 10 years ago (Stone & Wray, 2001) and in the meantime, several studies that focused on the evolution TF BSs in *Drosophila* and vertebrates could associate conservation and divergence of BSs with the conserved or species-specific presence of *cis*-elements, respectively (Bradley *et al*, 2010; He *et al*, 2011; Schmidt *et al*, 2010). Also *A. thaliana*-specific SEP3 BSs were associated with local nucleotide changes at the orthologous site of the CArG-box in *A. lyrata* (Muiño *et al*, 2016).

Alignments of some *A. alpina*-specific CArG-boxes to *A. thaliana* and other species suggested that those CArG-boxes were gained in the *A. alpina* lineage, rather than specifically lost in *A. thaliana* (Figure 13). Furthermore, the analysis of motif conservation in other species suggested that some conserved BSs are specifically conserved between *A. alpina* and *A. thaliana* and in consequence lost in other species because the correlation coefficient increases if *A. thaliana* is removed from the analysis (Figure 14). These two studies provide examples of gains and losses of BSs, however, which mechanism leading to diversification of binding is more common remains to be tested by future ChIP-seq studies in additional species.

Several studies identified the expansion of TEs carrying *cis*-elements as a rapid means to generate a high number of new TF BSs in vertebrates but not in *Drosophila* (Kunarso *et al*, 2010; Ni *et al*, 2012; Schmidt *et al*, 2012). Compared to humans, *Drosophila* has a much smaller genome and intergenic space is strongly reduced. This, in combination with a higher population size results in a lower tolerance of random mutations and a faster rate of removal of fixed TE insertions (González & Petrov, 2012). Variation of genome size and TE content in plants is

much more common than in vertebrates (Bennetzen *et al*, 2005; Dehal & Boore, 2005; Hawkins *et al*, 2009), suggesting that TE insertions in plants might be a mechanism causing high variation in the number of TF BSs between species. Indeed, *A. lyrata*-specific SEP3 BSs were associated with TE insertions (Muiño *et al*, 2016), corresponding to a bigger genome and higher number of TEs in *A. lyrata* compared to *A. thaliana* (Hu *et al*, 2011). Although *A. alpina* has an even bigger genome and higher TE content than *A. lyrata* (Willing *et al*, 2015), we did not find *A. alpina*-specific PEP1 BSs associated with TEs. It is noteworthy that the expansion of TEs provides just one mechanism of rapid multiplication of BSs, and for fixation of these, selection pressure has to coincide with a boost of TEs containing a specific *cis*-element. To determine if TE expansion plays a greater role in the evolution of TF BSs in plants, further comparative ChIP-seq studies will be required.

Despite a statistically significant association of conserved BSs with conserved CArG-boxes, the presence of a conserved CArG-box was not sufficient to predict conservation of binding. Close to 30 % of PEP1/FLC BSs with a CArG-box in both genomes did not show conserved binding. In humans, recent publications claim that in contrast to what was previously expected, the majority of species-specific binding events cannot be explained by sequence changes in *cis*-elements (Deplancke *et al*, 2016). It remains to be elucidated whether BSs of plant TFs generally show this more complex pattern of evolution. For 41 % of *A. thaliana*-specific BSs that have a CArG-box in both genomes, I found that the divergence of binding could potentially be explained by the presence or absence of a second *cis*-element in one of the genomes (Figure 16). This second motif might be bound by an interacting TF that could positively or negatively influence PEP1 binding in *A. alpina*. In addition, these conserved CArG-boxes in species-specific BSs were more frequently bound by other MADS-box TFs than average species-specific BSs (Figure 18). Furthermore, I found that conserved CArG-boxes in species-specific BSs were more widely conserved in other species compared to all CArG-boxes (Figure 17), suggesting that CArG-boxes that were conserved although FLC binding was not are important for additional processes. Taken together, these findings suggest that conserved CArG-boxes in non-conserved BSs are part of *cis*-regulatory modules (CRMs), which are regions containing BSs for several TFs in close proximity.

Various studies have supported the importance of interacting TFs that influence binding of its partners. In *Drosophila* and vertebrates, BSs that were clustered with BSs of other TFs in CRMs were preferentially conserved between species (He *et al*, 2011; Stefflova *et al*, 2013; Ballester *et al*, 2014). Furthermore, binding of TFs to BSs in CRMs was more strongly affected by

mutation of interacting TFs compared to binding to isolated BSs (Stefflova *et al*, 2013; Ballester *et al*, 2014). Other studies showed that sequence changes in neighboring BSs account for divergence in TF binding in *Drosophila* and rodents (Bradley *et al*, 2010; He *et al*, 2011; Heinz *et al*, 2013). In contrast, Tuch et *al*., showed that evolution of interactions between TFs in yeast can be rapid (Tuch *et al*, 2008) and between human and mouse, only 50 % of interactions between TFs are conserved (Ravasi *et al*, 2010). These findings suggest that not only the generation of new *cis*-elements but also new TF-TF interactions can drive evolution of TF binding in these species. Whether this plays a significant role in plants remains to be determined. The importance of interacting TFs for TF binding in *A. thaliana* has been shown in numerous publications. Binding of FLC to some BSs, for example, depends on the presence of SVP and *vice versa* (Mateos *et al*, 2015) and DNA binding of SVP in the ambient temperature flowering pathway depends on the presence of its interactor FLM (Posé *et al*, 2013b). Finally, repurposing of TF BSs (temporal or spatial variation of TF binding) was found to be very common between human and mouse (Denas *et al*, 2015). To test this possibility in plants, it will be interesting to compare PEP1 and FLC BSs not only at one developmental stage but at different time points as well as under different conditions and in specific tissues.

TFs could also influence binding of their partner proteins by recruiting them to the DNA. Several FLC BSs in *A. thaliana* did not contain a CArG-box motif but 48 % of these sites contained a G-box or TGGGCC motif, suggesting that binding might occur indirectly via interactors that bind to these motifs. In the remaining BSs without CArG-boxes, additional other motifs bound by other groups of interacting TFs might be present but not easily detectable due to a low number of events. Indirect binding might be a common phenomenon in *A. thaliana* as analysis of 27 ChIP-seq studies in this species revealed that SEP3 and FLM were the only TFs that contained a canonical motif in every BS (Heyndrickx *et al*, 2014). Also functionality of TF binding did not depend on the presence of a canonical motif in the BSs of the remaining 25 TFs (Heyndrickx *et al*, 2014). Specifically for MADS-box TFs it was shown that complexes of four MADS-box TFs can bind to sites that contain only one canonical CArG-box (which would be bound by one of the two dimers) (Melzer & Theissen, 2009; Smaczniak *et al*, 2012b). Since almost all *A. alpina* PEP1 BSs contained a CArG-box, indirect binding might be more common in *A. thaliana*. Alternatively, indirect interactions might not have been detected in *A. alpina* due to slight differences in the experimental conditions.

In conclusion, the presence of a conserved CArG-box correlates well with conservation of PEP1/FLC binding, however, the conserved CArG-box is neither necessary nor sufficient for

conserved binding. Therefore, direct testing of binding, rather than prediction by DNA sequence analysis, will be required in the future to get a more complex picture of the evolution of TF BSs in plant species and the role that TF networks play in plant developmental processes.

2.7.2 The regulation of flowering is the conserved core function of PEP1 and FLC

Genes related to flowering and flower development were found to be enriched among conserved target genes of PEP1 and FLC. *Pep1* and *flc* mutants in both species have a strong late-flowering phenotype (Michaels & Amasino, 1999; Sheldon *et al*, 1999; Wang *et al*, 2009b), suggesting that the regulation of flowering is also a major function of these TFs. This hypothesis is further supported by the finding that more of the conserved target genes, which were enriched in flowering-related genes, were differentially regulated in the *pep1-1* mutant (Figure 9). BSs of some flowering-related target genes were also conserved in other Brassicaceae species and in *T. hassleriana*, a member of the sister family Cleomaceae (Figure 21 B-D). Recently, FLC and its regulation by vernalization was also reported in monocot species, however a function of FLC in vernalization of monocots has not been defined genetically (Ruelens *et al*, 2013). Taken together, these findings suggest that the function of FLC in the regulation of flowering is the major function of FLC under standard experimental conditions and is deeply conserved in the Brassicaceae family and perhaps beyond. Future testing of FLC binding to flowering related genes in additional species will be crucial to validate this hypothesis.

Conservation of the core function despite generally high diversification of target genes of orthologous TFs in related species has also been postulated for SEP3 in *A. thaliana* and *A. lyrata* (Muiño *et al*, 2016) and for TFs in yeast (Tuch *et al*, 2008) and vertebrates (Conboy *et al*, 2007; Odom *et al*, 2007; Schmidt *et al*, 2010; Ballester *et al*, 2014). For example, BSs of liver development TF CEBPα that were shared between five vertebrate species were specifically enriched for genes related to liver development (Schmidt *et al*, 2010) and CRMs of different liver development TFs that were shared between species showed a higher enrichment of liver related GO-terms (Ballester *et al*, 2014), indicating conservation of an entire gene regulatory network. In contrast to this deep conservation of regulatory networks, other studies explained functional conservation by high degrees of compensatory binding, for example binding of two orthologous TFs to different sites in proximity of the same genes in both species resulting in an identical transcriptional output (Odom *et al*, 2007; Kunarso *et al*, 2010; Schmidt *et al*, 2010; Heinz *et al*, 2013; Denas *et al*, 2015). For instance, analysis of BSs of four liver-specific TFs FOXA2, HNF1A, HNF4A and HNF6 in human and mouse revealed that approximately two thirds of all common target genes did not have conserved BSs (Odom *et al*, 2007).

A. alpina PEP1 and *A. thaliana* FLC bound conserved BSs associated with three central floral integrator genes at the SAM, indicating that their evolutionarily conserved function is to inhibit floral induction at the meristem. SOC1 and SPL15 are floral promoters that integrate signals from various flowering pathways (Samach *et al*, 2000; Hepworth *et al*, 2002; Moon *et al*, 2003; Schwab *et al*, 2005; Helliwell *et al*, 2006; Searle *et al*, 2006; Wang *et al*, 2009a; Deng *et al*, 2011; Hyun *et al*, 2016) whereas SVP is a repressor of flowering (Lee *et al*, 2007; Fujiwara *et al*, 2008; Li *et al*, 2008). In contrast to *SOC1* and *SPL15*, *SVP* was not differentially expressed in *pep1*/*flc* mutant plants, however the role of PEP1/FLC in the regulation of floral repressor *SVP* might be fine-tuning or feed-back control to prevent precocious induction of flowering. Furthermore, a conserved BS was associated with *SEP3*, a master regulator of floral development (Pelaz *et al*, 2000; Honma & Goto, 2001), indicating that PEP1 and FLC might also have a conserved function in a later step following floral initiation. However, SEP3 has developmental stage specific functions (Pajoro *et al*, 2014) and thus might contribute to the function of FLC/PEP1 in the initiation of flowering. **F**inally, *PIF3* is a conserved target gene involved in the initiation of flowering. PIF3 acts in the ambient temperature pathway to induce *FT* expression (Galvão *et al*, 2015). In consequence, a third level of the conserved regulation of flowering by PEP1 and FLC might be in the ambient temperature pathway. However, PIF3 physically interacts with the phytochrome photoreceptors (Castillon *et al*, 2007). Thus, the role of FLC/PEP1 in the regulation of PIF3 might be related to light signaling but not flowering. In addition, PEP1 and FLC regulated genes that are involved in other flowering pathways by non-conserved binding to common target genes (compensatory binding): FLC binds to florigen *FT* (Helliwell *et al*, 2006; Searle *et al*, 2006), whereas PEP1 weakly binds to an ortholog of *TSF*, which is a paralog of *FT* with conserved function (Yamaguchi *et al*, 2005). Furthermore, both TFs bind to different BSs at *GA2OX8*, suggesting a role in the GA pathway. Finally, species-specific binding to additional flowering related genes was identified, indicating that another aspect of the function of PEP1 and FLC in the regulation of flowering is species-specific and might have evolved independently (see 2.7.3).

Conserved BSs, which are mainly associated with flowering-related genes, showed a very high overlap with BSs of other *A. thaliana* MADS-box TFs, which also have a function in flowering. This finding suggests that the general network that controls flowering, which involves PEP1/FLC and other interacting MADS-box TFs, is conserved between species. This hypothesis is further supported by the finding that the CArG-boxes in conserved BSs were extended by the ‘TTT’ trinucleotide preceding the core motif in both species. Since CArG-boxes are palindromic, the ‘TTT’ extension at the 5’ end is probably functional equivalent to

the ‘AAA’ extension at the 3’ end. The ‘AAA’ extension following the core motif was previously identified in BSs of SEP3, FLC, SVP, SOC1, AP3 and PI and the additional ‘TTT’ extension was identified in BSs of SOC1, SEP3 FLC, SVP, AP3 (Deng *et al*, 2011; Immink *et al*, 2012; Tao *et al*, 2012; Wuest *et al*, 2012; Pajoro *et al*, 2014; Mateos *et al*, 2015). The occurrence of this CArG-box with extended half site in all of these BSs of flowering-related MADS-box TFs might indicate that this part of the motif plays a role in the flowering network. Since MADS-box TFs act as tetramers of which one dimer binds a CArG-box (Schwarz-Sommer *et al*, 1992; Pellegrini *et al*, 1995; Egea-Cortines *et al*, 1999; Honma & Goto, 2001; Folter *et al*, 2005; Smaczniak *et al*, 2012a) this might imply that FLC and other flowering MADS-box TFs interact and thus influence each other’s BSs to be enriched for the trinucleotide extension. Pajoro *et al*. previously suggested that SEP3 binds different CArG-boxes at different developmental stages (Pajoro *et al*, 2014). The motif with both extensions is bound throughout development, whereas the one lacking the ‘TTT’ extension is only bound during later stages of floral development (probably when FLC is not expressed anymore). Taken together, the CArG-box with the ‘TTT’ extension might be preferentially bound by MADS-box TFs functioning in the conserved network regulating floral induction and early flower development.

The present study showed that FLC and PEP1 repressed a set of deeply conserved target genes to repress floral induction at the SAM. This knowledge paves the way for future investigation of the regulation of genes in a conserved flowering network in two species with different life histories. *SPL15* was one of these deeply conserved PEP1/FLC target genes. In *A. thaliana*, SPL15 integrates signals from three different flowering pathways: miR156 represses *SPL15* at the post-transcriptional level in the age pathway (Schwab *et al*, 2005; Hyun *et al*, 2016), FLC represses *SPL15* transcription in the vernalization pathway (Deng *et al*, 2011) and DELLA repressors inhibit SPL15 activity in the absence of GA (Hyun *et al*, 2016). SPL15 plays an important role in floral induction under non-inductive SD conditions but the repression of *SPL15* can be bypassed by the LD pathway (Hyun *et al*, 2016). Plants overexpressing miR156 which represses *SPL15*, flower slightly later in LDs but are very late flowering under SD conditions and this phenotype is also shown by *spl15* mutants (Schwab *et al*, 2005; Wang *et al*, 2009a; Hyun *et al*, 2016). The conditional role of the SPL15 pathway suggests that annual *A. thaliana* has evolved a mechanism of flowering rapidly in response to LDs and independently of SPL15. In *A. alpina*, *SPL15* is also regulated by vernalization and plant age because PEP1 and miR156 repress *SPL15* on the transcriptional and post-transcriptional level (Bergonzi *et al*, 2013; this study). Overexpression of miR156 prevents flowering of wild-type plants after vernalization despite down-regulation of *PEP1*. Also flowering of *pep1-1* mutant plants in LDs

is strongly delayed by overexpression of miR156, suggesting that in *A. alpina*, miR156 can block the vernalization response downstream of PEP1, probably at the level of SPL TFs (Bergonzi *et al*, 2013). It is possible, that de-repression of *SPL15* is essential for flowering of *A. alpina* in response to vernalization, as suggested by the restricted vernalization response of the *A. alpina spl15* mutant (preliminary results by Y. Hyun, personal communication). This would imply that *A. alpina* shares a conserved network of flowering time genes with *A. thaliana*, which has been adapted to different life histories. In *A. alpina*, *SPL15* would be essential for flowering, which can only proceed when the negative regulators of *SPL15*, PEP1 and miR156 have been repressed. By contrast, in annual *A. thaliana*, the *SPL15* pathway is not essential for flowering but can be bypassed by LDs, ensuring that flowering occurs within one year to allow the rapid cycling life-history. In order to compare *SPL15* regulation in *A. thaliana* and *A. alpina*, genomic *SPL15* was cloned in both species to create transgenic lines expressing the protein fused to a Venus fluorescent protein tag (see apendix). Spatial and temporal expression of *SPL15* will be analyzed in response to vernalization and the effect of FLC/PEP1 will be tested in constructs carrying mutated CArG-boxes in the FLC/PEP1 BSs (*mSPL15*). The interaction of age and vernalization pathway will be analyzed by comparing *SPL15* expression and its induction by vernalization in juvenile and adult plants. To dissect the effects of the different pathways the miR156 recognition sequence was mutated (*rSPL15*) and to investigate the interactions of FLC/PEP1 and miR156, this mutation was combined with a mutation in the CArG-box (*mrSPL15*). Finally, the effect of GA on SPL15 activity on *FUL* transcription will be measured in the different transgenic lines to provide information about the interaction of the three flowering pathways that are integrated by SPL15 in *A. thaliana* and *A. alpina*.

Besides a conserved function in the regulation of flowering, PEP1 and FLC seem to have other conserved functions as suggested by conservation of the BSs of several other genes throughout the Brassicaceae family. FLC was described to delay shoot maturation in *A. thaliana* by directly repressing *SPL15* and possibly also *SPL3*, although no differential expression of *SPL3* was detected (Deng *et al*, 2011). *SPL15* is among the target genes with the most deeply conserved FLC/PEP1 BS, suggesting that also PEP1 might have a function in regulating shoot maturation in *A. alpina*. However, since *SPL15* is also involved in the initiation of flowering, the function of PEP1 in regulating this gene might also be restricted to the flowering control.

Among the target genes with deeply conserved BSs was also *bZIP44* (Figure 21), a promoter of germination (Iglesias-Fernández *et al*, 2013). *bZIP44* is expressed during germination and activates transcription of a gene encoding MANASE7, which causes breakage of embryo

surrounding tissue during germination (Iglesias-Fernández *et al*, 2013). As a transcriptional repressor, FLC/PEP1 might repress transcription of *bZIP44*, and thereby repress germination, however, this remains to be tested experimentally and it cannot be excluded that *bZIP44* has additional roles later in development. Nevertheless, FLC was previously described to induce temperature dependent seed germination by positively regulating GA biosynthesis in cold imbibed seeds via the known flowering genes *FT* and *SOC1* (Chiang *et al*, 2009). Taken together, it seems that FLC is involved in two different pathways with opposite effects on germination, however, both effects might play a role at different developmental stages. The bZIP44 pathway was described to be active in germinating seeds whereas the pathway involving flowering genes is active during seed maturation and maternal FLC primes the seeds to germinate when imbibed (Chiang *et al*, 2009; Iglesias-Fernández *et al*, 2013). The conserved BSs at *bZIP44* suggest that this pathway is conserved between species, and PEP1 and FLC might act to repress germination before the first winter. Whether the other pathway involving deeply conserved target *SOC1* and *A. thaliana*-specific target *FT*, is also conserved in *A. alpina* remains to be tested. It might be a means of fine-tuning the timing of germination in *A. thaliana* to counteract the repression of germination before winter via the *bZIP44* pathway if conditions are favorable to allow more rapid cycling.

Another deeply conserved target gene is *BRC1*. *BRC1* is transcriptionally repressed by FLC and PEP1 and in *A. thaliana*, *BRC1* was described to repress branching by inhibiting outgrowth of axillary meristems (AMs) (Aguilar-Martínez *et al*, 2007). Together, these observations suggest that FLC and PEP1 activate branching, which is in contrast to the high branching phenotype observed in the *pep1-1* mutant (data not shown). BRC1 also interacts with FT to delay floral transition in AMs (Niwa *et al*, 2013). Therefore, FLC might repress *BRC1* to delay flowering of axillary shoots. In annuals this might change plant architecture which could be beneficial to facilitate seed dispersal in a way that first the main shoot and later the side shoots flower and then can freely spread their seeds. In perennial plants, this might prevent some side shoots from flowering to allow vegetative growth after floral induction of the SAM. Alternatively, consistent with its induction during vernalization (data not shown), *BRC1* might play additional roles in floral buds and regulate branching of the inflorescence. In barley, the *BRC1* ortholog *HvTb1* was associated with an increased number of spikelets due to increased male fertility (Ramsay *et al*, 2011). Previously, FLC was identified as a QTL causing reduced shoot branching by repressing AM formation in *A. thaliana* (Huang *et al*, 2013). This indicates that also the putative function of FLC in branching involves several pathways (including AM formation and outgrowth) with opposite effects. Although it is currently unknown, if the effect

on AM formation is conserved in *A. alpina*, it could explain the increased branching of the *pep1-1* mutant.

In conclusion, besides flowering, other life-history traits are regulated by PEP1 and FLC. It seems that for these different traits, PEP1 and FLC regulated some conserved target genes and additional species-specific BSs that might confer additional species-specific functions. For several traits, there was evidence, that PEP1/FLC had positive and negative effects on the same trait, possibly thereby fine-tuning developmental transitions. Combined regulation of different traits by FLC in winter annual accessions of *A. thaliana* or by PEP1 in perennial *A. alpina*, might allow plants to optimize their fitness. FLC might contribute to the optimization of the time of germination and by delaying shoot maturation and increasing the number of shoot branches, FLC might allow the plant to produce a higher number of flowering branches once flowering is induced after vernalization. In summer annual accessions, this regulation by FLC might not be favorable over a rapid completion of the life cycle, and in consequence many summer annuals are *flc* mutants. In this study, information on deeply conserved target genes of PEP1 and FLC gave insight on their conserved core function. In the future, ChIP-seq of less well characterized TFs in different species can be used as a tool to get information about their core functions based on deeply conserved binding events.

2.7.3 Species-specific functions of PEP1 and FLC reflect convergent evolution in adaptation to the environment

Comparison of functions associated with FLC and PEP1 target genes did not reveal any species-specific function despite the high divergence of both gene sets. In both species, target genes were mainly enriched for GO-terms related to flowering and the response to cold and gibberellins. PEP1 and FLC bound large sets of COR genes that hardly overlapped and repressed their cold induction (Figure 22; Figure 23). This indicates that PEP1 and FLC repress the response to intermittent cold using different mechanisms. Intermittent cold induces the cold acclimation response, which is an acquired tolerance to freezing temperatures after exposure to cold non-freezing temperatures (Thomashow, 1999). This cold response includes retardation of growth, representing a trade-off between stress tolerance and growth (Scheres & van der Putten, 2017). Thus, repression of the cold response by PEP1/FLC might influence the trade-off between freezing tolerance and growth under cold, but non-freezing temperatures. Indeed, in *A. alpina*, growth retardation was stronger in the *pep1-1* mutant. Cold but non-freezing temperatures could occur at the end of autumn before winter or in early spring. At these times of year, cold temperature might coincide with expression of *FLC/PEP1* which is not yet down-

regulated in autumn or expression is already increasing in the case of *PEP1* in spring. Therefore, PEP1 and FLC might be ideal regulators to ensure growth under cold but non-freezing conditions while freezing tolerance can be maximal in winter, towards the end of vernalization when flowers are formed, which is when *PEP1/FLC* is silenced. This specific selection pressure might explain why PEP1 and FLC independently evolved a regulatory role in the cold response. Interestingly, although PEP1/FLC were associated with the vernalization response, the idea of them functioning specifically in response to cold has not been proposed previously. Coupling of vernalization response and freezing tolerance was previously described in cereals. In wheat and rye, freezing tolerance is maximal at the beginning of vernalization and then decreases towards the time of flowering (Fowler *et al*, 1996). In barley, vernalization induces expression of floral promoter *VRN1* which itself directly represses *CBF* genes to reduce freezing tolerance (Deng *et al*, 2015). In contrast to *A. alpina*, which grows in the Alps, cereals might not encounter cold temperatures after or towards the end of winter. A recent study suggested an opposite trend, putatively the same as in *A. thaliana* and *A. alpina*, in *A. arenosa* populations, where non-vernalization requiring populations are more freezing tolerant (Baduel *et al*, 2016). It will be interesting to test whether this increased freezing tolerance is due to a non-functional FLC-pathway as we observed for *A. thaliana* and *A. alpina*.

Analysis of natural variation in *A. alpina* has revealed that Swedish accessions were more freezing tolerant and earlier flowering than Spanish accessions (Toräng *et al*, 2014). Taking into account that vernalization requirement due to active *PEP1* might be associated with a repression of cold tolerance, it is possible that in northern accessions, the selection pressure to increase cold tolerance caused selection of an inactive *pep1* allele. In consequence, early flowering of these accessions due to inactive *pep1* would be a side effect of increased cold tolerance. Whether this is a general trend in *A. alpina* accessions, could be tested by analyzing freezing tolerance and vernalization requirement in additional accessions. Also in *A. thaliana*, many natural early flowering accessions carry mutations in *FRI* or *FLC* (Johanson *et al*, 2000; Michaels *et al*, 2003) and northern accessions were found to be more freezing tolerant (Horton *et al*, 2016). A latitudinal cline in flowering time however, was only found in accessions carrying an active FLC pathway (Stinchcombe *et al*, 2004). There might be no association between flowering time and freezing tolerance because in general, rapid cycling summer annual accessions (*flc* or *fri* mutants) complete their life cycle before winter and therefore there is no selection pressure on freezing tolerance unlike in *A. alpina pep1* mutants.

As discussed above, the repression of floral induction at the meristem seems to be highly conserved between PEP1 and FLC. In addition, however, species-specific target genes of both TFs were involved in different flowering pathways. In both species, these non-conserved target genes were mainly involved in the photoperiod and GA pathways (Table A6). This suggests that, similar to the cold response, both species encountered similar selection pressure that triggered independent evolution of an additional PEP1/FLC function. Regulation of photoperiod and GA pathways in addition to regulating flowering at the SAM might contribute to fine-tuning of the time of floral induction under specific environmental conditions or cause a stronger block on flowering prior to vernalization. Whether PEP1 and FLC have an effect on floral induction via the photoperiod and GA pathways and whether this effect is similar in both species remains to be tested. Since the plant hormone GA regulates many other traits apart from flowering, PEP1 and/or FLC might also bind GA-related genes in order to regulate other traits such as for example plant architecture, which could be related to the annual or perennial life-history (Investigated and discussed in part 3). Also for other putatively conserved functions of PEP1 and FLC, like germination and branching, PEP1/FLC seem to have evolved to regulate different pathways regulating different aspects of ed between species.

Common functions of FLC and PEP1 that areone trait (see 2.7.2). It remains to be tested, whether these additional regulatory roles are conserv associated with non-conserved binding events most likely evolved independently in both species. Possibly, both species encountered similar selection pressure after the two lineages split and convergent evolution of additional PEP1/FLC functions represents adaptation to the environment. The Brassicaceae family originated 38 million years ago under tropical conditions by splitting from the sister family Cleomaceae (Couvreur *et al*, 2010). After separation of the basal Aethionemeae lineage, the core Brassicaceae underwent a period of diversification which was associated with global temperature decrease. Adaptation to these cooler conditions allowed species of the core Brassicaceae lineage to move to more northern latitudes where they are found nowadays (Couvreur *et al*, 2010). In consequence, functions of PEP1 and FLC that evolved independently might have evolved during the rapid radiation period of the core Brassicaceae and might be an adaptation to a cooler and seasonally changing climate. In this context, PEP1 and FLC might have independently evolved roles in the regulation of the trade-off between freezing tolerance and growth and they might contribute to optimally adjust the timing of germination, vegetative phase transition and start and end of the flowering phase to environmental conditions. In the future, it will be interesting to investigate species-specific functions of FLC orthologs in other

Brassicaceae species to test if they evolved similar functions to adapt to a seasonally changing environment.

Conservation of the core function of a TF is quite common in vertebrates, *Drosophila* and yeast (discussed above), however evolution of species-specific target genes as a mean of local adaptation was not commonly found. Only Borneman *et al*., suggested that rapid evolution of new BSs of the yeast developmental TFs Ste12 and Tec1 contributed to rapid specialization for distinct habitats (Borneman *et al*, 2007). Other studies focusing on vertebrate or *Drosophila* species led the authors to the conclusion that species-specific BSs evolve neutrally (Schmidt *et al*, 2010; He *et al*, 2011), whereas Muiño *et al* concluded from the SEP3 study that species-specific BSs evolved neutrally but might still be evolving to become functional (Muiño *et al*, 2016). In the present study, many target genes were not detected as differentially regulated in *pep1-1* in the RNA-seq experiment (Figure 9) indicating that binding might be non-functional. However, when analyzing expression of genes associated with GO categories that showed enrichment among target genes but were not enriched among DEG, we found differential expression in *pep1-1* for almost all genes tested. Many genes were affected by PEP1 in early stages of vernalization (see part 3), indicating that PEP1 binding to these genes is functional in the beginning of vernalization, before *PEP1* expression is silenced. COR genes were affected by PEP1 during cold (Figure 24). Many of these genes were expressed at very low levels under ambient temperature conditions and they are functional in cold, indicating that PEP1 binding is not non-functional but PEP1 regulates their expression only under conditions where these genes are functional. This might be true for many other target genes involved in condition-, stage- or organ-specific processes as the regulation of germination or branching at different stages of development. These condition-specific effects could be explained by condition specific expression of other factors that are required for TF activity. For example, another interacting TF might be required to induce changes in transcription, as was described for FLC and SVP (Mateos *et al*, 2015) or the function of a binding event can be to poise the promoter for a fast subsequent response to other factors as suggested by Para *et al*. (Para *et al*, 2014). Certainly some, but probably few, binding events are truly not associated with any change in transcription. One reason for this could be that the BS evolved neutrally and will either be lost again or the TF will evolve a regulatory function. Another explanation could be that some TF BSs are located in proximity of more than one gene although only one gene is regulated, which results in a certain number of false positives among the target genes. In the future, more detailed analysis of spatial and temporal gene expression will probably reveal condition-specific effects of PEP1 (and other TFs) on gene expression although binding appeared to be non-functional

under ambient conditions. In combination with investigation of condition-specific gene regulation, it will be interesting to assess the relevance of condition-specific binding by performing ChIP-seq experiments under different experimental conditions.

3 The role of PEP1 and Gibberellins in *A. alpina* development

3.1 Introduction

The FLC-SVP complex in *A. thaliana* regulates GA-related genes (Mateos *et al*, 2015) and the present study identified genes involved in GA metabolism and the response to GA to be enriched among *A. thaliana* FLC and *A. alpina* PEP1 direct targets (Figure 20). Genes involved in GA metabolism were much more strongly enriched among PEP1 compared to FLC target genes and no enrichment was detected among conserved target genes (Figure 20), suggesting that PEP1 and FLC might play different roles in the regulation of GA metabolism. For several species it was shown that vernalization induces the GA pathway (Hazebroek *et al*, 1993; Hisamatsu *et al*, 2004; Zanewich & Rood, 1995). In *A. thaliana*, however, altered GA levels did not affect the vernalization response (Chandler *et al*, 2000). In this study, the interaction between the GA pathway and PEP1 during the vernalization response of *A. alpina* was investigated and compared to *A. thaliana* to understand how variation in TF binding to GA-related genes affects regulation of flowering in two related species with different life histories.

3.2 PEP1 and FLC bind and regulate different sets of GA-related genes

Identification of PEP1 and FLC direct target genes revealed that both of them bind to genes involved in GA metabolism and signaling (Figure 20). Figure 28 lists GA-related genes that were bound or regulated by PEP1 or FLC. Both TFs targeted several genes involved in the GA pathway, but only *PIF3* and *GA2OX8* were commonly bound by PEP1 and FLC (Figure 28). *GA2OX8*, however, is not a conserved target gene, since PEP1 and FLC bound to different BSs in the orthologous loci (Table A5). PEP1 bound several genes encoding GA metabolic enzymes including GA degrading *GA2OX2* and *GA2OX8* and GA biosynthesis gene *GA3OX2* (Figure 1A, Figure 28). In addition, several genes encoding GA signaling components like GA-receptor *GID1B* were bound by PEP1 (Figure 1B, Figure 28). FLC was previously found to bind *GA3* which encodes an early GA biosynthesis enzyme (Mateos *et al*, 2015). In addition, several genes involved in GA metabolism including *GA3OX1*, *GA20OX2* and *GA2OX6* were differentially expressed in the *flc-3* mutant (Figure 1A, Figure 28). Interestingly, those genes encoding GA metabolic enzymes that were indirectly regulated by FLC were different from the

ones that were bound by PEP1 in *A. alpina*. Furthermore, FLC-specific target genes included several genes encoding various GA signaling components (Figure 1B, Figure 28).

gene	ID Aa	ID At	target *Aa*	target *At*	DEG *Aa*	DEG *At*
BOI	Aa_G148220	AT4G19700		bound		
DDF1	Aa_G319730	AT1G12610		bound		
EXP1	Aa_G786710	AT1G69530		bound		down SD leaves
GA20OX2	Aa_G598100	AT5G51810				up *svp-41*
GA2OX2	Aa_G655020	AT1G30040	bound			up SD leaves
GA2OX6	Aa_G6340	AT1G02400				up *svp-41*
GA2OX8	Aa_G37650	AT4G21200	bound	bound		
GA3OX1	Aa_G112460	AT1G15550				up leaves
GA3OX2	Aa_G312140	AT1G80340	bound			
GID1B	Aa_G163570	AT3G63010	bound			
GID1L3=GID1C	Aa_G251080	AT5G27320		bound		
KAO=GA3	Aa_G574380	AT5G25900		bound**		
MYB21	Aa_G570800	AT3G27810	bound			
PIF3	Aa_G73490	AT1G09530	bound	bound	up apex	
RGL2	Aa_G47610	AT3G03450		bound		
SPL8	Aa_G106150	AT1G02065	bound			
TEM1	Aa_G596330	AT1G25560		bound	up apex	up leaves
TEM2	Aa_G304200	AT1G68840		bound*	up apex	down

Figure 28 FLC and PEP1 bound and regulated genes involved in GA biosynthesis and signaling.

(A) List of GA-related genes that were bound by PEP1 or FLC or detected as DEG in *pep1-1* or *flc-3*, respectively. * indicates that FLC binding was only detected in the SD ChIP-seq experiment by Mateos *et al* (Mateos *et al*, 2015).** indicates that FLC binding was only detected in the ChIP-seq experiment performed by Deng *et al* (Deng *et al*, 2011). For DEG, the direction of differential expression in the mutant is given accompanied by experimental conditions, if the differential expression was only detected under specific conditions. *svp-41* indicates that DEG was only detected if *svp* was mutated in addition to *flc*. This list includes all genes that were directly or indirectly targeted by PEP1 or FLC and involved in GA metabolism, direct targets that are part of the GO-category GO:0009739: response to gibberellin stimulus and have a confirmed function in GA signaling as well as some additional direct and indirect targets that were selected based on publications describing their role in the response to GA.

Binding of PEP1 to GA-related target genes was confirmed by ChIP-qPCR (Figure 29A). For *PIF3* and *GA2OX8*, the target genes that were shared with FLC, binding was also validated in *A. thaliana* (Figure 29B). Expression of these PEP1 direct target genes was then analyzed by qPCR under the same conditions as used for the RNA-seq experiment. In *A. alpina*, grown for 2w under LD conditions, only *GA2OX2* and *PIF3* (in leaves) were up-regulated in the *pep1-1* mutant (Figure 30A). Expression analysis of the orthologs in *A. thaliana* under the same conditions revealed that, *GA2OX2*, *SPL8* and *GID1B* were up-regulated in *flc-3* although these genes were PEP1-specific target genes and not bound in *A. thaliana* (Figure 30B). The only conserved target gene *PIF3* was also up-regulated in the *A. thaliana flc-3* mutant. Except for

SPL8, however, expression changes in *flc-3* were minimal (Figure 30B). Differential expression of several FLC-specific target genes involved in GA-related processes was confirmed in a previous study (Mateos *et al*, 2015). Taken together, PEP1 and FLC seem to be involved in the regulation of GA-related processes but binding events and regulatory roles were not conserved.

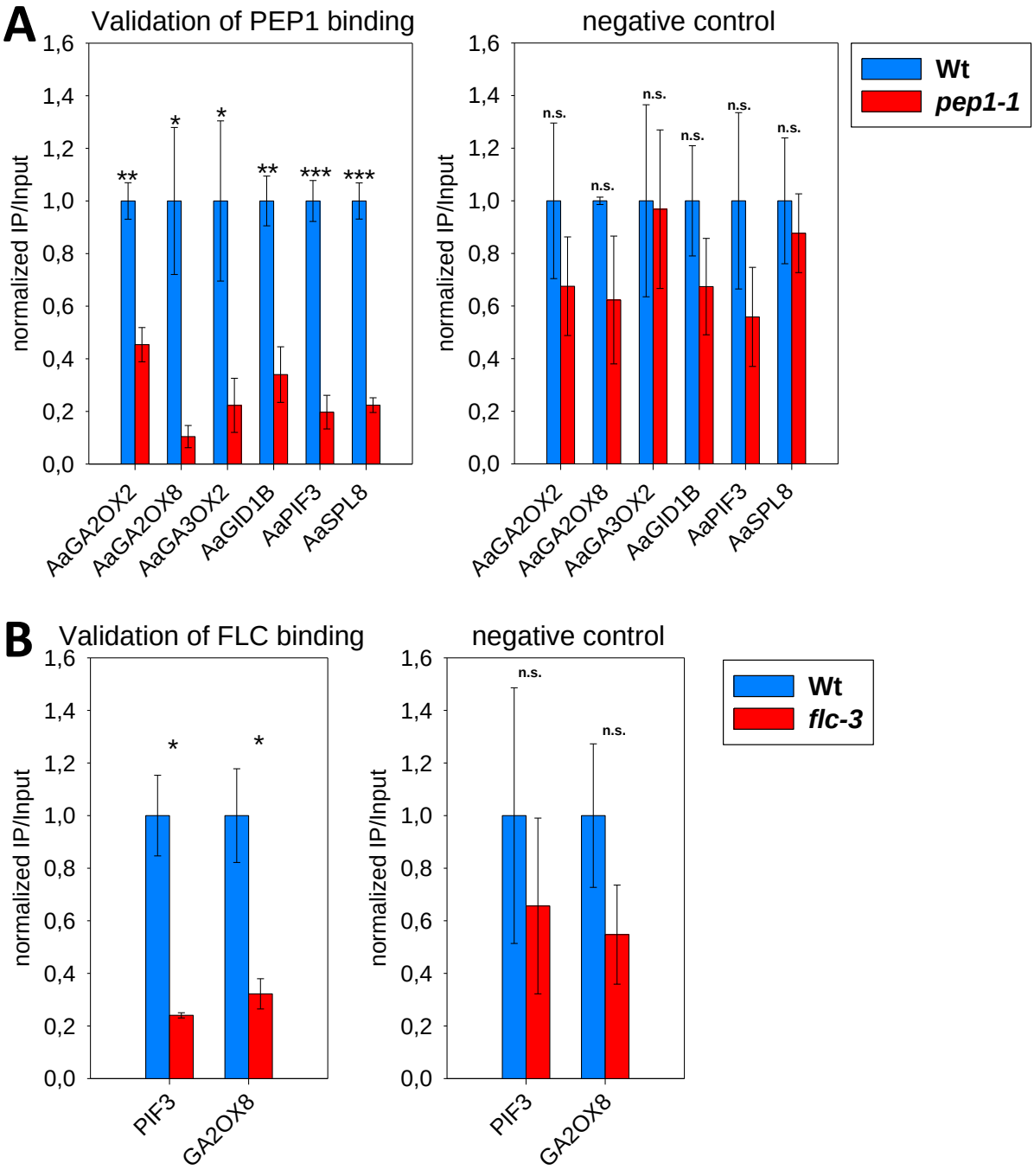


Figure 29 Validation of PEP1 and FLC binding to selected GA related-target genes.

(A) Validation of PEP1 binding to selected GA-related target genes by ChIP-qPCR. **(B)** Validation of FLC binding to *PIF3* and *GA2OX8*. For each target, fold-enrichment of the IP sample relative to its input is shown. Negative controls were performed using primers not flanking predicted BSs (1-2 kb distance to BS). Plants were grown for 2w in LDs, harvesting was performed at ZT 8. Data are shown as mean ± SEM (n = 3 biological replicates). Asterisks indicate significant enrichment in wild-type compared to the mutant (n.s. not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; Student's t-test). Primers are listed in the appendix.

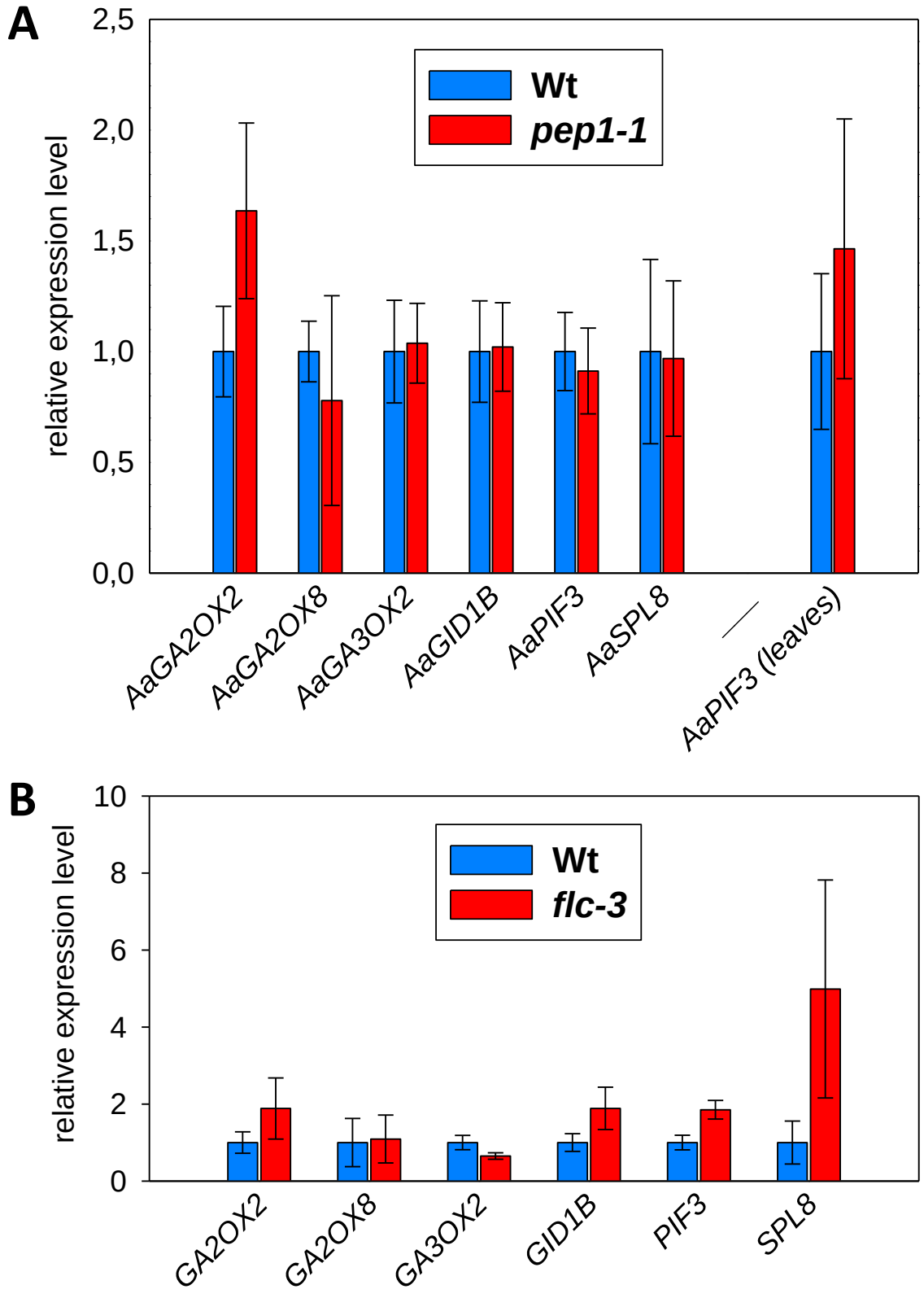


Figure 30 Expression of GA-related target genes in *pep1-1* and *flc-3* mutants.

Expression of selected PEP1 direct target genes (as in Figure 30) using qPCR. Plants were grown under the same conditions as for ChIP-seq and RNA-seq experiments (2 w in LDs, harvesting was performed at ZT 8). Data are shown as mean ± SEM (n = 4 biological replicates). If not indicated otherwise, expression was analyzed in apices of Wt and mutant in both species and all genes were normalized to *PP2A*. Final values were normalized to expression in Wt. **(A)** *A. alpina*. **(B)** *A. thaliana*.

3.3 The *pep1* mutant phenocopies a GA-treated plant

PEP1 bound several genes involved in GA biosynthesis and GA signaling (Figure 28, Figure 29A). Given that PEP1 represses transcription of its target genes (Figure 3), this suggests that PEP1 represses expression of GA-related genes. For a few GA-related PEP1 direct target genes, differential expression in *pep1-1* was detected under ambient conditions (**Figure 30**A), for other genes, the regulatory role of PEP1 might be restricted to specific conditions. Differential regulation of GA-related genes in the *pep1-1* mutant evokes the hypothesis that *pep1-1* has altered GA levels or GA signaling which could affect GA-related phenotypes. To test this hypothesis, the *pep1-1* mutant was examined for phenotypes that might be caused by altered GA responses. In wild-type seedlings, GA treatment caused hypocotyl elongation, whereas treatment with PAC, which inhibits GA biosynthesis and thereby strongly decreases GA levels, prevented hypocotyl elongation (Figure 31A). The *pep1-1* mutant had longer hypocotyls compared to the wild-type, resembling GA-treated wild-type plants. Both genotypes responded to exogenous GA, suggesting that GA levels or GA signaling were elevated in *pep1-1*, but not saturated (Figure 31A). Similarly, GA treatment led to increased plant height in the wild-type and the height of *pep1-1* was strongly increased compared to wild-type (Figure 31B). PAC treatment prevented internode elongation and abolished the difference between *pep1-1* and wild-type plants (Figure 31B), indicating that the *pep1-1* mutant phenotype requires GA. Chlorophyll content and plant diameter, phenotypes that are affected by GA in *A. thaliana* (Koornneef & van der Veen, 1980; Schomburg *et al*, 2003; Griffiths *et al*, 2006; Mitchum *et al*, 2006; Rieu *et al*, 2008b, 2008a; Porri *et al*, 2012), did strongly not change in response to GA but were clearly affected by PAC treatment (Figure 31C, D). This indicates that the levels of GA or GA signaling required to regulate these traits are close to saturation under LD conditions in *A. alpina*. The *pep1-1* mutant did not strongly differ from wild-type in these traits (Figure 31C, D), suggesting that PEP1 is not involved in regulating these traits.

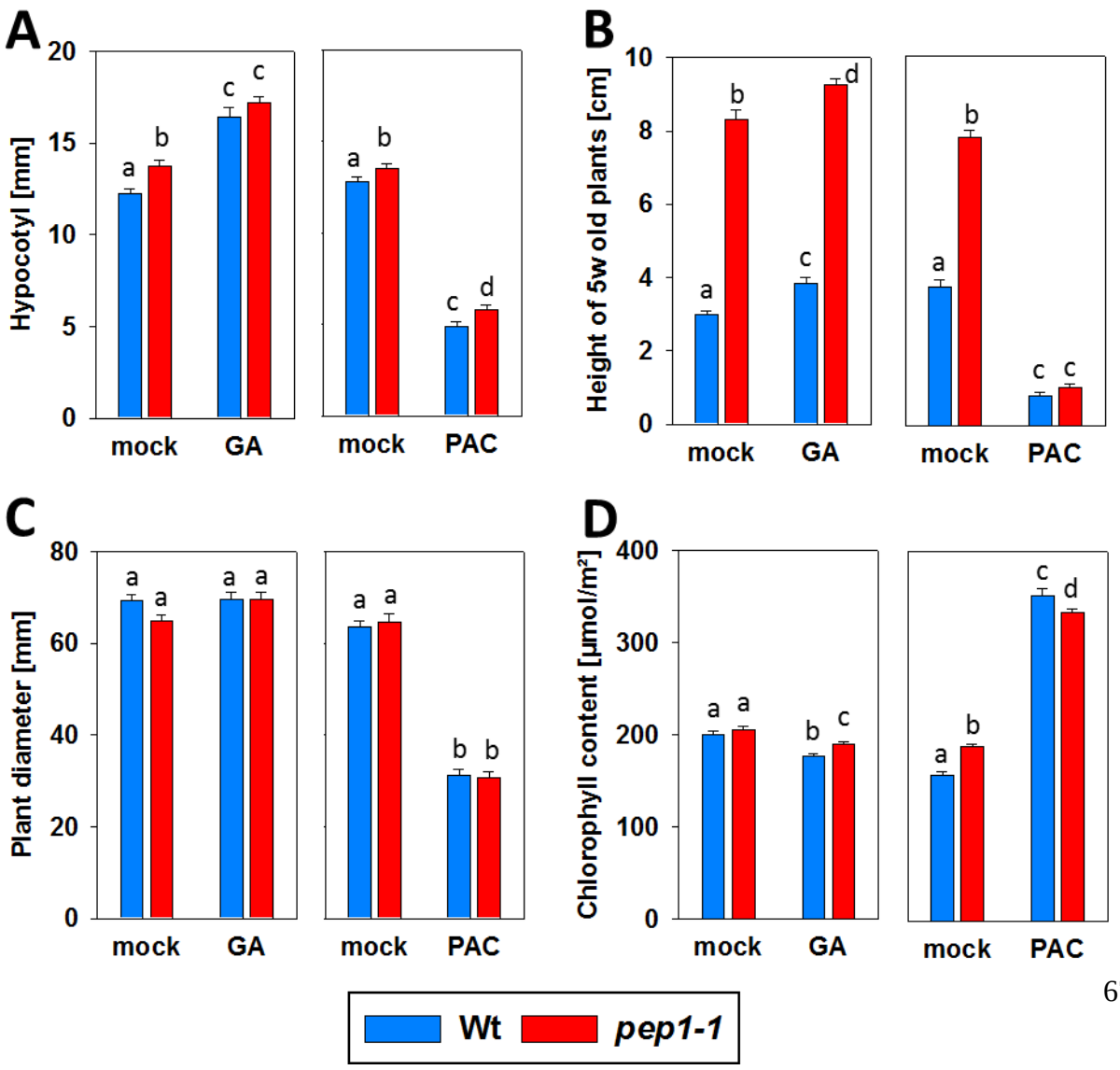
In *A. thaliana*, GA strongly promotes flowering under SD conditions (Wilson *et al*, 1992) and has a weak effect under LD conditions (Griffiths *et al*, 2006). In *A. alpina*, flowering is not induced under SD conditions but compared to the Pajares wild-type progenitor, which does not flower prior to vernalization, *pep1-1* is clearly early flowering in LDs (Wang *et al*, 2009b). GA is required for flowering of *pep1-1*, because PAC treatment delayed flowering (Figure 31E), however the levels of GA in *pep1-1* are not limiting for flowering time as GA applications did not accelerate flowering (Figure 31E). Possibly, part of the early-flowering phenotype of *pep1-1* is due to increased levels of GA or GA signaling, which is then saturated in the mutant under LD conditions. However, since the wild-type only flowers after vernalization, it is not possible

to directly compare the effects of GA on flowering in the mutant and wild-type. In the wild-type, GA cannot promote flowering without vernalization (PhD Thesis of Renhou Wang). After vernalization, however, when PEP1 is down-regulated (similarly to the *pep1-1* mutant), GA did not promote flowering in the wild-type but PAC treatment caused a delay in flowering of around 10 days, similar to the effect in *pep1-1* (Figure 31E). These results suggest that GA is needed for floral induction in *pep1-1* and after vernalization but it was not limiting under our experimental conditions.

In *A. thaliana*, other flowering-related traits are also regulated by GA (Koornneef & van der Veen, 1980; Hay *et al*, 2002; Jasinski *et al*, 2005; Griffiths *et al*, 2006; Rieu *et al*, 2008b), therefore I investigated the effect of GA on bolting and the total number of siliques produced at the main shoot in *A. alpina* wild-type and *pep1-1* mutant. GA treatment did not affect the number of siliques produced at the main shoot or the final height of the main shoot in either genotype but PAC application caused a reduction of silique number and final height in wild-type and *pep1-1* (Figure 31F-G). In addition, PAC caused floral reversions in more than 50 % of wild-type plants (percentage of reverting plants is printed in Figure 31G). This indicates that, as for other phenotypes described above, GA is necessary but not limiting for bolting and normal flower development under LD conditions. Compared to the wild-type, *pep1-1* had fewer siliques on the main shoot (Figure 31F). However, due to the different flowering behaviors, *pep1-1* and Paj were not compared in the same experiments. Also PAC and GA treatments were performed in independent experiments and comparison of the results for the mock-treated samples indicates that there was variation between replicates (Figure 31F). In conclusion, GA availability and slight differences in environmental conditions between experiments seem to affect the number of siliques produced on the main shoot and differences in the flowering behavior of *pep1-1* versus wild-type make it difficult to assess whether PEP1 affects the extent of flowering on the main inflorescence. The *pep1-1* mutant had a slightly increased final height (Figure 31G). Considering that both genotypes flowered after approximately 10 weeks in LDs, which in the wild-type is interrupted by the vernalization period where almost no new nodes are produced, and *pep1-1* had fewer siliques on the main shoot, *pep1-1* did have fewer internodes than the wild-type and height per internode was increased in *pep1-1*. This indicates that PEP1 represses stem elongation not only at the vegetative stage (Figure 31A, B) but also after bolting. Further traits that are known to be regulated by GA in *A. thaliana*, like germination, leaf initiation and leaf shape, trichome formation, fertility and seed development (Koornneef & van der Veen, 1980; Schomburg *et al*, 2003; Griffiths *et al*, 2006; Mitchum *et*

al, 2006; Rieu *et al*, 2008b, 2008a) did not show any obvious differences between *pep1-1* and wild-type and therefore were not further investigated.

In summary, *pep1-1* showed several phenotypes that resembled a GA-treated wild-type plant. These phenotypes suggest that PEP1 negatively regulates GA signaling or GA biosynthesis, in accordance with binding to genes that are involved in these processes. This effect of PEP1 seems to be temporally or spatially restricted since not all GA-regulated phenotypes were affected. The fact that PAC treatment completely abolished the phenotypic effect of PEP1 on plant height (Figure 31B) shows that the *pep1-1* phenotype requires GA and that GA signaling does not occur in *pep1-1* independently of GA. GA treatment still affected *pep1-1* phenotypes, even although the GA response in wild-type plants under LD conditions was very limited (e.g. very weak response for plant height, Figure 31B) which indicates that GA levels under these conditions were close to saturation. This would imply that the effect in *pep1-1* was due to elevated GA signaling, however, it cannot be excluded that elevated levels of GA in *pep1-1* prior to the treatment (at early seedling stage) affected the phenotype.



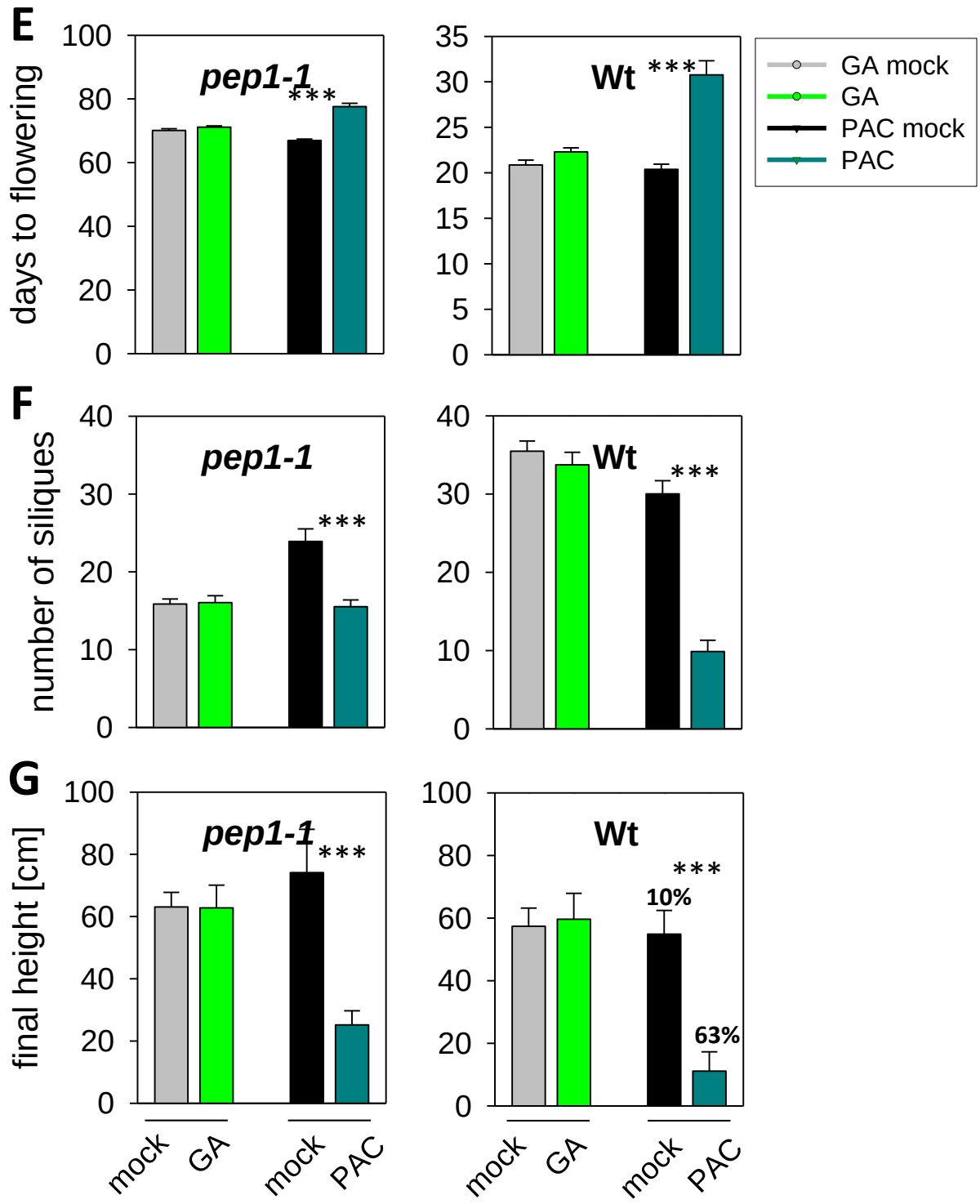


Figure 31 GA-related phenotypes of the *pep1-1* mutant.

Phenotypes of Wt vs. *pep1-1* mutant plants and effect of GA/PAC treatment. **(A)** Hypocotyl length of plants grown for 11 days in LD. 3 independent biological replicates, n ≥38. **(B)** Height of plants grown for 5 w in LD. GA/mock: 3 independent biological replicates. n ≥33. PAC/mock: 2 independent biological replicates. n ≥32. **(C)** Chlorophyll content. Plants were grown for 6 w in LD and measurements were performed on the 7th true leaf. 3 independent biological replicates. n ≥ 43. **(D)** Plant diameter. Plants were grown for 3 w in LD. 2 independent biological replicates. n ≥21. **(E)** Flowering time. Plants were grown in LDs. Wt plants were vernalized for 12 w when 5.5 w old. (Left) *pep1-1*, total number of days until first flower opened. GA/mock: 2 independent biological replicates. n ≥25. PAC/mock: 2 independent biological replicates. n ≥33. (Right) Wt, days after vernalization until first flower

opened. GA/mock: 2 independent biological replicates. n ≥20. PAC/mock: 2 independent biological replicates. n ≥30. **(F)** Total number of individual siliques on the main shoot (siliques on branches not included). Plants were grown as in E. (Left) *pep1-1*, GA/mock: 2 independent biological replicates. n ≥21. PAC/mock: 2 independent biological replicates. n ≥29. (Right) Wt, GA/mock: 2 independent biological replicates. n ≥19. PAC/mock: 2 independent biological replicates. n ≥29. **(G)** Final height of the main shoot. Plants were grown as in E. (Left) *pep1-1*, GA/mock: 2 independent biological replicates. n ≥21. PAC/mock: 2 independent biological replicates. n ≥29. (Right) GA/mock: 2 independent biological replicates. n ≥19. PAC/mock: 2 independent biological replicates. n ≥29. Percentage above the bars indicates percentage of reverting inflorescences that could not be scored: PAC 19 out of 30, PAC mock 3 out of 29. All error bars represent SEM. For all phenotypes, n describes the total number of replicates after combining all biological replicates. Letters in A-D indicate statistically different groups determined by two-way analysis of variance and multiple comparisons using the Bonferroni-t-test method that were performed within genotypes and within treatments. Groups were defined as statistically different when $P \leq 0.05$. Asterisks in E-F indicate significant difference between treatments within genotypes (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; Student's t-test).

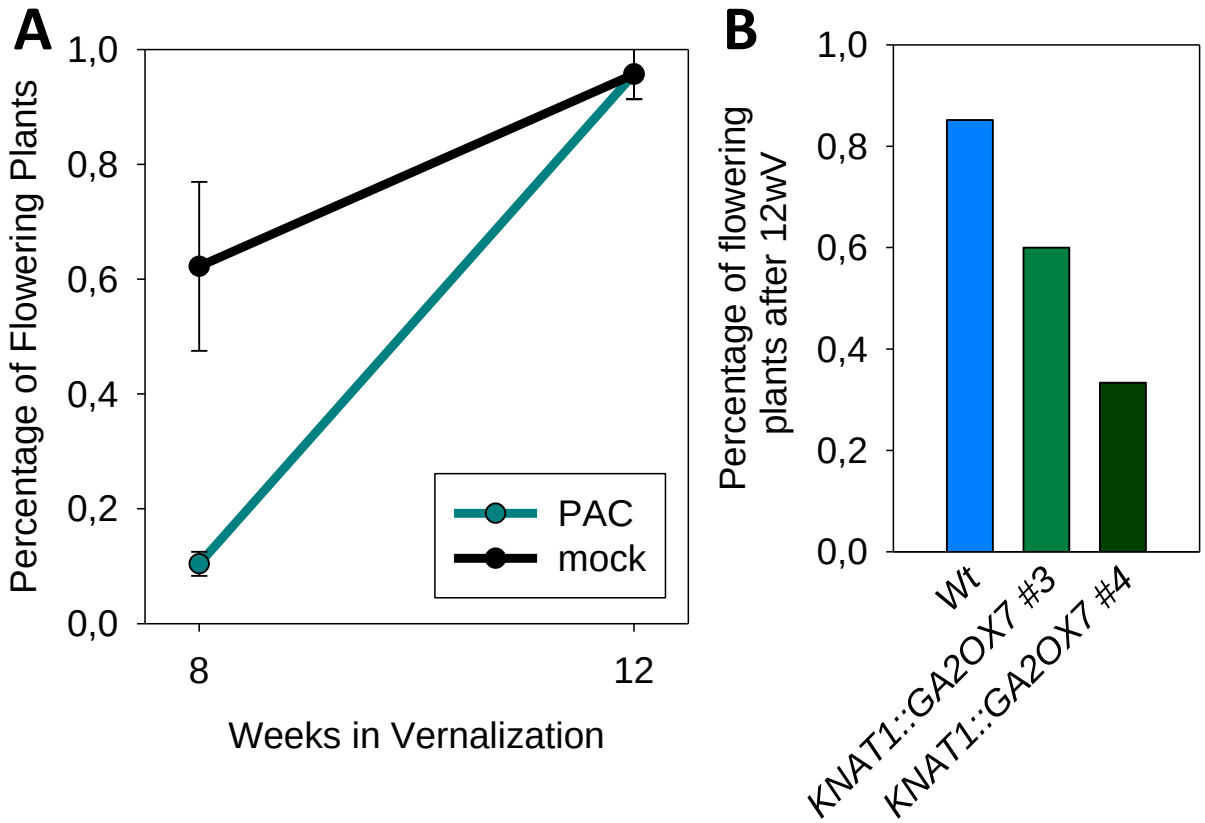
3.4 GA promotes flowering during vernalization in *A. alpina*

Phenotypic analysis of the *pep1-1* mutant suggested that PEP1 negatively regulates GA biosynthesis or signaling. In wild-type plants, GA applications caused internode elongation (Figure 31B) but did not induce flowering in non-vernalized plants (PhD Thesis of Renhou Wang) or promote flowering of vernalized plants under LD conditions (Figure 31E). As Pajares wild-type plants undergo floral transition in vernalization (Wang *et al*, 2009b, 2011), it was analyzed whether GA affects floral induction during vernalization. Reduction of GA levels by PAC application during an 8 week vernalization treatment reduced the number of plants that flowered after vernalization (Figure 32A). However, after 12 weeks of vernalization, which is a more complete vernalization treatment that induces flowering in 100 % of wild-type plants, all PAC-treated plants also flowered (Figure 32A). These results suggest that GA promotes floral induction during vernalization but that this is only defected by PAC treatments when vernalization is at a threshold level. This hypothesis was further supported by analysis of transgenic plants, expressing the GA degradation enzyme GA2OX7 from the meristem-specific *KNAT1* promoter (Lincoln *et al*, 1994). Reduction of GA content in the meristem by the *KNAT1::GA2OX7* transgene was previously shown to strongly delay flowering in *A. thaliana* (Porri *et al*, 2012). Also in *A. alpina*, the *KNAT1::GA2OX7* transgene suppressed floral induction during vernalization. While over 80 % of wild-type plants flowered after 12 weeks of vernalization, only 30-60 % of two different transgenic lines flowered under these conditions (Figure 32B). This result demonstrates the importance of GA for flowering of *A. alpina* in vernalization, and suggests that the transgenic approach reduces GA levels more significantly than PAC treatments.

Analysis of the effect of reduced GA levels on gene expression during vernalization showed that, while the reduction of GA content by PAC treatment did not affect *PEP1* expression, the induction of floral marker genes *LFY*, *FUL* and *AP1* was delayed (Figure 32C). This indicates

that floral induction was delayed, corresponding to the observed flowering phenotype (Figure 32A). In *A. thaliana*, GA acts through the regulation of SPL15 activity to promote *FUL* expression (Hyun *et al*, 2016). Interestingly, in *A. alpina*, PAC application also caused delayed induction of *SPL15* transcription during vernalization (Figure 32C). *SPL15* expression could either be directly affected by GA or the effect could be a consequence of a reduced size of the meristem due to delayed floral induction, which would cause a reduced expression domain of *SPL15* (Hyun *et al*, 2016). Expression of *SHOOT MERISTEMLESS (STM)* was measured as a marker for the size of the meristem (Long *et al*, 1996). *STM* expression was slightly reduced in the PAC-treated samples at the end of vernalization (Figure 32C), suggesting that meristem size was reduced. Therefore, reduced meristem size could explain the lower *SPL15* mRNA levels but only at the end of vernalization. In *A. thaliana*, *SPL15* is regulated by the SVP-FLC complex (Mateos *et al*, 2015). *SVP* expression was reduced in PAC-treated apices at all time points during vernalization (Figure 32C). A reduction of *SVP* expression by PAC is in contrast to a repressive effect of GA on *SVP* expression in *A. thaliana* (Li *et al*, 2008) but might explain the reduction of *SPL15* expression observed in this experiment.

Taken together, these results suggest that GA acts during vernalization to promote floral induction. Possibly, PEP1 represses GA signaling or biosynthesis prior to vernalization and vernalization causes silencing of PEP1 and thereby flowering is promoted (at least in part) via the GA pathway.



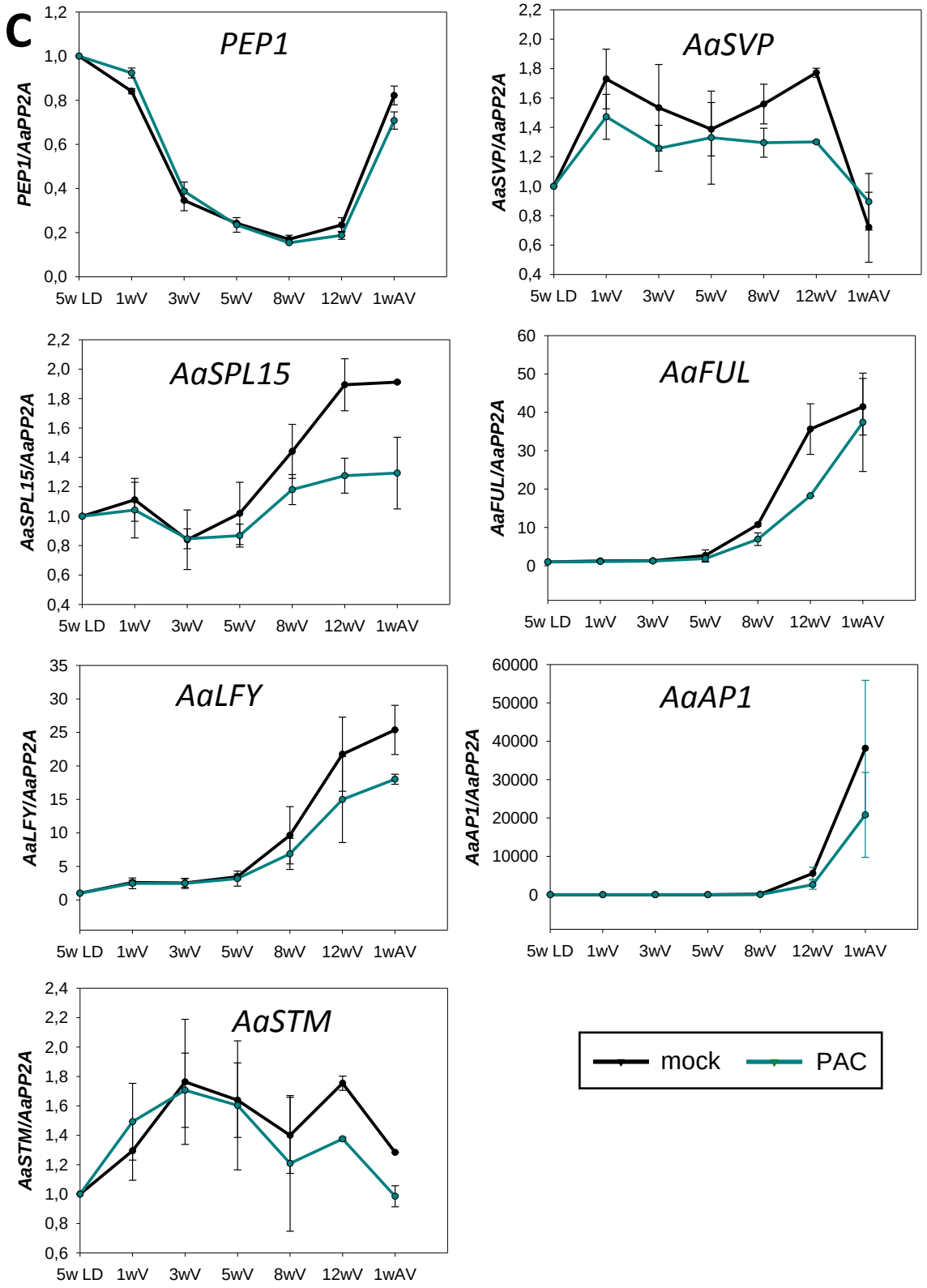


Figure 32 Role of GA in the induction of flowering during vernalization.

(A) Percentage of flowering plants in LD after vernalization. Wt plants were grown for 5.5 w in LD and then vernalized for 8 w or 12 w. During vernalization, plants were weekly treated with PAC or mock. Pooled data from 2 independent biological replicates. n ≥45. Error bars are SEM from the 2 biological replicates. **(B)** Effect of *KNAT1::GA2OX7* transgene on flowering. Percentage of flowering plants after 12 w of vernalization. Two independent transformants (at least heterozygous for *KNAT1::GA2OX7*) compared to Wt. n ≥15. **(C)** Expression

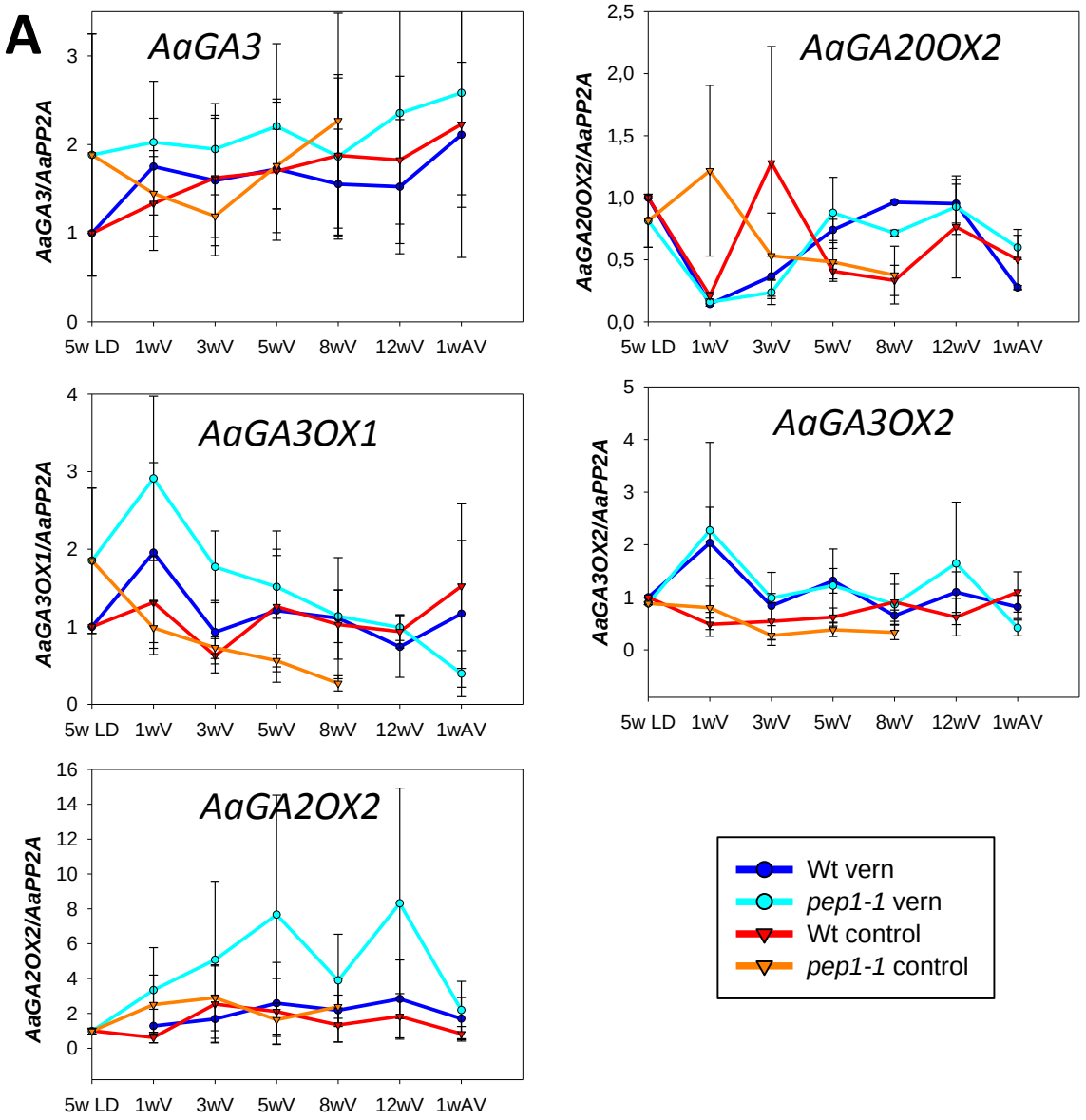
analysis by qPCR of floral marker genes during vernalization in Wt that were weekly treated with PAC or mock during vernalization. Plants were grown for 5.5 w in LD and then transferred to 4°C for 12 w and then shifted back to LD. Data are shown as mean ± SEM (n = 2 biological replicates). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment. For phenotypic experiments A and B, n describes the total number of replicates after combining all biological replicates.

3.5 PEP1 represses induction of genes involved in GA metabolism and signaling at early stages of vernalization

GA promoted floral induction during vernalization (Figure 32). Several genes involved in GA metabolism and GA signaling were among PEP1 direct target genes (Figure 28, Figure 29A). To test whether the repression of PEP1 during vernalization correlates with increased expression of GA-related target genes that might promote floral induction during vernalization, the expression of genes involved in GA metabolism and signaling (Figure 28) was analyzed during vernalization. All genes encoding GA metabolic enzymes that were listed in Figure 28 were analyzed. Figure 33 shows expression patterns of genes that were found to be regulated by vernalization or by PEP1. Several genes encoding GA biosynthetic enzymes were induced by vernalization in apices and leaves. Those included PEP1 direct target gene *GA3OX2* and indirectly regulated genes such gene *GA3*, which encodes an enzyme that acts early in GA biosynthesis (Helliwell *et al*, 1998), as well as *GA20OX2* and *GA3OX1*. Expression of each gene was also increased in *pep1-1* mutants (Figure 33). PEP1 direct target gene *GA2OX2*, which encodes a GA degrading enzyme, was induced by vernalization only in the *pep1-1* mutant (Figure 33). Cold induction of genes encoding GA biosynthesis enzymes occurred mainly prior to and during floral induction (for comparison see expression of floral marker genes Figure 32C, Figure 33), suggesting that vernalization might cause an increase in GA levels that promotes floral induction during vernalization. PEP1 seems to have a repressive effect on this pathway that might prevent premature induction of flowering by shorter vernalization periods.

In addition, expression levels of genes encoding GA signaling components that were direct PEP1 targets or regulated by PEP1 (Figure 28) were analyzed during vernalization. Transcript levels of the GA receptor gene *GID1B* increased with time to similar levels in vernalized wild-type and *pep1-1* and non-vernalized *pep1-1* (Figure 34A). *SPL8* showed a similar pattern but induction of *SPL8* in vernalized wild-type was delayed compared to *pep1-1* (Figure 34A). The induction of *SPL8* and *GID1B* might be restricted to floral meristems, since it correlates with floral induction (see expression of floral marker genes Figure 32C) and does not seem to be a response to vernalization prior to floral induction as observed for genes encoding GA metabolic enzymes (Figure 33). *PIF3* expression in leaves and apices resembled the pattern observed for genes encoding GA metabolic enzymes. *PIF3* was induced by cold and induction was stronger

in *pep1-1* (Figure 34). Also *TEMPRANILLO1* (*TEM1)* and *TEM2* showed a transient increase of expression during vernalization, however, these genes reached higher levels in the wild-type and induction was observed throughout the whole period of vernalization (Figure 34A). This positive effect of PEP1 on *TEM* gene expression is likely to be an indirect effect, since *TEM1* and *TEM2* were not among PEP1 direct target genes (Figure 28). In *A. thaliana*, the *TEM* genes were shown to negatively regulate expression of *GA3ox* genes (Osnato *et al*, 2012). Therefore, down-regulation of transcription of the *TEM* genes in *pep1-1* might cause up-regulation of *GA3OX1*, which also is not directly targeted by PEP1.



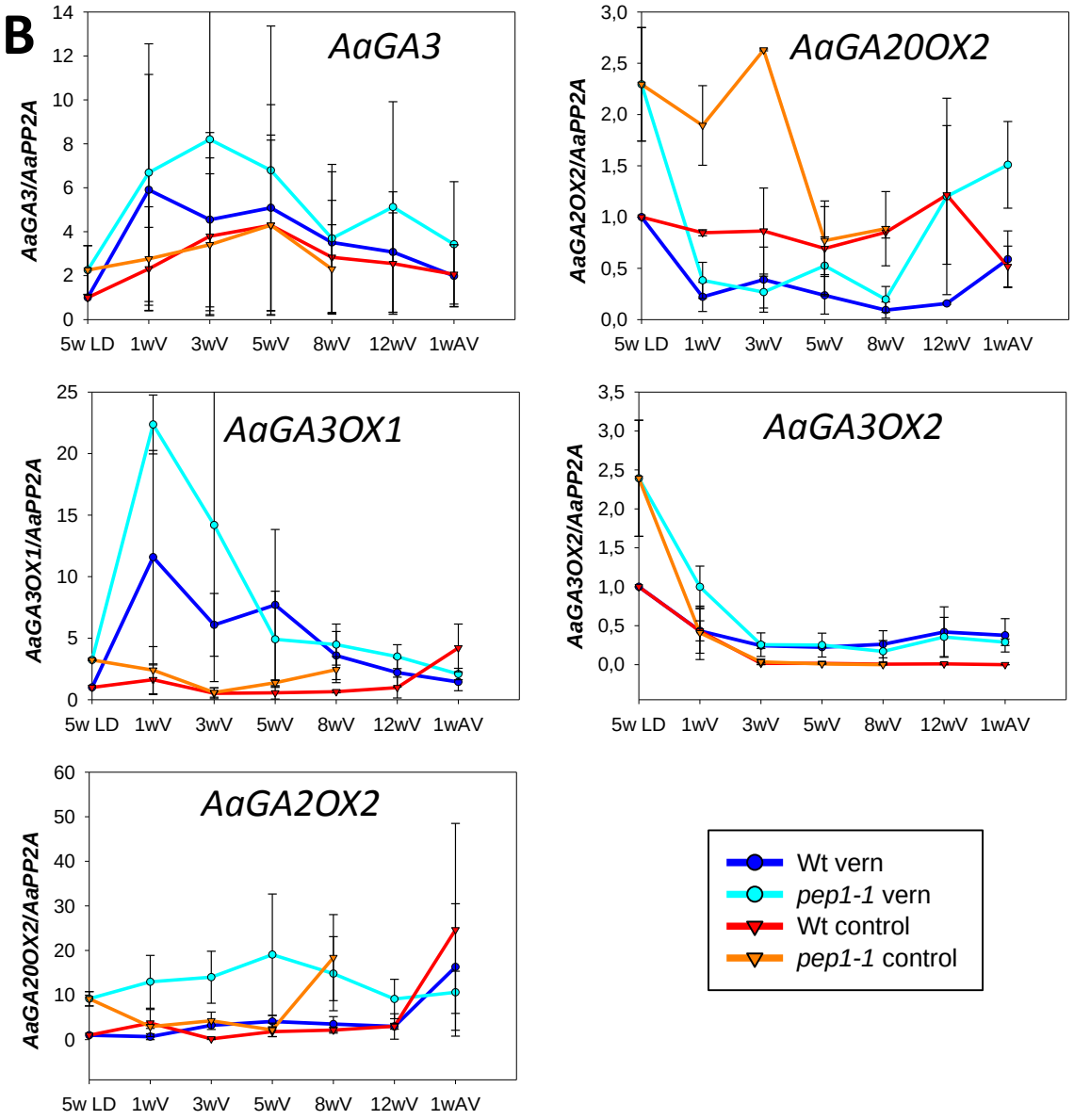


Figure 33 Effect of *PEP1* and vernalization on the expression of genes encoding GA metabolic enzymes.

Expression analysis of genes involved in GA metabolism during vernalization in *pep1-1* vs. Wt. Plants were grown for 5.5 w in LD and then transferred to 4°C for 12 w or kept under control conditions (SD, 21°C) and then shifted back to LD. Samples were taken at ZT8. Data are shown as mean ± SEM (n = 2 biological replicates). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment. **(A)** Apices. **(B)** Leaves.

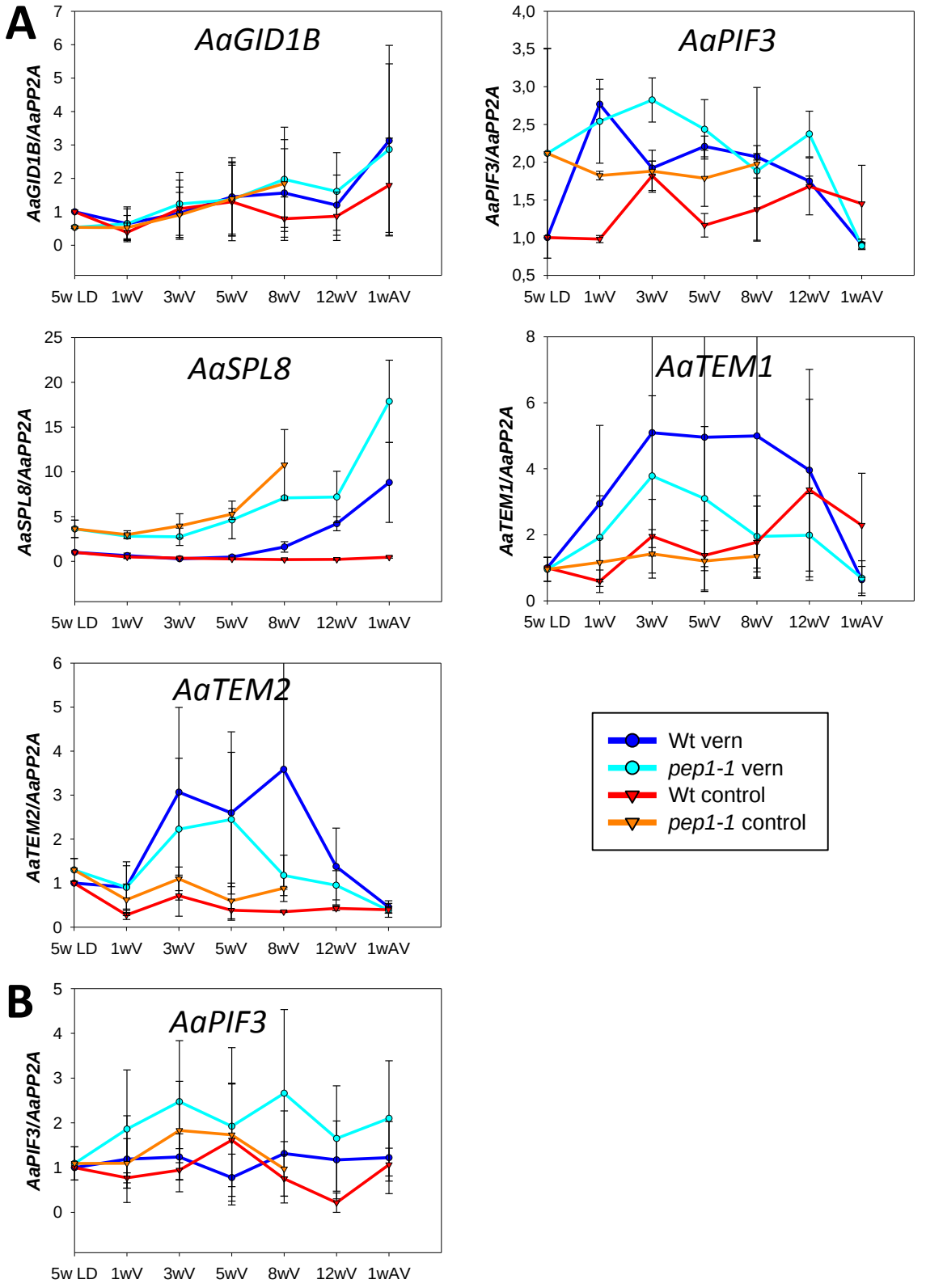


Figure 34 Effect of *PEP1* and vernalization on the expression of genes encoding GA signaling components.

Expression analysis of genes involved in GA signaling during vernalization in *pep1-1* vs. Wt. Plants were grown for 5.5 w in LD and then transferred to 4°C for 12 w or kept under control conditions (SD, 21°C) and then shifted back to LD. Samples were taken at ZT8. Data are shown as mean ± SEM (n = 2 biological replicates). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment. **(A)** Apices. **(B)** Leaves.

The effect of vernalization and PEP1 on expression levels of GA-related genes was also analyzed in juvenile plants that do not flower after vernalization, to elucidate which effects might be directly related to floral induction. Expression levels of *GA3OX2* and *GA2OX2*, two PEP1 direct target genes with opposite functions in GA metabolism, were induced towards the end of vernalization in juvenile plants and PEP1 repressed this induction for GA biosynthesis gene *GA3OX2* but not for GA degrading *GA2OX2* (Figure 35A). This is in contrast to the effects of vernalization and PEP1 in adult plants, where vernalization induced expression levels of *GA3OX2* and *GA2OX2* prior to floral induction and PEP1 had a repressive effect on induction of *GA2OX2* but not on induction of *GA3OX2* (Figure 33). These findings suggest that vernalization and PEP1 have different effects on PEP1 direct target genes that are involved in the regulation of GA levels, depending on plant age. The GA biosynthetic genes *GA3* and *GA3OX1* that were indirectly regulated by PEP1 showed similar patterns in juvenile and adult plants (Figure 33; Figure 35A), indicating that their induction by prolonged cold and the repressive effect of PEP1 on that is not specific to the induction of flowering in adult plants. As a consequence, only in adult plants, PEP1 and vernalization might cause an increase in GA levels at the beginning of vernalization, preceding floral induction.

GID1B, which encodes a GA receptor, was transiently and strongly induced by prolonged cold in juvenile *pep1-1* but only slightly induced in the wild-type (Figure 35B). In adults, *GID1B* expression correlated with the formation of floral buds and was not dependent on the genotype (Figure 34). These results suggest that PEP1 functions specifically in juvenile plants to repress *GID1B* induction by cold. *SPL8* expression patterns in juvenile and adult plants were very similar but in juvenile plants, expression levels were much lower (Figure 34; Figure 35B), indicating that high levels of *SPL8* expression are specific to floral induction. Expression levels of the *TEM*s were transiently induced in juvenile as well as in adult plants, but expression reached higher levels in adult plants and PEP1 had a positive effect on *TEM* induction in adults, whereas PEP1 negatively influenced *TEM* induction in juvenile plants (Figure 34; Figure 35B). This suggests that PEP1 represses flowering via the *TEM*s specifically in adult plants.

Taken together, it seems that several regulatory functions of vernalization and PEP1 that affect genes involved in GA biosynthesis and GA signaling are specific to the process of floral induction, since they could only be observed in adult plants. In summary, several GA metabolism and signaling genes were induced during vernalization and PEP1 affected this. Some of these effects were specific to adult plants, suggesting that vernalization and PEP1 interact to modulate GA levels and GA signaling during vernalization to induce flowering.

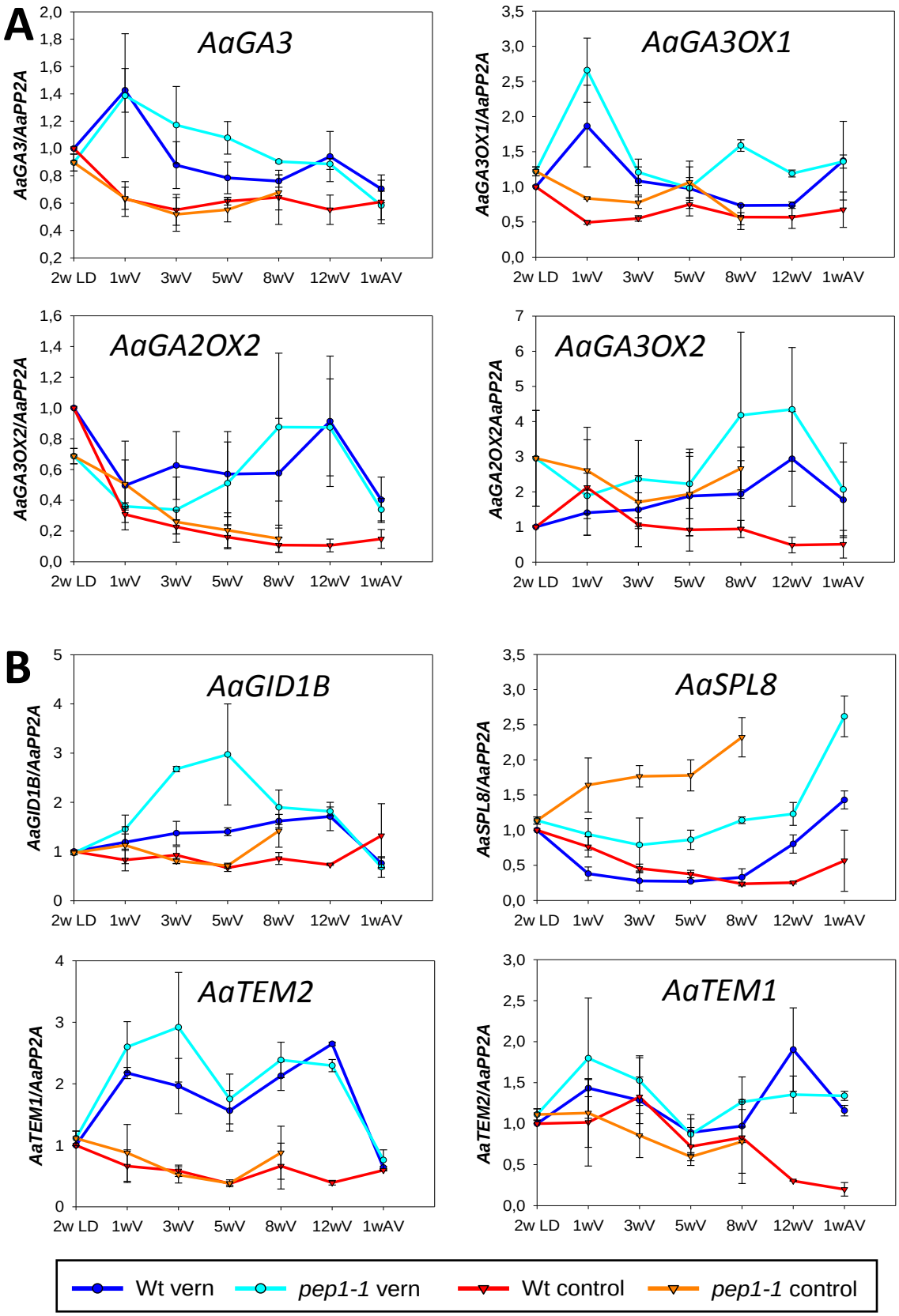


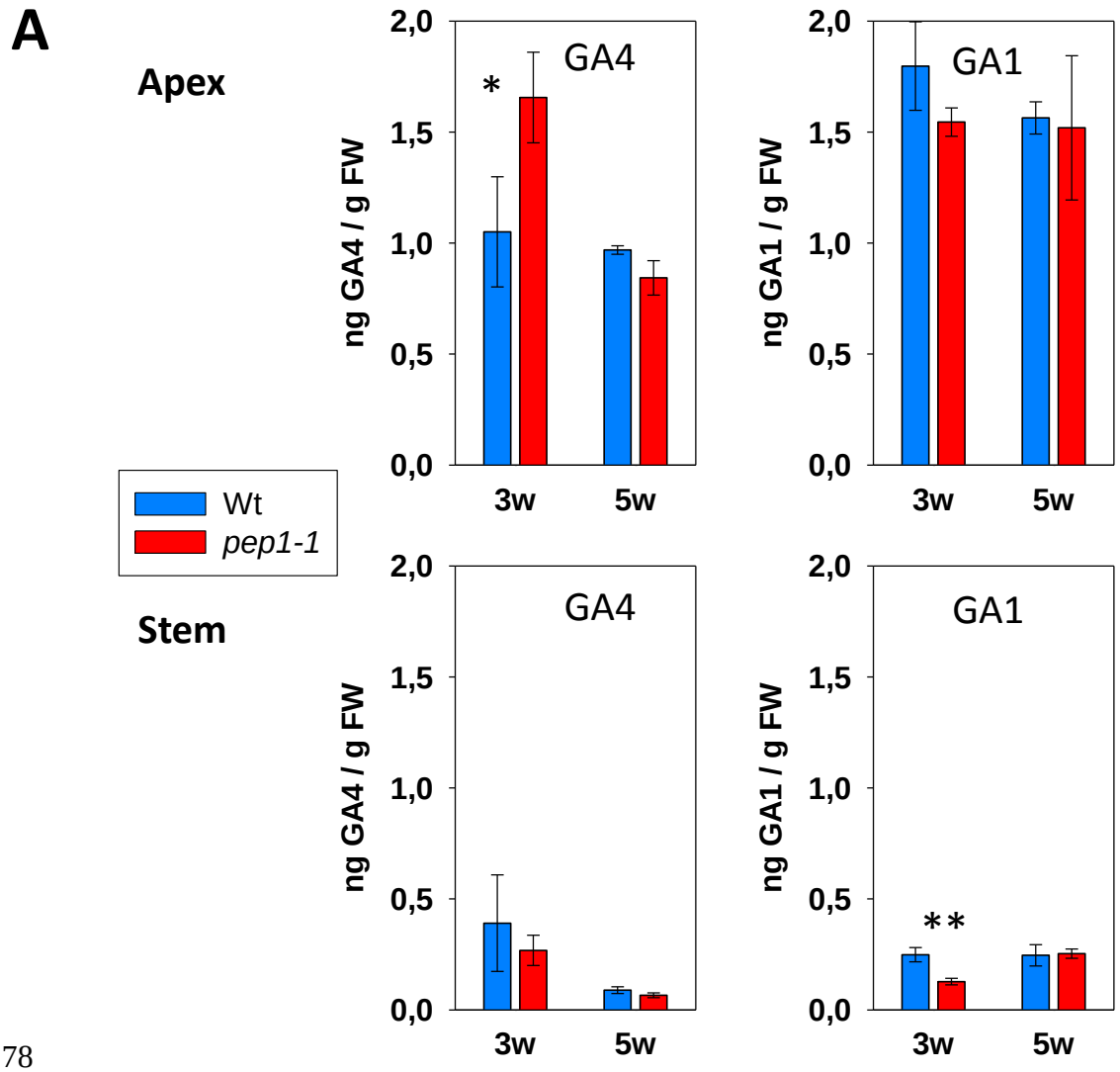
Figure 35 Effect of *PEP1* and vernalization on the expression of GA-related genes in juvenile plants.

Expression analysis of genes involved in GA metabolism during vernalization in *pep1-1* vs. Wt. Plants were grown for 2 w in LD and then transferred to 4°C for 12 w or kept under control conditions (SD, 21°C) and then shifted back to LD. Samples were taken at ZT8. Data are shown as mean ± SEM (n = 2 biological replicates). Expression

was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment. **(A)** Genes encoding GA metabolic enzymes. **(B)** genes encoding GA signaling components.

3.6 PEP1 does not cause a general increase of GA levels and GA levels decrease during vernalization

PEP1 was found to bind and regulate genes encoding GA metabolic enzymes (see above) and the *pep1-1* mutant phenotype resembles a GA-treated plant (Figure 31), suggesting that PEP1 might have a negative effect on GA levels. To investigate whether increased GA levels could explain the observed internode phenotype of 5-week old *pep1-1* mutants, levels of active GAs were measured in stem and apical samples (containing internodes prior to elongation) of 3-week and 5-week old plants. The *pep1-1* mutant had significantly higher levels of GA4 in apices compared to wild-type at the age of 3 weeks but not after 5 weeks (Figure 36A). Levels of active GA in stem tissue did not strongly differ between genotypes, *pep1* had only slightly less GA1 in 3-week old stems (Figure 36B). A transient and spatially restricted increase of the GA4 level in apices of 3-week old *pep1-1* could be responsible for the increased stem length observed in 5-week old plants (Figure 31B).



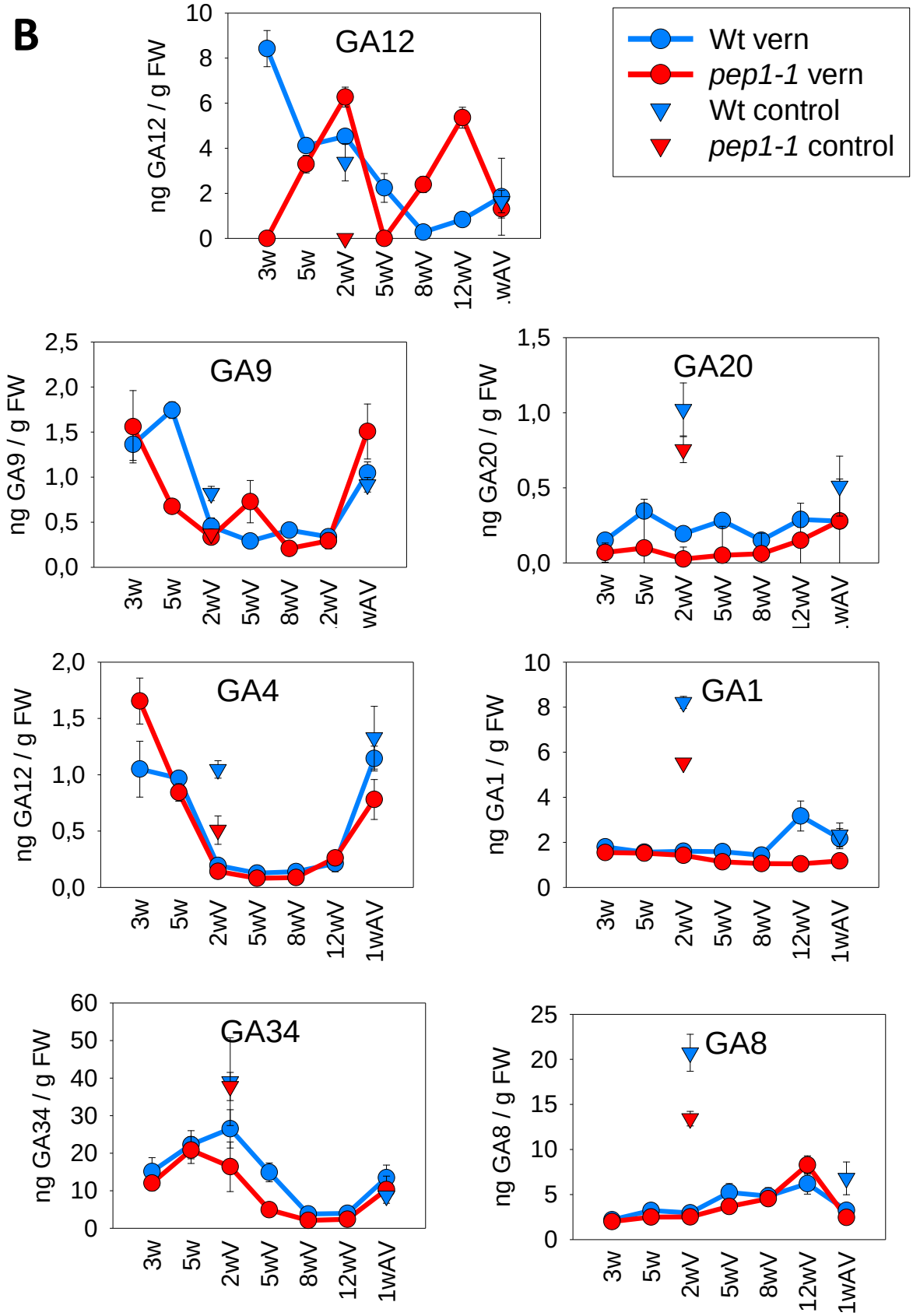


Figure 36 Effects of *PEP1* and vernalization on GA levels.

(A) Levels of active GAs in apices and stems of *pep1-1* and Wt after 3 w and 5 w in LD. **(B)** Levels of GA precursors (GA12, GA9, GA20), active GAs (GA4, GA1) and GA degradation products (GA34, GA8) during vernalization. Plants were grown for 5.5 w in LD and then transferred to 4°C for 12 w or kept under control conditions (SD, 21°C) and then shifted back to LD. Data are shown as mean ± Stdev. (n = 3 biological replicates,

except apices of Paj and *pep1-1* 5 w LD n = 2; *pep1-1* 5 w LD stem: n = 1). Asterisks in A indicate significant difference between treatments within genotypes (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; Student's t-test).

Furthermore, it was tested whether the induction of GA biosynthetic enzymes during vernalization correlated with increased GA levels. Unexpectedly, levels of GA4 were reduced in cold. GA1 did not show a cold response (Figure 36B). The *pep1-1* mutant did not contain altered levels of GA compared to wild-type. Interestingly, *A. alpina* contained slightly higher levels of GA1 compared to GA4 (Figure 36B), which is in contrast to *A. thaliana*, where GA4 is the major active GA (Xu *et al*, 1997; Eriksson *et al*, 2006). GA4 and GA1 (GA1 only in wild-type) levels slightly increased towards the end of the vernalization period but levels were still lower than in non-vernalized control plants (Figure 36B). This slight increase might be related to the induction of flowering, however it is not comparable to the tremendous increase of GA4 in apices of *A. thaliana* prior to floral induction under short days (Eriksson *et al*, 2006). In summary, the induction of GA biosynthesis genes by vernalization that is stronger in *pep1-1* (Figure 33), did not correlate with a detectable increase of GA levels during cold or in the *pep1-1* mutant. The reduced GA4 level in cold seems to occur due to regulation of a very early step in GA biosynthesis, since also the level of GA12, the common precursor of GA4 and GA1, was reduced (Figure 36B). In consequence, changes in expression of *GA3OX*es and *GA20OX*es do not seem to cause the observed changes in GA levels. Interestingly, several precursors of active GA and the degradation products GA34 and GA8 accumulated to much higher levels than active GA1 and GA4 (Figure 36B), indicating that regulatory steps occur on additional levels which might include spatially restricted differences in levels of different GAs and changes in abundance of GA metabolic enzymes that are not detectable by qPCR.

In summary, GA levels (Figure 36) and expression levels of genes encoding GA metabolic enzymes (Figure 28, Figure 30, Figure 33) did not show strong correlations and in contrast to the strong GA-related phenotypes, *pep1-1* had only slightly altered GA levels compared to the wild-type.

3.7 PEP1 negatively regulates GA signaling

PEP1 was found to bind and regulate genes encoding GA signaling components (see above) and the *pep1-1* mutant phenotype resembled a GA-treated plant (Figure 31), suggesting that sensitivity to GA might be increased in *pep1-1*. The response of *pep1-1* and wild-type to GA was tested by weekly applying PAC to prevent endogenous GA biosynthesis, and simultaneously treating with different concentrations of GA. Plant height measured after 6.5 weeks increased more strongly in *pep1-1* compared to wild-type. In the *pep1-1* mutant, plant

height was increased at all concentrations tested and this effect was statistically significant for the highest concentration of GA (Figure 37A). The slope of the linear regression curve was around 1.5 fold higher for *pep1-1* compared to the wild-type (Figure 37A), indicating that the plant height increases more strongly with an increasing concentration of GA. Thus *pep1-1* responds more strongly to a given concentration of GA. The increase of plant diameter with increasing GA concentration, however, was similar in both genotypes (Figure 37B). Thus, GA signaling seems to be increased in *pep1-1* but this effect is not universal but restricted to certain developmental processes and thereby could explain the observed *pep1-1* phenotypes.

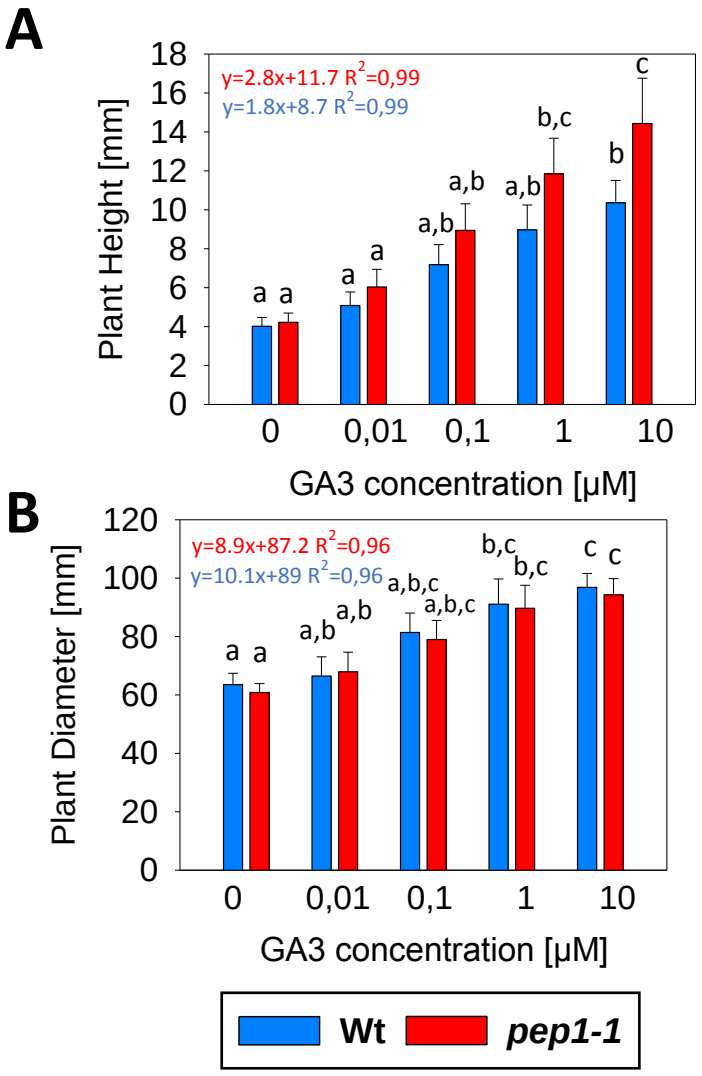


Figure 37 Effect of *PEP1* on GA signaling.

(A) Plant height after 6.5 w in LD. **(B)** Plant diameter after 6 w in LD. *pep1-1* and Wt plants were treated simultaneously with PAC to inhibit synthesis of endogenous GA and different concentrations of GA3 once per week to investigate the effect of the genotype on the response to GA. 2 independent biological replicates (total number of replicates after combining all biological replicates: $n \geq 23$). Letters indicate statistically different groups determined by two-way analysis of variance and multiple comparisons using the Bonferroni-t-test method that were performed within genotypes and within treatments. Groups were defined as statistically different when $P \leq$

0.05. Linear regression curves and R^2 are printed in blue for Wt and in red for *pep1-1*. Linear regressions were calculated using a logarithmic scale for the GA concentration (log_{10}(GA concentration)).

3.8 PEP1 regulates induction of GA-related target genes but not GA levels in intermittent cold

PEP1 was involved in the response to short cold exposure (Chapter 2.5) and regulated GA-related genes during vernalization (Figure 33, Figure 34). In *A. thaliana*, exposure to intermittent cold leads to growth retardation caused by a reduction of GA levels due to reduced expression levels of *GA2OX*es (Achard *et al*, 2008). To elucidate if the response to short periods of cold temperature in *A. alpina* involves changes in the expression of GA-related genes and if PEP1 is involved in this, expression of GA metabolism and signaling genes was analyzed after exposure to short periods of cold. Therefore, plants were grown for 2 weeks in SDs at 21°C and then, at ZT4, transferred to 4°C for up to 24 h. As previously described in *A. thaliana*, in *A. alpina GA2OX* genes were induced in response to cold (Figure 38). Cold induction of the PEP1 direct target gene *GA2OX2* did not differ between genotypes, however, induction of *GA2OX1* and *GA2OX6* (which were not bound by PEP1 (Figure 28)) was more pronounced in the wild-type (Figure 38), indicating that PEP1 has an indirect positive effect on their expression in cold. Interestingly, unlike in *A. thaliana*, genes encoding GA biosynthesis enzymes were also induced by short cold treatments in *A. alpina* and PEP1 had a repressive effect on this (Figure 38), similar to what was observed during vernalization (Figure 33). Higher levels of mRNAs of GA biosynthetic enzymes and lower levels of *GA2OX* mRNAs in *pep1-1* suggest that *pep1-1* has higher levels of GA compared to the wild-type after the exposure to short periods of cold temperature. Simultaneous induction of genes encoding GA biosynthetic and GA degrading enzymes in cold-exposed *A. alpina*, suggests that the decrease of GA levels in cold and thus the retardation of growth in *A. alpina* might be reduced compared to *A. thaliana*.

Since PEP1 and prolonged exposure to cold positively affected expression levels of several genes encoding GA signaling components (Figure 34), I also analyzed their expression in response to intermittent cold. Transcript levels of genes encoding GA signaling components were induced after exposure to a few hours of cold in *A. alpina* and the induction was more pronounced in *pep1-1* (Figure 39), similar to the effect of a few weeks of cold (Figure 34). Interestingly, PEP1 had the opposite effect on induction of the *TEM* genes in response to intermittent cold compared to prolonged cold. While PEP1 positively affected induction of *TEM1* and *TEM2* after prolonged exposure to cold, PEP1 had a repressive effect on their induction after short exposure to cold (Figure 34, Figure 39).

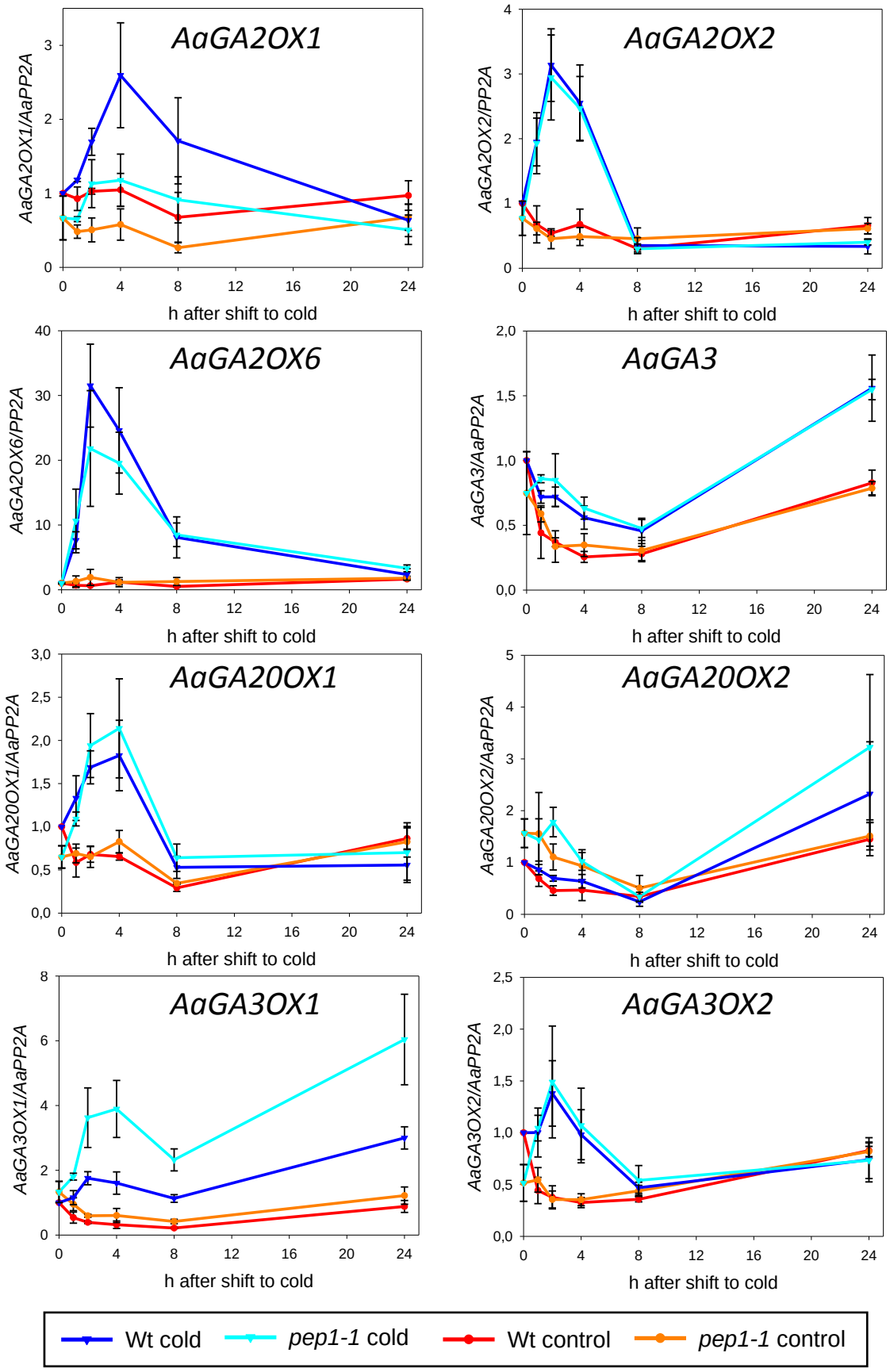

AaGA2OX1
AaGA2OX1/AaPP2A
AaGA2OX2
AaGA2OX2/PP2A
AaGA2OX6
AaGA2OX6/PP2A
AaGA3
AaGA3/AaPP2A
AaGA20OX1
AaGA20OX1/AaPP2A
AaGA20OX2
AaGA20OX2/AaPP2A
AaGA3OX1
AaGA3OX1/AaPP2A
AaGA3OX2
AaGA3OX2/AaPP2A
h after shift to cold
Wt cold
pep1-1 cold
Wt control
pep1-1 control

Figure 38 Effect of *PEP1* and short cold treatment on the expression of genes encoding GA metabolic enzymes.

Expression analysis of genes encoding enzymes involved in GA metabolism after short cold treatments of up to 24h in seedlings of *pep1-1* vs. Wt. Plants were grown for 2 w in SD at 21°C and then then at ZT4 (0h sample), transferred to 4°C for 24 h or kept under control conditions (SD, 21°C). Data are shown as mean ± SEM (n = 3 biological replicates). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment.

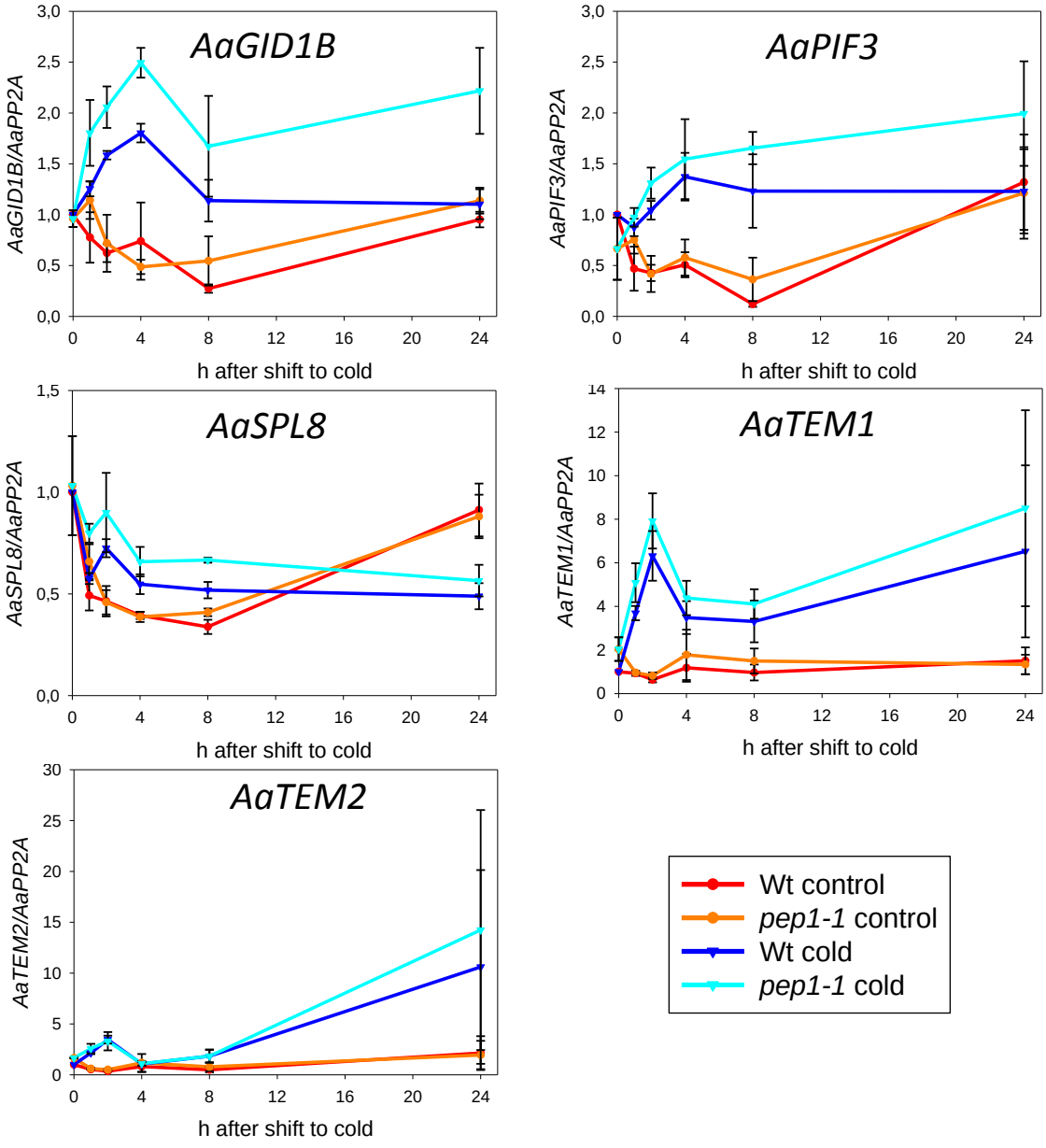


Figure 39 Effect of *PEP1* and short cold treatment on the expression of genes encoding GA signaling components.

Expression analysis of genes involved in GA signaling after short cold treatments of up to 24h in seedlings of *pep1-1* vs. Wt. Plants were grown for 2 w in SD at 21°C and then at ZT4 (0h sample), transferred to 4°C for 24 h or kept under control conditions (SD, 21°C). Data are shown as mean ± SEM (n = 3 biological replicates; except Wt and pep1-1 0h and 4h control n=2). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment.

In summary, short periods of cold caused increased expression of genes encoding GA signaling components and GA metabolic enzymes and PEP1 might have a negative effect on GA levels and GA signaling under cold stress conditions. To test whether intermittent cold affects GA levels in wild-type or the *pep1-1* mutant, levels of active GAs were analyzed after exposure to short-term cold. The GA1 content did not change in response to 4h cold treatment. The level of GA4 decreased at 4°C in both genotypes compared to control samples that were kept at 21°C for 4h, however the decrease in cold was not detectable if compared to the starting point of the experiment (0h) (Figure 40). The difference in GA levels between 4h control conditions and 4h cold but not between those samples and the 0h start sample suggest that GA levels slightly increase towards ZT8 (when the 4h samples were taken) and that cold reduces the levels of GA4. The *pep1-1* mutant had slightly if not significantly lower levels of GA4 at all time points but the change in response to cold was similar in both genotypes (Figure 40). A reduction of GA4 in response to cold was previously described in *A. thaliana* (Achard *et al*, 2008) and corresponds to the reduction of growth during cold in *A. alpina*, in particular even lower GA levels in *pep1-1* could explain the stronger growth retardation in *pep1-1* (Figure 33). As observed for the correlation between expression of genes encoding GA metabolic enzymes and GA levels during vernalization, also after short-term cold exposure, the increased levels of mRNAs encoding GA biosynthesis enzymes and reduced levels of mRNAs encoding GA degrading enzymes in *pep1-1* do not correspond to the observed lower GA levels in *pep1-1* compared to wild-type. Gene expression levels would rather suggest higher GA levels in *pep1-1* after exposure to short periods of cold temperature (Figure 38, Figure 40).

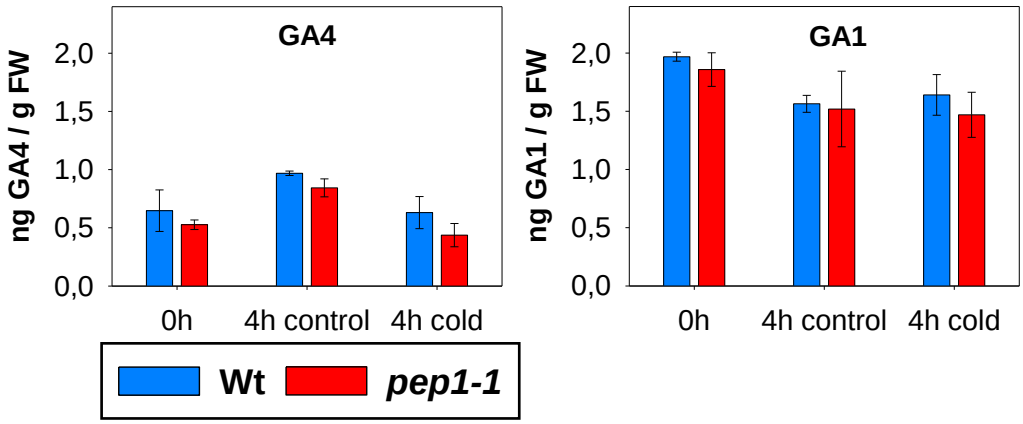
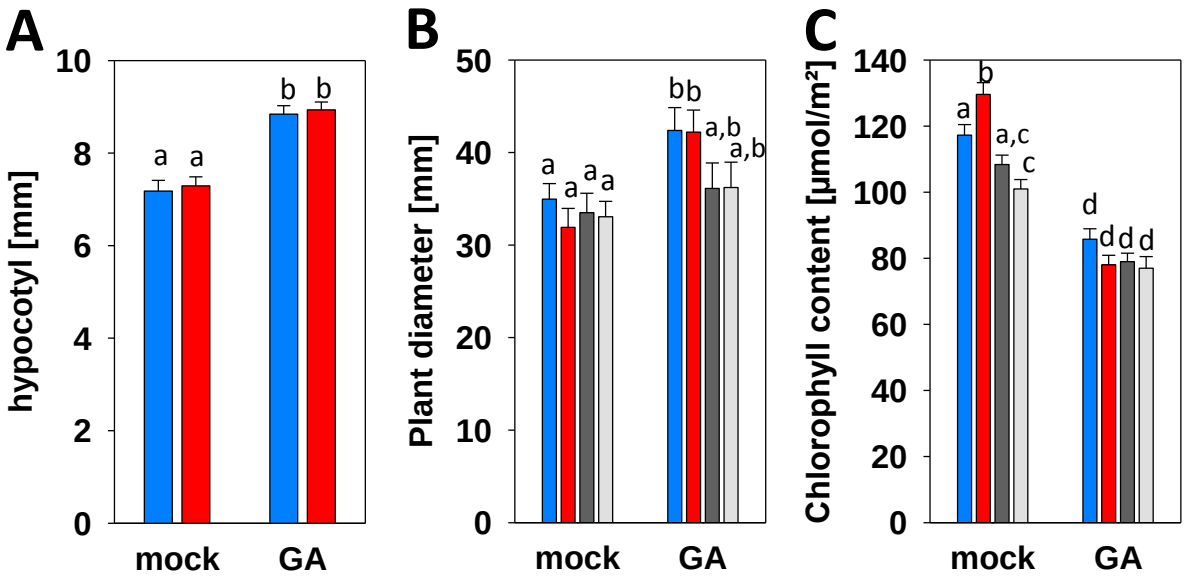


Figure 40 Effects of *PEP1* and short cold treatment on GA content.

Levels of active GAs after short exposure to cold. Plants were grown for 2 w in SD at 21°C (0 h) and then transferred to 4°C at ZT4 for 4 h (4h cold) or kept under control conditions (SD, 21°C) (4h control). Data are shown as mean ± Stdev. (n = 3 biological replicates, except Paj and *pep1-1* 0 h cold: n = 2). Letters indicate statistically different groups determined by two-way analysis of variance and multiple comparisons using the Bonferroni-t-test method that were performed within genotypes and within treatments. Groups were defined as statistically different when $P \leq 0.05$.

3.9 The regulation of GA biosynthesis/signaling by FLC in *A. thaliana* diverges from the role of PEP1 in *A. alpina*

A. alpina PEP1 and *A. thaliana* FLC bound and regulated different sets of GA-related genes (Figure 28). The *A. alpina pep1-1* mutant showed several GA-related phenotypes (Figure 31) that might be caused by differential expression of GA-related genes. Analysis of the *flc-3* mutant phenotype did not reveal any GA-related phenotypes in comparison to *ColFRI*$^{+}$ (hereafter referred to as wild-type). GA treatment caused an increased hypocotyl length, rosette diameter and a decreased chlorophyll content, but no strong differences between *flc-3* and wild-type could be observed (Figure 41A-C). In *A. thaliana*, FLC acts in a protein complex with the related MADS-box TF SVP (Li *et al*, 2008). SVP was shown to reduce GA levels by reducing expression of *GA20OX2* and the SVP-FLC complex regulates several GA-related genes (Andrés *et al*, 2014; Mateos *et al*, 2015). To test whether GA-related phenotypes of the *flc-3* mutant could be masked by functional redundancy with *SVP*, the *svp-41* mutant (in the *ColFRI*$^{+}$ background, see methods) as well as the double mutant *flc-3 svp-41* were included in the phenotypic analysis. Rosette diameter and chlorophyll content of the double mutant did not differ from the *svp-41* single mutant (Figure 41B, C), suggesting that *SVP* has the main effect on GA-related phenotypes.



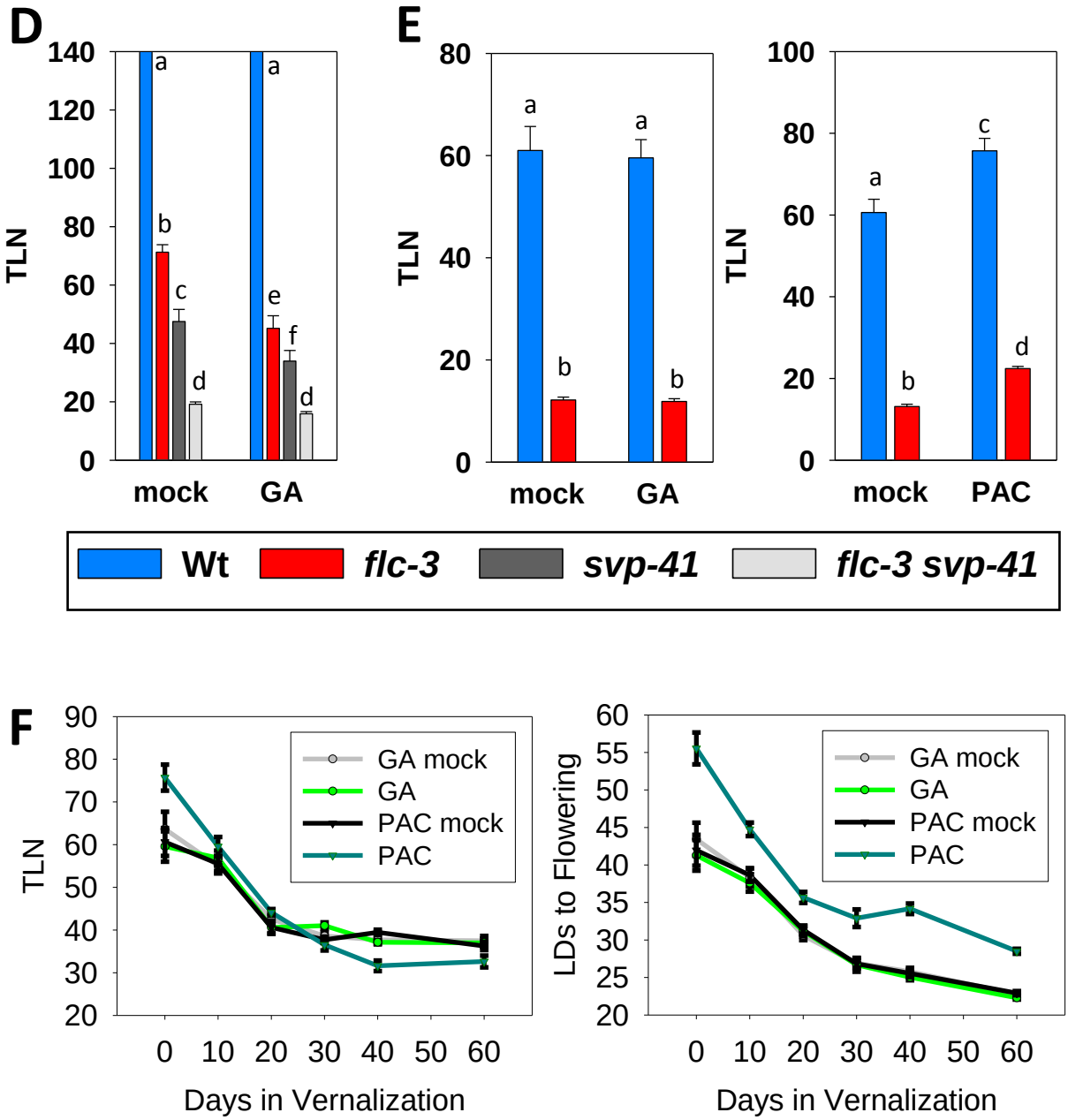


Figure 41 GA-related phenotypes of the *Arabidopsis flc-3* mutant.

Phenotypes of *ColFRI+* (Wt) vs. *flc-3* and *svp-41* and *svp-41 flc-3* mutant plants and effect of GA/PAC treatment. All genotypes are *FRI*$^{+}$. **(A)** Hypocotyl length of plants grown for 11 days in LD. 3 independent biological replicates, n ≥44. **(B)** Chlorophyll content. Plants were grown for 4 w in SD and measurements were performed on the 6th true leaf. 2 independent biological replicates. n ≥16. **(C)** Plant diameter. Plants were grown for 4 w in SD. 2 independent biological replicates. n ≥17. **(D)** Flowering time in SD. 2 independent biological replicates. n ≥18. **(E)** Flowering time in LD. 2 independent biological replicates. n ≥23. **(F)** Flowering time in LD after different periods of vernalization. Plants were grown for 10 days in LD prior to vernalization. Left: TLN, Right: Number of LDs to flowering (all days countend except vernalization time was substracted), scored as first flower bud visible by eye, days in vernalization were not counted. 2 independent biological replicates. n ≥21. For all experiments, plants were treated with GA/PAC twice per week throughout their life span. All data points are mean ± SEM. For all phenotypes, n describes the total number of replicates after combining all biological replicates. Letters indicate statistically different groups determined by two-way analysis of variance and multiple comparisons using the Bonferroni-t-test method that were performed within genotypes and within treatments. Groups were defined as statistically different when P ≤ 0.05. In D, TLN of the wild-type was set to 140 which was the leaf number at the end of the experiment when no wild-type plant flowered and was thus the minimal TLN possible.

Next, the effects of *FLC* and *SVP* on flowering time were analyzed in SDs, since GA only has a minor effect on flowering under LDs (Wilson *et al*, 1992; Griffiths *et al*, 2006). The *svp-41* mutant responded less to GA than the *flc-3* mutant, and the GA response was further reduced in the double mutant (Figure 41D), suggesting that *FLC* and *SVP* redundantly control flowering in response to GA but *SVP* plays the major role. Wild-type plants did not flower in SDs with or without GA (Figure 41D). Thus, functional FLC blocks the acceleration of flowering by GA. However, GA might act downstream of FLC repression and since the plants are not flowering in SD the function of GA might not come into effect. To test if FLC might repress the GA pathway, the effect of GA on flowering in wild-type was compared to *flc-3* also in LDs. Treatment with exogenous GA did not affect flowering time in LDs in wild-type or *flc-3*, but PAC treatment delayed flowering in both genotypes to similar extents (Figure 41E), indicating that GA promotes flowering in both genotypes. These findings suggest that the FLC- and the GA-mediated flowering responses are partly independent. GA acts independently of FLC since both genotypes showed a similar response to PAC and the wild-type did not show a response to GA or a stronger response to PAC, there is no evidence that FLC acts through repressing the GA biosynthetic pathway. To test whether GA or a reduction of GA levels affect the vernalization response of *A. thaliana*, the effects of GA and PAC on the vernalization response were analyzed. After germination, plants were treated twice per week with GA or PAC until flowering occurred. GA treatment did not cause an altered flowering response after different periods of vernalization (measured by total leaf number or by the number of days the plant was exposed to LDs until the flower bud was visible by eye) (Figure 41F). PAC treatment delayed the number of days until flowering independently of the time of vernalization and did not affect the total leaf number (Figure 41F), indicating that the vernalization response is not affected by the GA content.

Expression of several genes encoding GA metabolic enzymes was increased by vernalization in *A. alpina* and this induction was affected by PEP1 (Figure 33). Among these genes, some were not bound by PEP1 but were detected as direct or indirect target genes of FLC (Figure 28). Analysis ofngenes encoding GA metabolic enzymes during vernalization in *A. thaliana* did not reveal any increased expression in response to prolonged exposure to cold (Figure 42). In contrast, *GA3*, *GA20OX2*, *GA3OX1* and *GA3OX2* were more highly expressed under warm control conditions. In addition, FLC had a positive effect on expression of *GA3*, *GA20OX2* and *GA3OX2* and only *GA3OX1* was repressed by FLC, which corresponds to the effect that was observed for PEP1 in *A. alpina* (Figure 42, Figure 33).

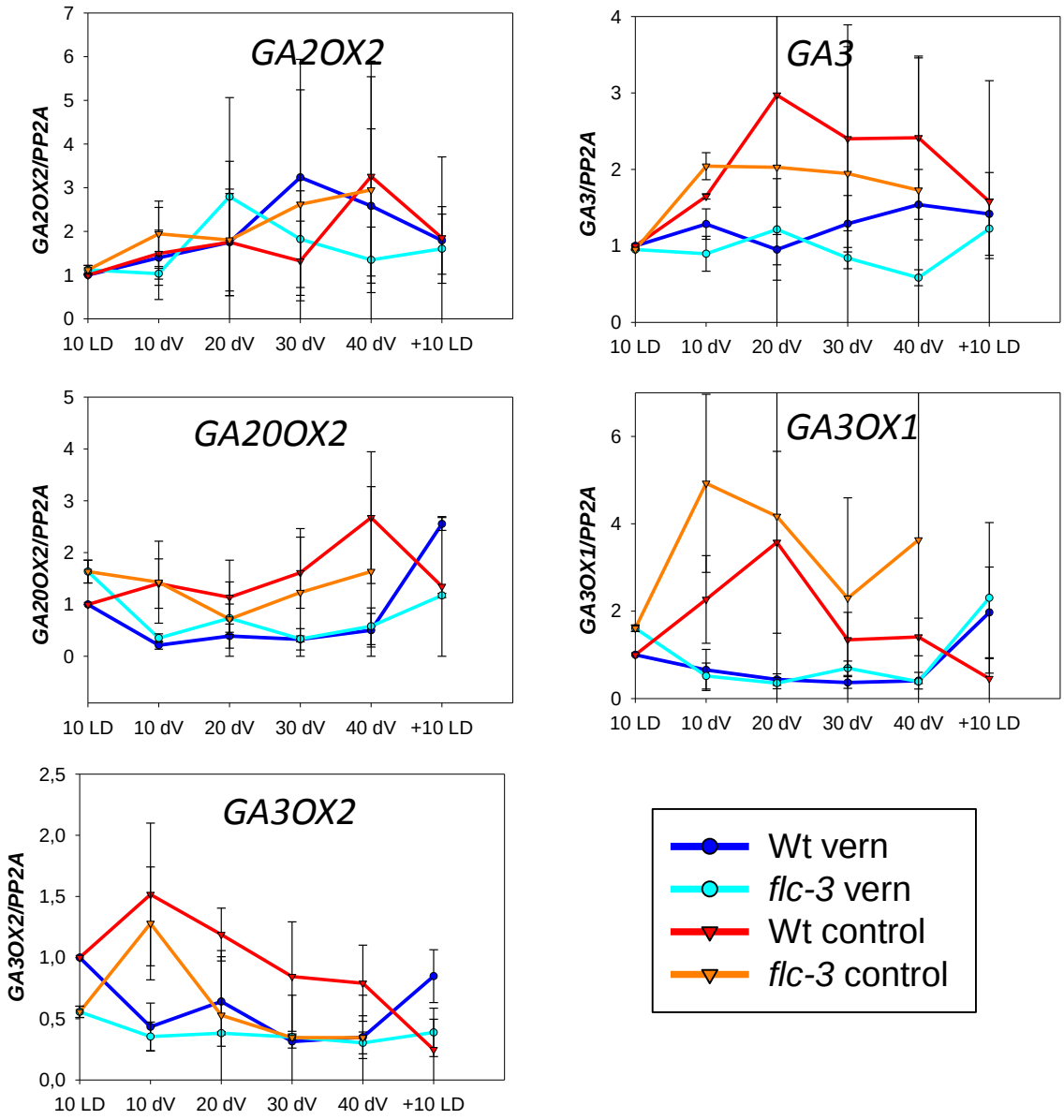


Figure 42 Effect of *FLC* and vernalization on the expression of genes encoding GA metabolic enzymes.

Expression analysis of genes involved in GA metabolism during vernalization in apices of *flc-3* vs. *ColFRI*$^{+}$ (Wt). Plants were grown for 10 days in LD and then transferred to 4°C for 40 days or kept under control conditions (SD, 21°C) and then shifted back to LD. Samples were taken at ZT8. Data are shown as mean ± SEM (n = 2 biological replicates). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment.

The GA signaling genes *GID1B*, *PIF3*, *SPL8*, *TEM1* and *TEM2* were induced during vernalization in *A. thaliana* (Figure 43). In *A. thaliana*, *SPL8* and *GID1B* expression was transiently increased in response to cold, in contrast to the progressive increase in *A. alpina*, and FLC did not have an effect on this, corresponding to the fact that they were not bound by FLC (Figure 43, Figure 34). The *TEM* genes were more strongly up-regulated in the wild-type, as was observed in *A. alpina*, however only FLC, not PEP1, directly bound to *TEM1* and *TEM2* (Figure 43, Figure 37, Figure 28). *PIF3* was the only conserved target gene of PEP1 and FLC that is involved in GA signaling, however in *A. thaliana*, *PIF3* showed stronger cold induction

in the wild-type whereas the opposite effect was found in *A. alpina* (Figure 28, Figure 43, Figure 37).

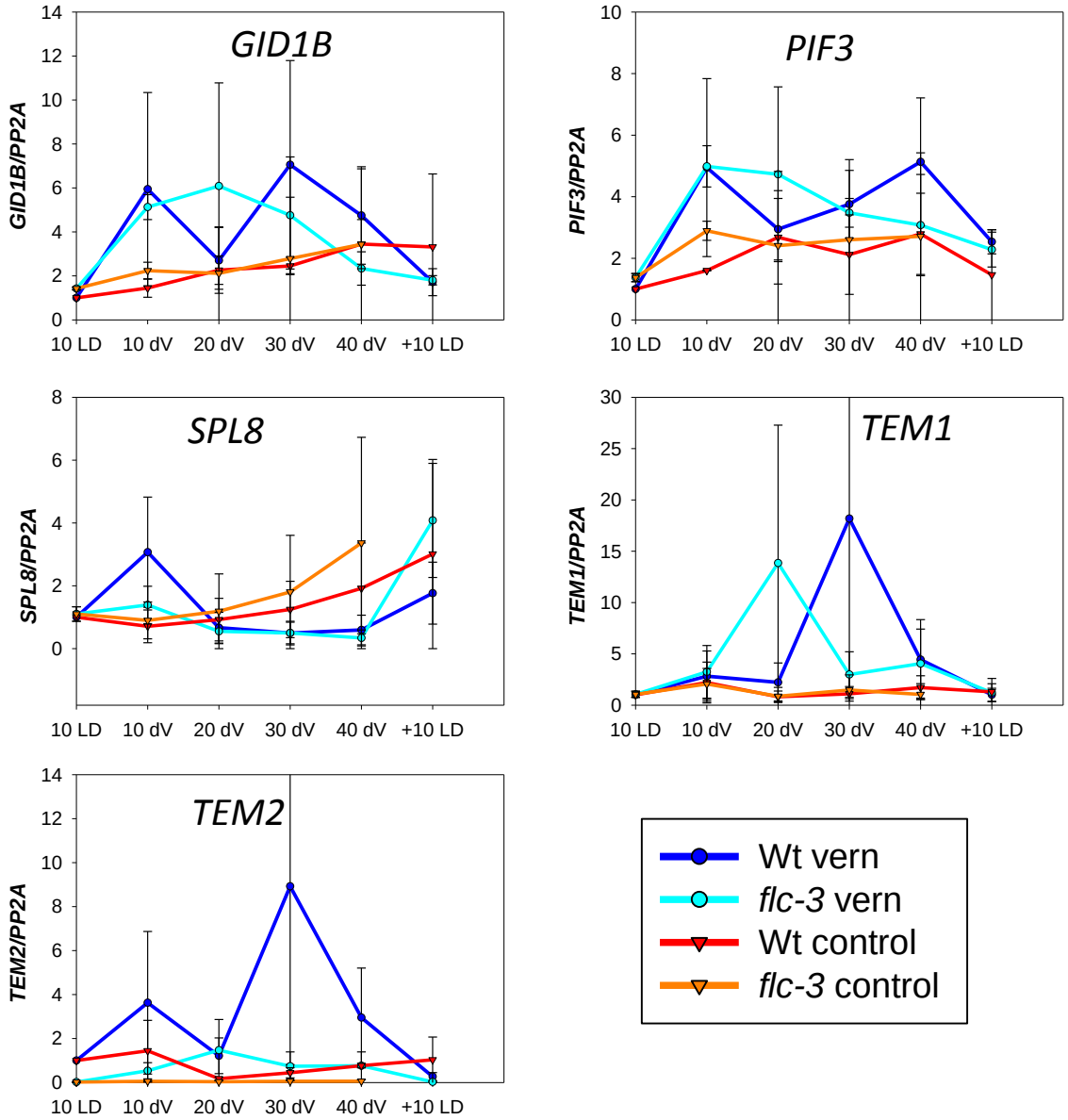


Figure 43 Effect of *FLC* and vernalization on the expression of genes encoding GA signaling components. Expression analysis of genes involved in GA signaling during vernalization in apices of *flc-3* vs. *ColFRI*$^{+}$ (Wt). Plants were grown for 10 days in LD and then transferred to 4°C for 40 days or kept under control conditions (SD, 21°C) and then shifted back to LD. Samples were taken at ZT8. Data are shown as mean ± SEM (n = 2 biological replicates). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment.

PEP1 and FLC seem to play a similar role in regulating cold induction of COR genes and this function probably arose by convergent evolution of BSs (see part 2.5). In *A. alpina*, PEP1 also affected cold induction of genes encoding GA metabolic enzymes (Figure 38). Since FLC also

regulated genes encoding GA metabolic enzymes, expression of these genes in response to intermittent cold was analyzed in *flc-3* compared to wild-type. As previously published (Achard *et al*, 2008) and as found in *A. alpina* (Figure 38), expression of GA-degrading *GA2OX*es was found to be increased by cold treatment (Figure 44). Like PEP1, FLC had a positive effect on cold induction of *GA2OX6*, whereas in contrast to PEP1, FLC negatively influenced cold induction of *GA2OX1* (Figure 44). Genes encoding GA biosynthetic enzymes that were cold induced in *A. alpina* were rather down-regulated in response to cold in *A. thaliana*. Only *GA20OX1* (Figure 44), which is published to be up-regulated as a result of feedback regulation of the up-regulation of *GA2OX*es (Achard *et al*, 2008), was cold-induced in *A. thaliana*.

Finally, the interaction of intermittent cold and FLC on the expression of selected genes encoding GA signaling components was investigated. Expression of several genes encoding GA signaling components was increased by short cold treatment, including *DDF1*, *GID1B*, *PIF3*, *TEM1* and *TEM2* while the DELLA *RGL2*, which was a direct FLC target was down-regulated in the cold (Figure 45). FLC affected the cold response of several of its direct target genes. *PIF3* and *TEM1* were more strongly up-regulated in *flc-3*, while *TEM2* responded more strongly to cold in the wild-type (Figure 45). The effect of FLC on *PIF3* and *TEM2* but not *TEM1* was similar to what was observed in *A. alpina* (Figure 39). In summary, FLC seems to be involved in regulating the induction of GA signaling components in response to short-term cold exposure, but the effect differed from what was observed for PEP1 in *A. alpina* (Figure 39, Figure 45). Only the repressive effect on cold induction of the conserved target gene *PIF3* seems to be conserved between species (Figure 39, Figure 45).

Overall, *flc-3* did not show any GA-related phenotypes, in contrast to *A. alpina pep1-1*. Vernalization treatment reduced expression of genes encoding GA metabolic enzymes, rather than increasing it as in *A. alpina.* However, vernalization caused an increase in expression of different genes encoding GA signaling components and this was affected by FLC, but the effects of FLC and PEP1 on genes encoding GA signaling components during vernalization differed. Short cold treatments caused increased expression of genes encoding GA degrading enzymes, as in *A. alpina*. However, in contrast to *A. alpina*, GA biosynthetic genes were not induced. Short cold treatments also caused up-regulation of genes encoding GA signaling components and FLC affected this but the effects were different from vernalization and from the effect of PEP1 in *A. alpina*.

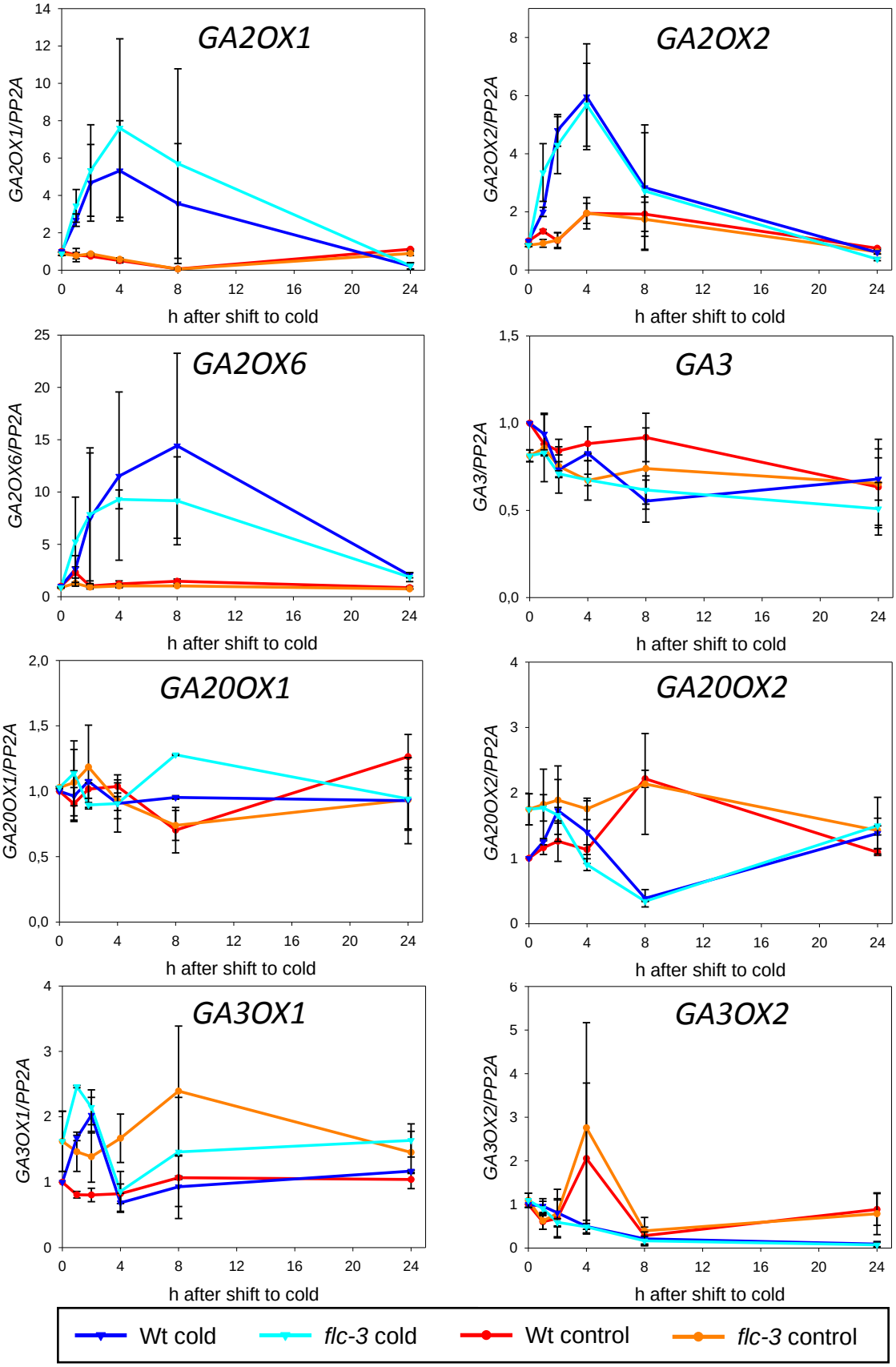

GA2OX1
GA2OX1/PP2A
h after shift to cold
GA2OX2
GA2OX2/PP2A
h after shift to cold
GA2OX6
GA2OX6/PP2A
h after shift to cold
GA3
GA3/PP2A
h after shift to cold
GA20OX1
GA20OX1/PP2A
h after shift to cold
GA20OX2
GA20OX2/PP2A
h after shift to cold
GA3OX1
GA3OX1/PP2A
h after shift to cold
GA3OX2
GA3OX2/PP2A
h after shift to cold
Wt cold
flc-3 cold
Wt control
flc-3 control

Figure 44 Effect of *FLC* and short cold treatment on the expression of genes encoding GA metabolic enzymes.

Expression analysis of genes involved in GA metabolism after short cold treatments of up to 24h in seedlings of *flc-3* vs. *ColFRI*$^{+}$ (Wt). Plants were grown for 2 w in SD at 21°C and then then at ZT4 (0h sample), transferred to 4°C for 24 h or kept under control conditions (SD, 21°C). Data are shown as mean ± SEM (n = 2 biological replicates). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment.

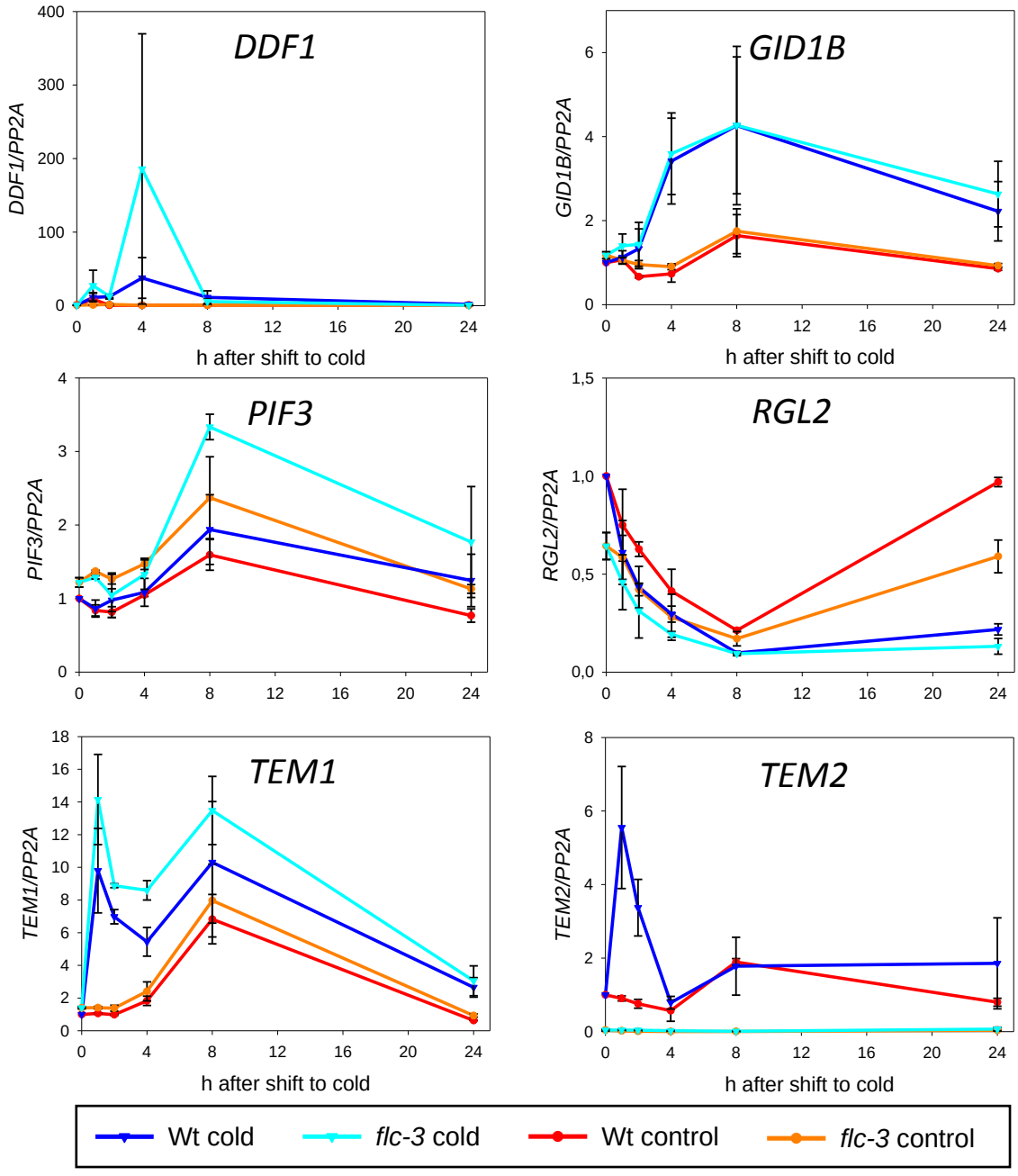


Figure 45 Effect of *FLC* and short cold treatment on the expression of genes encoding GA signaling components.

Expression analysis of genes involved in GA signaling after short cold treatments of up to 24h in seedlings of *flc-3* vs. *ColFRI*$^{+}$ (Wt). Plants were grown for 2 w in SD at 21°C and then at ZT4 (0h sample), transferred to 4°C for 24 h or kept under control conditions (SD, 21°C). Data are shown as mean ± SEM (n = 2). Expression was normalized to PP2A. Each experiment was normalized to expression in Wt at the start of the experiment.

3.10 Summary

PEP1 and FLC bound and regulated different genes related to GA metabolism and signaling. The *A. alpina pep1-1* mutant showed several phenotypes resembling a GA-treated plant and GA promoted floral induction during vernalization in the wild-type. Vernalization induced expression levels of genes involved in GA biosynthesis, degradation and different aspects of GA signaling in *A. alpina* and PEP1 had a repressive effect on this. Similarly, the exposure of *A. alpina* to intermittent cold caused induction of genes encoding GA metabolic enzymes and GA signaling components, which was counteracted by PEP1. GA levels, however did not increase but decreased in response to prolonged or short cold treatment but higher GA4 levels in apices of 3-week old *pep1-1* might partly explain the increased internode elongation of *pep1-1*. In addition, *pep1-1* showed increased GA responsiveness for internode elongation but not plant diameter. In consequence, locally enhanced GA signaling could explain the observed GA-related phenotypes of *pep1-1* (Figure 46).

In contrast to *pep1-1*, *flc-3* did not show any GA-related phenotypes. Unlike in *A. alpina*, vernalization of *A. thaliana* caused a reduction of expression of genes encoding GA metabolic enzymes and FLC did not have a general effect on this. As previously published (Achard *et al*, 2008), short exposure to cold temperatures caused induction of genes related to GA degradation, as in *A. alpina*. However, in contrast to *A. alpina*, GA biosynthesis genes were not induced at the same time as those involved in GA degradation. Prolonged and short exposure to cold temperatures resulted in induction of genes encoding GA signaling components and FLC had a negative effect on this. This role was similar to what was observed for PEP1 but the majority of genes encoding GA signaling components they regulated and the mechanisms (direct versus indirect regulation) diverged (Figure 46).

In conclusion, PEP1 and FLC both regulated GA-related genes, most likely as a result of convergent evolution, which in *A. alpina* and possibly also in *A. thaliana*, results in the negative regulation of certain GA-related processes but not in systemically increased GA responses.

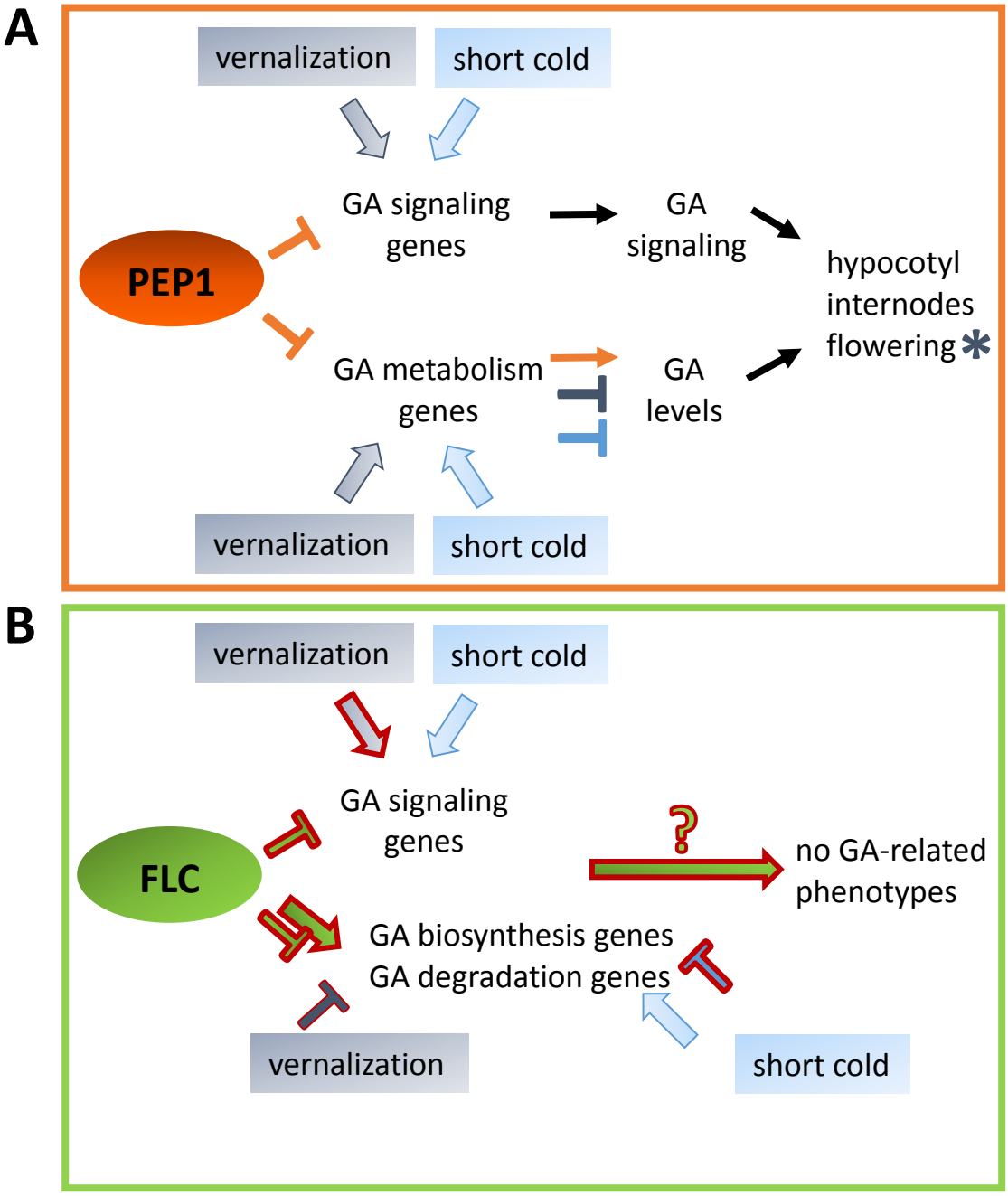


Figure 46 Model summarizing the diverging effects of PEP1 and FLC on GA signaling and GA biosynthesis

(A) *A. alpina* PEP1 represses the increased expression of genes involved in GA metabolism and GA signaling observed during vernalization and short periods of cold temperatures. GA signaling is increased in *pep1-1* and GA levels are increased in apices of 3w old *pep1-1*. Prolonged and short cold treatments reduce GA4 levels and PEP1 does not affect this. This network might cause GA-related phenotypes of *pep1-1* and the induction of flowering during vernalization (symbolized by *). **(B)** *A. thaliana* FLC represses the increased expression of genes involved in GA signaling observed during prolonged and short cold. Effects of FLC or of prolonged and short cold on genes encoding GA metabolic enzymes are not conserved between the two species. No GA-related phenotypes of *flc-3* could be detected. Arrows represent positive regulation, bars represent negative regulation. Red frames indicate non-conserved function. Orange: effect of PEP1; green: effect of FLC; dark blue: effect of vernalization; light blue: effect of intermittent cold.

3.11 Discussion

3.11.1 The role of GA during vernalization in *A. alpina*

In this study, we found that GA acted during vernalization to promote floral induction in *A. alpina*, although previously it was shown that GA applications do not induce flowering prior to vernalization (PhD Thesis of Renhou Wang). This suggests that the requirement for vernalization has a repressive effect on the GA flowering pathway or on a signaling component downstream of that pathway, so that GA can only induce flowering once vernalization is proceeding. Hence, vernalization requirement is epistatic to the GA pathway, but induction of flowering in vernalization involves GA. Previous studies also found that vernalization acts through the GA pathway in different species. In *Eustoma grandiflorum*, *Brassica napus* and *Thlaspi arvense*, vernalization was found to cause an increase in GA levels (Hazebroek *et al*, 1993; Zanewich & Rood, 1995; Mino *et al*, 2003; Hisamatsu *et al*, 2004). In *T. arvense*, GA treatment can also overcome vernalization requirement (Metzger, 1985), suggesting that here, vernalization acts mainly if not exclusively through the GA pathway. Similarly, in *Raphanus sativus* GA can overcome the vernalization requirement in LDs (Suge & Rappaport, 1968), suggesting that vernalization acts primarily through the GA pathway or the GA pathway can act to bypass requirement for vernalization. In contrast, in the model species *A. thaliana*, GA was not found to affect the vernalization response (Chandler *et al*, 2000; Figure 41F) but GA promotes flowering in summer annual accessions and after vernalization (Wilson *et al*, 1992; Griffiths *et al*, 2006; Figure 41F). In this species, the GA pathway might induce flowering downstream of the vernalization pathway, for example by activating flowering genes that are repressed before vernalization. Since in *A. thaliana*, floral induction occurs after vernalization (Moon *et al*, 2003), such an interaction of the two pathways would not require a role of GA during vernalization, as was found in *A. alpina*.

In this study, we found that in *A. alpina*, genes encoding GA biosynthesis enzymes were transiently increased in expression in the early stages of vernalization. This included enzymes of early and late steps in the GA biosynthesis pathway, similar to what was found in *E. grandiflorum* (Mino *et al*, 2003). The fold change in transcript levels in *E. grandiflorum* was higher (around 10x change) than in *A. alpina* (around 2x change), however this might be explained by the fact that the authors only identified one copy of the *GA3OX* and *GA20OX* genes (Mino *et al*, 2003), whereas in *A. alpina*, as in *A. thaliana*, these genes are part of gene families that function partly redundantly (Mitchum *et al*, 2006; Rieu *et al*, 2008b). In consequence, the observed expression changes in *A. alpina* and their effects on development might be spatially or temporally restricted. In contrast to *A. alpina* and *E. grandiflorum*, in *T.*

arvense, vernalization only increased the expression of genes involved in the early steps of GA biosynthesis, suggesting a different interaction between vernalization and the GA metabolism pathway (Hazebroek & Metzger, 1990; Hazebroek *et al*, 1993).

Despite increased expression of genes encoding GA biosynthetic enzymes, we found that levels of active GA1 and GA4, as well as their precursors and degradation products were decreased during vernalization and only a minor increase of active GAs occurred around the time of floral induction in later stages of vernalization (Figure 36B). Reduced GA levels during vernalization are in contrast to published results for other species (Hazebroek & Metzger, 1990; Hisamatsu *et al*, 2004) but the increase of active GAs at the end of the vernalization period resembles findings for winter canola (Zanewich & Rood, 1995). However, in *A. alpina*, GA levels increased very weakly at the end of vernalization and were still lower compared to non-vernalized controls (Figure 36B) unlike in winter canola (Zanewich & Rood, 1995) and in contrast to the tremendous increase of GA4 levels in apices of *A. thaliana* prior to floral induction under SDs (Eriksson *et al*, 2006). The increase in *A. thaliana* might be important not only for floral induction but also for bolting, which is closely associated with flowering in this species. By contrast, in *A. alpina* internode elongation already occurs at the vegetative stage, so a weaker and very local increase of GA levels might be sufficient to induce flowering and to further promote bolting. Alternatively, GA might play a role in fine-tuning the timing of floral induction during vernalization in *A. alpina*. Therefore, locally higher levels of GA might promote flowering in response to shorter periods of vernalization, but this effect could be weak and highly variable depending on slight changes in growth conditions.

Previously, a few studies described a reduction of GA levels in response to cold, however these effects might not be associated with floral induction. In *R. sativus*, levels of active GAs were reduced during vernalization and were increased in LD after vernalization when bolting occurred (Nakayama *et al*, 1995). In *A. thaliana*, GA levels decrease in response to intermittent cold, that, in contrast to prolonged cold, delays flowering (Achard *et al*, 2008). This observed reduction of the GA content was in contrast to increased transcription of GA biosynthesis genes during vernalization. It is possible that enzyme activity is reduced by low temperatures and elevated expression levels of GA genes are a way of compensating for this to prevent an even stronger decrease of GA content. Such compensation might be cold induced or it might be due to feedback regulation by decreased GA levels on transcription of genes encoding biosynthetic enzymes. In *A. thaliana*, low GA levels were described to positively feedback on expression levels of *GA20OX1-3* and *GA3OX1* (Chiang *et al*, 1995; Phillips *et al*, 1995; Mitchum *et al*,

2006; Rieu *et al*, 2008b) and several GA biosynthesis enzymes were found to be up-regulated by a feedback mechanism of lower GA levels during intermittent cold (Achard *et al*, 2008). Indeed, the fold change of gene expression identified by Achard *et al*. was similar to our results for *A. alpina*. Interestingly, levels of precursors of active GAs were always higher than active GAs but both curves followed the same pattern, suggesting that the relative amount that was converted to active GAs was constant. This would imply that the levels of GA biosynthesis enzymes are not strongly affecting GA levels or that tight feedback regulation ensures constant reaction rates. Strikingly, in cold only GA4 but not GA1 levels were reduced. A possible explanation could be that concentration and relative reaction rates of GA20OXes and GA13OXes were changed so that the absolute amount of GA12 that was 13-hydroxylated and processed to GA1 did not change while the amount of GA12 that was oxidized by GA20OXes to enter the pathway to GA4 was reduced. A similar scenario was previously described for tobacco, where ectopic expression of citrus *GA20OX* causes increased GA4 but not GA1 levels, which was explained by competition of GA20-Oxidase and GA13-Hydroxylase for the substrate GA12 (Vidal *et al*, 2001). Further experiments will help to conclude if temporally and spatially restricted changes in GA biosynthesis genes cause local changes in GA levels that could affect flowering. It will be required to analyze in more detail the expression patterns of the genes involved, for instance by investigating localization of fluorescently labelled proteins expressed from endogenous regulatory sequences. In addition, the relevance of feedback regulation of GA levels on genes encoding GA metabolic enzymes should be tested *in vivo* by applying exogenous GA prior to gene expression analysis.

We also found that vernalization increased expression of several genes encoding GA signaling components in *A. alpina*, suggesting that vernalization might influence the GA pathway by regulating the expression of GA signaling components. Indeed, several of these genes might promote flowering during vernalization in *A. alpina*. PIF3 acts in the ambient temperature pathway to induce *FT* expression in *A. thaliana* (Galvão *et al*, 2015) and *PIF3* expression was induced at the time of floral induction during vernalization in *A. alpina*. *GID1B*, which was induced in apices at the time of flower development, encodes a GA receptor in *A. thaliana* that functions partly redundantly with two other receptors but is particularly involved in flower initiation and development (Griffiths *et al*, 2006; Nakajima *et al*, 2006; Suzuki *et al*, 2009). Also *SPL8* was induced in apices at the time of flower development and in *A. thaliana SPL8* promotes GA-mediated steps later during flower morphogenesis (Zhang *et al*, 2007). In addition, vernalization increased expression of *SPL15*. In *A. thaliana*, *SPL15* integrates not only the age and vernalization pathways to induce flowering, but its activity is repressed at the post-

translational level by DELLA proteins and thereby GA signals are integrated by SPL15 as well (Hyun *et al*, 2016). In *A. alpina*, we found additional regulation of *SPL15* mRNA levels by GAs, indicating that signals from the GA pathway are also integrated at the level of *SPL15* transcription in this species (Figure 32C). This implies that GA might be required for SPL15 to induce flowering in parallel to the vernalization and age pathways to allow floral induction in *A. alpina*. TEM1 and TEM2 are floral repressors that repress expression of *FT* (Castillejo & Pelaz, 2008) and *GA3OX1/2* (Osnato *et al*, 2012). Expression levels of *TEM1/2* were induced during the whole period of vernalization and thus did not correlate with the timing of floral induction. However, *TEM1* and *TEM2* might act to prevent precocious induction of flowering after short periods of cold exposure. Analysis of the vernalization response in mutants or overexpressors of these genes encoding GA signaling components will be required to test their role in the interaction of the vernalization and GA pathways. Furthermore, *in situ* hybridization of floral marker genes and GA-related PEP1 target genes in apical samples might reveal when and how GA acts to induce flowering during vernalization. In addition, it will be interesting to test if the effect of GA on flowering-related traits is restricted to the vernalization period or if GA also acts after vernalization to affect flowering-related traits that appear later such as silique number and bolting.

3.11.2 The role of PEP1 in the regulation of GA

The genome-wide ChIP-seq and RNA-seq studies revealed that PEP1 regulates expression of GA-related genes and we found several, but not all, GA-related traits were affected in the *pep1-1* mutant. In *pep1-1*, internode elongation was increased, which correlated with elevated GA levels in young apices. Furthermore, our data suggest that PEP1 might repress flowering by negatively regulating the GA pathway.

PEP1 did not globally affect GA-related traits, since only some phenotypes were affected. Furthermore, additional factors need to be considered, as expression levels of target genes were not perfectly complementary to *PEP1* expression levels. This suggests that PEP1 acts in the complex network that regulates GA activity to regulate different GA-related traits. Repression of internode elongation by PEP1 might be beneficial in herbaceous perennial plants because only flowering shoots (which are those that do not express *PEP1*) would elongate, making flowers more accessible for cross-pollination and facilitating seed dispersal. After flowering, these shoots die and internode elongation would again be repressed in the remaining vegetative branches which would increase the stability of the plant. In consequence, increased internode elongation in *pep1* might strongly destabilize the plant, however, many natural *pep1* mutants

were previously identified so these can survive in some environments (Albani *et al*, 2012). It is possible that, depending on the habitat, natural growth conditions including cold temperatures (see part 2) and nutrient limitation, might strongly restrict growth and thereby counteract the effect of the *pep1* mutation. Thus, a possible disadvantageous effect of the *pep1* mutation on plant architecture would only become apparent under specific growth conditions. Also disadvantageous effects on plant architecture might be compensated for by the longer flowering duration and increased number of flowering branches in the *pep1* mutant, which increases seed yield (Wang *et al*, 2009b; Albani *et al*, 2012).

Repression of the GA flowering pathway during the early stages of vernalization by PEP1 is interesting because it shows that PEP1 is not only repressed by vernalization but that it has specific functions in the early stages of cold exposure. In this way, PEP1 might fine-tune the timing of floral induction to prevent precocious flowering before winter has passed. This interaction of two flowering pathways, GA and vernalization, might have evolved in the *A. alpina* lineage, which is in a different clade of the Brassicaceae than *A. thaliana* (Willing *et al*, 2015). This might be an adaptation to the alpine environment where cold periods, followed by warmer days might occur in autumn and should not induce flowering at this time. In addition, in contrast to rapid cycling *A. thaliana*, perennial *A. alpina* could tolerate not flowering after particularly mild winters, since flowering occurs repeatedly in the perennial life cycle and therefore does not need to occur each year. Divergence in interaction of flowering pathways between annual *A. thaliana* and perennial *A. alpina* was previously observed. In *A. thaliana*, the age pathway involving miR156 and miR172 are coupled and plants can flower under inductive LD conditions before miR156 levels have declined, which allows rapid cycling (Wang *et al*, 2009a; Wu *et al*, 2009). In *A. alpina*, these two pathways act in parallel with the result that the plant has to reach a certain age to flower and it needs vernalization (Bergonzi *et al*, 2013). Possibly, the GA pathway acts as another parallel pathway in the flowering network of *A. alpina.*

The *pep1-1* mutant showed increased mRNA levels of genes encoding GA biosynthetic enzymes and the GA degrading enzyme *GA2OX2*. Unlike in the wild-type, also *GA2OX2* was cold induced and cold induction of GA biosynthesis genes was stronger in *pep1-1*. Interestingly, only *GA2OX2* and *GA3OX2* were directly targeted by PEP1, up-regulation of the other GA biosynthesis genes must be due to indirect effects of PEP1. Simultaneous up-regulation of GA degrading and GA biosynthesis genes in *pep1-1* might be a result of feedback regulation as described for *A. thaliana* in intermittent cold and could occur in different cells (Achard *et al*,

2008). This scenario might imply that *pep1-1* has increased levels of both types of enzymes during vernalization and GA turnover is faster but absolute levels are not changed, which corresponds to what we observed during vernalization (Figure 36B). Alternatively, both types of genes might be induced in a cell-type specific manner causing local changes of GA levels. Such a scenario could explain why we detected increased GA levels only in apices of young *pep1-1* plants but not in other tissues, which might be associated with the observed longer internode phenotype of the mutant. GA levels in the SAM were previously shown to have a strong effect on internode elongation in *A. thaliana* (Porri *et al*, 2012). Increased GA levels in apices of *pep1-1* might be due to elevated levels of expression of *GA3OXes* and *GA3*, whereas elevated levels of *GA2OX2* expression might have a compensating effect in other tissues. Additional tissue specific increases of GA levels might be restricted to a low number of cells, and therefore not be detectable in our samples. For example, higher GA levels in a restricted number of cells in the meristem of *pep1-1* might contribute to its early-flowering phenotype. This hypothesis is in line with early studies in oat and pea which showed that in general, GA levels are high where GA is acting (Kaufman *et al*, 1976; Smith *et al*, 1992) and several studies detected a strong increase of GA levels in the apex at the time of floral induction (Zanewich & Rood, 1995; Talon & Zeevaart, 1990; Eriksson *et al*, 2006). Alternatively, different forms of active GA might be involved in regulating flowering in *A. alpina* as was described for *Lolium temulentum*, where GA5 and maybe GA6 increase at the SAM and induce flowering (King *et al*, 2001).

In conclusion, the effects of PEP1 on genes encoding GA metabolic enzymes might be restricted to specific cells and compensatory effects might ensure spatial restriction of changes in GA levels. In the future, detailed analysis of spatial expression of the network of GA-related genes will be crucial to understand the effect of PEP1 on GA metabolism. Interestingly, as discussed in section 2.7.2, PEP1 might also have positive as well as negative effects on germination or branching. These compensatory effects of PEP1 might allow fine-tuning of developmental processes as adaptation to specific environmental conditions and they might explain why many natural *pep1* mutant plants survive in their habitat without showing detrimental phenotypes.

Besides regulating GA levels, we also found that PEP1 reduced GA signaling to repress internode elongation (Figure 37A). Orthologs of several PEP1 target genes that were more highly expressed in the mutant were shown to promote growth in *A. thaliana*. PIF3 is repressed by DELLAs and in the presence of GA promotes hypocotyl elongation (Feng *et al*, 2008). *PIF3*

was up-regulated in apices and leaves of *pep1-1*, where it might contribute to the increased elongation of the hypocotyl and internodes and maybe additional phenotypes in the leaves that were not identified. These phenotypes might also be explained by increased activity of the GA receptor *GID1B* that is more highly expressed in *pep1-1*, which most likely causes a general increase in GA signaling that could cause many phenotypes (Griffiths *et al*, 2006; Nakajima *et al*, 2006). As discussed in the previous section, de-repression of genes encoding GA signaling components during vernalization might enhance the activity of GA in floral induction during vernalization. Interestingly, in the *pep1-1* mutant, the transcripts of the candidate genes *PIF3*, *GID1B*, *SPL8* and *SPL15* were present at higher levels compared to the wild-type, indicating that PEP1 functions early in vernalization to repress GA-mediated floral induction. *TEM1* and *TEM2*, which are negative regulators of floral induction (Castillejo & Pelaz, 2008; Osnato *et al*, 2012), were less strongly induced in *pep1-1*, suggesting that PEP1 positively regulates their expression to repress floral induction in early vernalization. To genetically confirm the function of these PEP1 targets in *A. alpina* and to test if PEP1 regulates GA-related traits by regulating these genes, it will be necessary to analyze mutants or overexpressors of these genes in wild-type and *pep1-1* background and to investigate their spatial expression patterns. Ideally this would include mutation of PEP1 BSs to confirm direct links between PEP1 function and the observed phenotypes. The CRISPR-Cas9 technology permits such reverse genetic approaches in *A. alpina*. Given that PEP1 regulates a high number of GA-related genes, it will be important to consider that PEP1 might not modify a certain phenotype by regulating individual genes but rather by modifying the activity of the whole gene network.

3.11.3 Conservation and divergence of PEP1/FLC function in the regulation of GAs

PEP1 and FLC both bound a set of GA-related target genes, but the two sets were hardly overlapping. As discussed in section 2.7.3, most TF binding events affect gene expression, at least under specific conditions. Thus, we hypothesize that PEP1 and FLC affect GA metabolism and signaling. Indeed, we found evidence that PEP1 regulates plant architecture by affecting GA-mediated internode elongation and PEP1 might regulate GA-mediated floral induction during vernalization. For *flc-3*, however, we did not find any GA-related phenotypes and no role of GA during vernalization could be identified in *A. thaliana* (Figure 41) (Chandler *et al*, 2000).

Like PEP1, FLC might negatively regulate the GA pathway to repress flowering before vernalization. For example, FLC might regulate floral induction by reducing GA levels since it bound to *GA3*, which encodes an enzyme that acts early in the biosynthetic pathway. However,

we did not observe increased expression of this gene in apices of *flc-3* or after vernalization. The increase of GA4 in apices of *A. thaliana* prior to floral induction in SDs was explained by transport of early intermediate GA12 (Eriksson *et al*, 2006; Regnault *et al*, 2015). Thus, GA3 might act outside of apices to increase levels GA12. Moreover, FLC might regulate floral induction by modulating GA signaling. FLC target genes included genes encoding components of the GA signaling pathway that have been associated with flowering like PIF3 (Galvão et al, 2015), DDF1 (Magome et al, 2004), GID1C (Griffiths et al, 2006; Suzuki et al, 2009), RGL2 (Cheng et al, 2004) and TEM1/2 (Castillejo & Pelaz, 2008; Osnato et al, 2012). Most of these genes were induced during vernalization rather than at the time of floral induction after vernalization and furthermore, not strongly differentially expressed in flc-3 prior to vernalization (Figure 43). More detailed analysis of spatial and temporal expression patterns might unravel if there are local changes in expression of these genes that could affect flowering.

Besides floral induction, GA was described to regulate other flowering-related phenotypes of *A. thaliana* that occur after floral induction. GA is required for bolting (Koornneef & van der Veen, 1980; Griffiths *et al*, 2006; Rieu *et al*, 2008b), flower development (Achard *et al*, 2004) and finally GA induces determinacy of the inflorescence meristem (Hay *et al*, 2002; Jasinski *et al*, 2005). It is possible that FLC regulates GA activity to influence these phenotypes, but due to the very early-flowering phenotype of the *flc-3* mutant, those phenotypes might not be detectable by comparing wild-type to *flc-3* under standard experimental conditions. FLC might delay bolting of side shoots to increase cross pollination and seed dispersal, similarly to PEP1 *A. alpina* (discussed above). FLC could potentially also repress GA-mediated reduction of chlorophyll production in flowering plants, which would lead to reduced amounts of chlorophyll after floral induction to allow re-allocation of resources to the seeds. Several FLC target genes could potentially affect bolting and chlorophyll content. *GA3* might affect GA levels (Helliwell *et al*, 1998), *GID1C* and *RGL2* might affect GA signaling in general (Lee *et al*, 2002; Tyler *et al*, 2004; Nakajima *et al*, 2006) and *PIF3* promotes growth and reduces chlorophyll content (Leivar & Monte, 2014). Further phenotypes that might be regulated by GA and by FLC before vernalization might not be obvious by visual comparison of wild-type and *flc* mutant, because the early floral transition of *flc-3* changes plant architecture soon after germination.

In conclusion, it seems that PEP1 and FLC regulate GA metabolism and signaling by targeting different genes within the GA network. These functions probably evolved by convergent evolution in response to similar selection pressure but in the context of different life histories

and flowering behaviors. The GA flowering pathway might be a convenient tool for rapid and subtle modification of flowering behavior during adaptation to changing environments, since it plays highly divergent roles in the control of flowering in different species (summarized in the introduction). In *A. alpina* and *A. thaliana*, not only the effect of PEP1 and FLC diverges between species but also the GA pathway itself. While in *A. thaliana* there is a strong increase of GA4 in the apex prior to floral induction under SDs (Eriksson *et al*, 2006), we only measured a weak change of GA in apices of *A. alpina* during flowering in response to vernalization. In addition, the major active GA in *A. thaliana* is GA4 (Xu *et al*, 1997; Eriksson *et al*, 2006), while we identified higher levels of GA1 in *A. alpina* (Figure 36). Which form of active GA plays the major role varies between plant species (Metzger, 1990; Smith *et al*, 1992; Jordan *et al*, 1995; Kobayashi *et al*, 1988; Lange *et al*, 2005) and in some cases even between developmental stages (Kobayashi *et al*, 1988; King *et al*, 2001; Zhu *et al*, 2006).

To get more information about the role of PEP1 and FLC in GA metabolism and signaling, it will be necessary to analyze local effects of PEP1 and FLC on gene expression. Analysis of different genes within the network will be crucial to understand the effect of PEP1 and FLC on the whole network. As a starting point, *A. thaliana GA2OX2* and *GA3OX1* were cloned, fused to Venus and transformed into *A. thaliana* plants to allow protein localization studies (see appendix). To better understand the function of these genes and the effect of PEP1/FLC it will be required to study protein localization in both species. In addition, analysis of mutants and overexpressors, including mutated versions of the genes that cannot be bound by PEP1/FLC, will allow unraveling differences and similarities in the GA gene networks and the role of PEP1 and FLC in the two species. As already mentioned in part 2.7.2, also in this context, it will be interesting to analyze conservation of the network involving FLC/PEP1, *SPL15* and GA in *A. alpina* and *A. thaliana.* In *A. thaliana*, SPL15 links regulation of the two miRNAs miR156 and miR172 since miR156 represses *SPL15* on the post-transcriptional level and SPL15 itself promotes expression of *MIR172b* (Hyun *et al*, 2016). In perennial *A. alpina*, regulation of miR156 and miR172 is not mechanistically coupled so that the plant needs to have a certain age and vernalization to be competent to flower (Bergonzi *et al*, 2013). This competence might largely be conferred by SPL15 (Bergonzi *et al*, 2013; Y. Hyun, personal communication). Possibly, in *A. alpina*, also the GA pathway is required during vernalization to ensure sufficient *SPL15* transcription and perhaps activity of SPL15 by triggering DELLA degradation. Thus, in perennial *A. alpina*, three parallel pathways might be needed to induce flowering, whereas in *A. thaliana*, activity of a single pathway induces flowering which facilitates the rapid cycling life-history. Analyzing the effects of vernalization, PEP1/FLC, GA and plant age on *SPL15*

expression levels and protein activity will reveal how conserved or divergent connections between different subnetworks contribute to the regulation of flowering in the background of the different life histories of *A. alpina* and *A. thaliana* (see appendix).

3.11.4 The role of PEP1/FLC and GA in the cold-stress response

Analysis of gene expression in response to intermittent cold revealed that cold increased expression of *GA2OX* genes, which encode GA degradation enzymes, in *A. alpina* and *A. thaliana* (Figure 38; Figure 44). In *A. thaliana*, intermittent causes a decrease of GA levels leading to reduced growth, by inducing expression of *GA2OX1*, *3* and *6* (Achard *et al*, 2008). This reduction of growth during the cold stress response is the result of a trade-off where plant growth is traded off against stress tolerance (Herms & Mattson, 1992; Alpert, 2006; Scheres & van der Putten, 2017). In our study, we found additional induction of *GA2OX2* in both species. In *A. alpina*, but not in *A. thaliana*, we also discovered cold-induction of GA biosynthesis genes *GA3*, *GA20OX1* and *GA3OX1/2* (Figure 38). Achard *et al*. (2008) detected up-regulation of *GA20ox1/3* and *GA3OX2* in response to cold due to feedback regulation of reduced GA levels. The degree of feedback regulation might depend on the GA content and in general might be stronger in *A. alpina* than in *A. thaliana*. It is noteworthy, that also in response to vernalization we detected up-regulation of GA biosynthesis genes in *A. alpina* but not in *A. thaliana*. Perhaps, in *A. thaliana*, cold causes more effective growth repression by strongly reducing GA levels, while in *A. alpina*, buffering of GA levels is stronger. In that way, growth repression by cold in *A. alpina* might be weaker, allowing more growth under cool, non-freezing temperatures, which could possibly be an adaptation to the alpine habitat. Indeed, we detected a weak decrease of GA4 after 4h of cold treatment in *A. alpina* (Figure 40), while in *A. thaliana* GA1 and GA4 strongly decreased after 4h in cold (Achard *et al*, 2004). However, both experiments were performed under different experimental conditions so comparisons should be treated with caution.

Differences in cold responses of genes encoding GA metabolic enzymes do not only exist between species. In *A. thaliana*, cold responses of genes encoding GA metabolic enzymes were shown to depend on the type of cold treatment and on the developmental stage. In contrast to the effect of intermittent cold and vernalization on seedlings (Achard *et al*, 2008; Figure 42; Figure 44), cold exposure of imbibed seeds causes induction of *GA3OX1* and *GA20OX2/3*, while expression levels of *GA2OX2* and *GA3OX2* decrease. This results in increased GA levels that promote germination (Yamauchi *et al*, 2004). Taken together, GA levels are regulated by

networks of genes that feed back on each other and seem to be easily adjustable to adapt to different environmental and developmental conditions.

In addition, we found that exposure to intermittent cold increased expression of genes encoding GA signaling components in *A. alpina* and *A. thaliana*. The sets of signaling genes that were affected were similar in both species but not identical and parts of the effects might also be due to feedback regulation of low GA levels (Middleton *et al*, 2012). In conclusion, exposure to cold affected the expression of a network of GA-related genes including subnetworks regulating GA metabolism and different aspects of GA signaling in both species. This interaction between cold and GA-related genes seems to be rapidly evolving leading to variation between species and between growth conditions. Growth and stress tolerance are traded-off against each other, allowing flexibility in the adaptation to the environment (Herms & Mattson, 1992; Alpert, 2006; Scheres & van der Putten, 2017). Thus, variation between species in the interaction between cold and the GA network probably probably represents adaption of plant growth to different environmental and endogenous signals.

Many of the GA-related genes that were induced by short exposure to cold temperatures in *A. alpina* were regulated by PEP1. In the *pep1-1* mutant, genes involved in GA biosynthesis and signaling were increased in expression more strongly while the effect on genes encoding GA degrading enzymes was less strong. Simultaneously increased expression levels of GA biosynthesis genes and decreased levels of GA degrading genes suggest that *pep1-1* has higher GA levels in cold. Alternatively, the observed differences in gene expression might be the result of stronger feedback regulation of low GA levels. Decreased GA levels negatively feed back on transcription of genes encoding GA degrading enzymes and positively affect genes encoding GA biosynthesis enzymes (Middleton *et al*, 2012). This would imply that *pep1-1* has lower GA levels compared to the wild-type. Confirming neither of these hypotheses, the change of GA levels in response to cold was similar in both genotypes (Figure 40). In the first part of this study, we detected enhanced cold-stress response in *pep1-1* mutants as measured by higher expression levels of COR genes and decreased growth in cold. The latter could be explained by reduced GA levels in *pep1-1* compared to the wild-type. Cold might cause a very weak or spatially and temporally restricted reduction of GA levels, which was not detectable in our experiments. This would most likely involve a different mechanism than the induction of *GA2OX* genes which was described by Achard *et al*. (Achard *et al*, 2008) because *GA2OX* expression levels were decreased, not induced in *pep1-1* compared to wild-type. Alternatively, cold might affect growth in *pep1-1* independently of changes in GA levels. Although the effect

of *CBF*s on GA levels was found in several species (Shan *et al*, 2007; Achard *et al*, 2008; Zhou *et al*, 2014), a possible alternative mechanism for this was described in *A. thaliana*, where cold induces for instance *ZAT10* and *ZAT12* which repress growth when overexpressed (Park *et al*, 2015). These two genes were not among PEP1 direct or indirect targets but *CZF2* (also known as *ZAT6)*, another candidate gene that was identified but not more deeply analyzed in the same study (Park *et al*, 2015), was regulated by PEP1 (Table A7).

If expression changes of genes encoding GA metabolic enzymes in *pep1-1* were due to increased levels of feedback regulation, as was suggested above, this would imply that *pep1-1* has more strongly reduced GA levels in intermittent cold compared to wild-type. Thus, in the wild-type, PEP1 would act to buffer changes in GA levels in response to intermittent cold. Interestingly, *GA2OX2* and *GA3OX2*, the only direct targets of PEP1 did not show this putatively increased feed-back effect in *pep1-1*. This might indicate that in the wild-type, PEP1 directly modifies expression levels of these genes to counteract changes of GA levels to maintain GA homeostasis in intermittent cold. PEP1 would directly repress expression of *GA2OX2* but positively affect *GA3OX2* which would both result in higher GA levels compared to *pep1-1*. Thus in sum, these genes would lack the regulation by PEP1 in *pep1-1* but simultaneously encounter a stronger feedback effect of GA levels and therefore, expression levels would seem unchanged compared to the wild-type. In summary, PEP1 seems to repress the cold response and might buffer changes of GA metabolism in intermittent cold. Both effects might contribute to maintain normal growth and development in intermittent cold prior to frost as an adaptation to alpine growth conditions. Thus, PEP1 modifies the trade-off between cold stress tolerance and growth. The putative effect of PEP1 on GA metabolism in intermittent cold seems to be specific to these conditions because before and during vernalization, PEP1 seems to repress the GA pathway.

In *A. thaliana*, FLC did not generally affect cold induction of GA degrading genes (Figure 44). While *GA2OX6* was more strongly induced by cold in the wild-type, *GA2OX1* was more strongly induced in *flc-3*. We found stronger induction of COR genes by intermittent cold in *flc-3* (see part 2), and higher expression of *CBF1* might have caused higher expression of *GA2OX1* to reduce GA levels and thereby growth, as was observed in *CBF*-overexpressing plants (Achard *et al*, 2008). FLC also affected cold induction of several genes encoding GA signaling components (Figure 45). This might be a feedback effect of low GA levels (Middleton *et al*, 2012) but as several of these genes were direct targets of FLC, it is possible that FLC regulates these genes to affect GA signaling in intermittent cold. For example cold induction of

DDF1 was increased in *flc-3*. DDF1 represses growth under salt stress by activating transcription of GA degrading *GA2OX7* (Magome *et al*, 2008). Possibly it also influences the trade-off between growth and stress tolerance under under cold stress conditions which would be part of the increased cold-stress response in *flc-3*.

In summary, it seems that different subnetworks of the GA network, like GA biosynthesis, GA degradation and signal transduction, play different roles in cold stress in *A. alpina* compared to *A. thaliana* and PEP1/FLC independently evolved different functions in regulating these networks. New connections of components of different gene networks that regulate stress responses and growth might facilitate rapid adaptation to changing environmental conditions by influencing the trade-off between growth and stress tolerance. The evolution of new TF BSs as for PEP1/FLC might have provided a relatively rapid mechanism for this to occur. To further characterize the roles of PEP1 and FLC in GA signaling during the cold-stress response, a first step will be investigation of feedback effects of GA application on GA-related genes in wild-type and *pep1*/*flc* mutants in both species.

4 Conclusions and perspective

This study describes the first comparative ChIP-seq study coupled with gene expression analysis in knock-out mutants for orthologous TFs in two related plant species. BSs of *A. alpina* PEP1 and *A. thaliana* FLC were highly divergent and this divergence of BSs was attributed to the species-specific occurrence of short DNA binding motifs. We identified the control of flowering and flower development as conserved core functions that were associated with conserved BSs and, according to mutant analysis, was the main function under standard growth conditions of these TFs. Species-specific functions were related to responses to hormones and environmental stimuli. In these responses, PEP1- and FLC-specific target genes were involved in similar processes, suggesting that these functions evolved independently to confer responses to similar environmental conditions but in the context of different life histories. Both TFs were involved in the response to intermittent cold and to GA. In *A. alpina*, GA was found to act during vernalization to promote floral induction. PEP1 negatively regulated the GA network in *A. alpina* to suppress internode elongation, putatively to increase stability of vegetative branches prior to flowering, and most likely to suppress flowering during the early stages of vernalization. This interaction between the vernalization and GA pathway in *A. alpina* provides a previously unknown connection of two flowering pathways that might be beneficial in

preventing flowering after short periods of vernalization in *A. alpina* before the alpine winter is over. In the perennial background, less strong induction of flowering by vernalization might be tolerable, since plants have the chance to reproduce repeatedly. Also *A. thaliana* FLC seems to regulate the GA pathway but the role of FLC and the role of GA in floral induction were not conserved. Furthermore, both TFs repressed the cold response, putatively to maintain growth under cold but non-freezing conditions prior to vernalization. Additionally, short periods of cold affected the GA network and whereas FLC does not seem to play a role in this process, PEP1 seems to buffer GA levels in response to intermittent cold in *A. alpina*. This effect of PEP1 might influence the trade-off between growth and cold response allowing growth to proceed in cold but non-freezing temperatures as an adaptation to the alpine habitat. In conclusion, this study allowed the identification of species-specific interactions between gene networks that regulate development and the abiotic stress response to environmental stimuli. These new connections of networks possibly represent adaptations to different habitats and life histories of the two species. This example illustrates how the evolution of new TF BSs provides a mechanism to connect gene networks as a rapid way of adaptive evolution in plants. These adaptive traits might represent sensitive adjustments to slight changes of growth conditions since PEP1/FLC targeted multiple rather than single genes within the networks.

Here we showed that comparative ChIP-seq for orthologous plant TFs can be a powerful tool to identify the conserved core function of two TFs as well as new species-specific functions and interactions between developmental and environmental response pathways. In the future, this approach can be used to functionally characterize less well characterized TFs and their role in phenotypic variation between related species. To obtain more information on the evolution of BSs and functions of the FLC TF, this comparative ChIP-Seq study could be extended to additional species. We identified conservation of FLC binding motifs outside of the Brassicaceae family in *T. hassleriana*, however, this species inhabits semi-tropical regions and therefore does not encounter prolonged cold that could trigger the vernalization response. It will be interesting to unveil the function of the putative FLC ortholog in *T. hassleriana*. Moreover, FLC orthologs have been identified in monocots (Ruelens *et al*, 2013). In cereals, however, vernalization induces flowering by induction of the *VRN1* gene (Yan *et al*, 2003), leaving the function of putative FLC orthologs unclear. In addition, it will be interesting to perform ChIP-seq and gene expression analysis in specific tissues and under specific environmental conditions. Such approaches might illustrate the dynamics of FLC/PEP1 binding profiles and might reveal additional functions such as for example roles in the perennial life-history of *A. alpina* that become apparent specifically in adult plants. Analysis of TF BSs under specific

environmental conditions will be particularly interesting since several species-specific functions of PEP1 and FLC seem to be responses to environmental stresses. In addition to condition-specific gene regulation, stress-specific DNA binding might contribute to these functions. In addition, FLC and PEP1 seem to regulate networks of COR and GA-related genes and most likely cause temporally and spatially restricted expression changes of different components within these networks. Therefore, detailed expression analysis like *in situ* hybridization or confocal microscopy of proteins with fluorescent tags for several components of the gene networks in combination with studies of putative feedback mechanisms (e.g. of changed GA levels) will be required to better characterize the role of PEP1/FLC in cold and GA responses. Furthermore, we found evidence that, especially for the conserved targets, PEP1 and FLC shared BSs with other MADS-box TFs that are involved in the regulation of flowering. To better characterize the evolution of the role of FLC/PEP1 within this network of flowering TFs and to investigate how they affect each other's functions, it will be interesting to perform ChIP-seq studies for different MADS-box TFs in the presence and absence of their interaction partners, similar to the study by Mateos *et al.* (Mateos *et al*, 2015).

In order to study conservation of interactions between different flowering pathways, *SPL15* might be a particularly interesting target for further investigation. *SPL15* was identified as a conserved target gene with deeply conserved CArG-box motifs. This suggests that *SPL15* has a conserved function in floral induction, not only in *A. thaliana* and *A. alpina*, two species that have distinct life histories and flowering behaviors. In *A. thaliana*, SPL15 was shown to induce flowering by promoting transcription of *FUL* and *miR172* in the SAM (Hyun *et al*, 2016). In addition, SPL15 activity is regulated by plant age post-transcriptionally by miR156 and on the transcriptional level by vernalization and by GA on the level of protein activity (Schwab *et al*, 2005; Deng *et al*, 2011; Hyun *et al*, 2016). In *A. alpina*, SPL15 also integrates signals from the age and vernalization pathway and both signals are absolutely required for flowering, in contrast to *A. thaliana* (Bergonzi *et al*, 2013; this study). There is genetic evidence that *SPL15* might be a main player in the vernalization response of *A. alpina* (Bergonzi *et al*, 2013; Y. Hyun, personal communication). In addition, in *A. alpina*, GA affected *SPL15* transcript accumulation during vernalization and promoted flowering during vernalization. Furthermore, PEP1 was found to repress the GA response in *A. alpina*. Taken together, these findings lead to the hypothesis that GA is also required to promote *SPL15* transcription and possibly activity to induce flowering during vernalization in *A. alpina*. Future experiments will compare the role and regulation of SPL15 in *A. thaliana* and *A. alpina*. Mutants of *spl15* and their response to vernalization and GA/PAC treatment will be analyzed to test if GA acts through SPL15 to induce flowering

during vernalization. Furthermore, we will analyze spatial and temporal expression patterns of *SPL15* and different mutant versions of the gene that cannot be regulated by miR156 or by PEP1/FLC. In addition, SPL15 activity will be monitored by analyzing *FUL* transcript accumulation by *in situ* hybridization. This combination of different methods will allow to further characterize the role of PEP1 in the regulation of GAs as well as the function of GA during vernalization in *A. alpina*. Finally, these studies will help to understand how conserved different flowering pathways are between species and how these subnetworks are interconnected in two closely related species with different flowering behaviors in the background of annual or perennial life-history.

5 Material and Methods

Plant material, growth conditions and phenotypic analysis

The *A. alpina* Pajares accession used as wild-type and the *pep1-1* mutant were described in Wang *et al*, 2009. The *KNAT1::GA2OX7* transgene, which promotes meristem-specific expression of *GA2OX7*, which encodes a GA degrading enzyme and was described to cause strongly decreased GA levels in *A. thaliana* (Lincoln *et al*, 1994; Schomburg *et al*, 2003; Porri *et al*, 2012), was transformed into the *pep1-1* mutant by floral dip (Clough & Bent, 1998) according to the common lab protocol for *A. alpina* floral dipping (T1 seeds were kindly provided by J. Mateos). F1 plants were crossed to Pajares and T3 plants homozygous for wild-type *PEP1* were identified by genotyping using a CAPS marker designed by Y. Hyun. Therefore, a PCR using the primers HY88 and HY89 was followed by a restriction digest with the enzyme AseI (New England BioLabs) for gel electrophoresis analysis. Presence of the *KNAT1::GA2OX7* transgene was identified based on the strong GA-deficient phenotype (dwarf and dark green). For *A. thaliana*, Col-0 with the introgressed *FRI SF2* allele (*ColFRI*$^+$) was used as wild-type to ensure high expression of FLC (Lee *et al*, 1994). The *flc-3* mutant in the *ColFRI*$^+$ background was described in Michaels & Amasino, 1999. Furthermore, the *svp-41* mutation (Hartmann *et al*, 2000) was introduced into the *ColFRI*$^+$ background (Mateos *et al*, 2015) (hereafter referred to as *svp-41*) and the resulting *svp-41 ColFRI*$^+$ mutant was crossed to *flc-3* to obtain the *flc-3 svp-41* double mutant (Mateos *et al*, 2015) (hereafter referred to as *flc-3 svp-41*).

Seeds were stratified on moist soil at 4°C in darkness for 2-4 days. Plants were grown at a light intensity of about 200 µmol/(m^2*s) under LD conditions (16 h light/ 8 h dark) or SD conditions (8 h light/ 16 h dark) at 21°C and 60-70 % humidity. A. thaliana plants for grown for 10 days

under LD prior to vernalization. A. alpina plants were grown for 2 weeks (juvenile plants) or 5.5 weeks (adult plants) under LD prior to vernalization. Vernalization and short cold treatments were performed at 4°C under SD conditions (8 h light (17 µmol/ (m^2*s))/ 16 h dark). For the short-term cold experiments, plants were grown under SD conditions for 2 weeks and shifted to cold conditions for up to 24h at ZT4, when the *CBF* response to cold temperatures is maximal (Fowler *et al*, 2005). For all experiments, trays where shifted weekly to avoid positional artefacts.

Plant height and diameter of plants with elongated internodes was measured using a ruler. To determine diameter and hypocotyl length of seedlings, plants were photographed together with a size standard and measurements were performed using the Image-J 1.43u software (Wayne Rasband National Institutes of Health, USA). Chlorophyll content was measured using the SPAD-502 leaf chlorophyll meter (Markwell *et al*, 1995). For each data point (1 plant), the average of three technical repetitions of measurements on the same leaf was created. For *A. thaliana*, measurements were performed on the 6th true leaf and for *A. alpina* on the 7th true leaf. Flowering time in *A. thaliana* was assessed by counting the total leaf number at the main shoot which represents the developmental stage of the plant. In addition, in the vernalization time course experiment, the number of days that the plant was exposed to LDs until the flower bud was visible by eye was counted. For *A. alpina*, flowering time was determined by the number of days until the first flower opened since PAC-treated plants were too compact to precisely determine TLN. The extent of flowering in *A. alpina* plants was assessed by counting the number of individual siliques at the main shoot without including siliques at side branches. All experiments were performed in independent biological replicates. The number of technical and biological replicates for each experiment is indicated in the figure legends. In order to calculate the GA response, a linear regression curve where x is $\log_{10}$(GA concentration) and y is the phenotype was calculated for each genotype. The slope of the regression curve is the phenotypic change per amount of GA (logarithmic scale). All data points are represented as mean ± SEM. Analysis of variance was performed using the SigmaStat 3.5 software.

Chromatin immunoprecipitation

For ChIP experiments, wild-type and *pep1-1*/*flc-3* mutant plants were grown for 2 weeks in LD and 2 g above ground tissue was harvested at ZT 8. For *A. alpina*, 1 µl of PEP1 antiserum (Albani *et al*, 2012) was used. For *A. thaliana*, 2 µl of FLC antiserum (kindly provided by C. Helliwell) (Deng *et al*, 2011) was used for the ChIP-seq experiments and 5 µl of novel FLC antiserum (Agrisera, kindly provided by R. Richter) which was created using the previously

described epitope (Sheldon *et al*, 2000) and tested for specificity by Western blot was used for the ChIP-qPCR experiments. ChIP was performed as described by Gendrel *et al*. (Gendrel *et al*, 2002). For ChIP-qPCR, 3 independent biological replicates were performed and samples were purified using the PCR clean-up Gel purification kit (Macherey-Nagel) and eluted in 20 µl water. Samples were diluted 1:10 and 3 µl were used for qPCR with the SYBR green master mix (Bio-Rad) and primers listed in Table A8. qPCR was performed in three technical replicates for each biological replicate. Data are represented as normalized fold change of IP divided by Input ($2^{(-IP/Input)}$) where Wt was set to 1. Significance of the difference between genotypes was defined as $p \geq 0.05$ after Student‘s t-test. ChIP-seq was performed by J. Mateos and R. Richter as described by Mateos et al. (Mateos *et al*, 2015). 2 and 3 independent biological replicates were performed for *A. thaliana* and *A. alpina*, respectively.

ChIP-seq data were analyzed by P. Madrigal. Low quality (Phred quality score ≥ 13 which is probability of the base called to be incorrect ≤ 0.05, in at least 90% of the bases called) and duplicated Illumina sequence reads were filtered out using Parallel-QC v1.0 (Zhou *et al*, 2013b). Reads were then mapped to the reference assembly V3 of *A. alpina* Pajares (Willing *et al*, 2015) and *A. thaliana* tair 10, respectively using Bowtie v2.0.2 under default parameters (Langmead *et al*, 2009). The software PeakRanger (Feng *et al*, 2011) was used to identify read-enriched regions in the genomes (tools ‘ranger’ and ‘wig’ were used with P value < $1e^{-6}$, q-value (FDR) < 0,01, rest of parameters default). The reads were extended to an average fragment size of 300 bp and MULTOVL v1.2 (with parameters ‘-L 1 -u -m 2 -M 0 -s multovl -f BED’) (Aszódi, 2012) resulted in the identification of 173 high confidence peaks present in at least two replicates of *A. alpina* and 377 high confident peaks present in the two *A. thaliana* replicates. Finally, we filtered by peak length only keeping peaks shorter than 1500 bp and we obtained final sets of 156 PEP1 peaks in *A. alpina* and 297 FLC peaks in *A. thaliana*, respectively. These peaks were annotated to the respective genomes using the Bioconductor package CSAR (Muiño *et al*, 2011). Target genes were defined as containing a peak region in a distance spanning 3 kb upstream from the start of the gene to 1 kb downstream from the end of the gene for *A. thaliana* and 5 kb upstream from the start of the gene to 3 kb downstream from the end of the gene for *A. alpina*, respectively. The IGV software was used for visualization of peaks and target genes (Thorvaldsdóttir *et al*, 2013).

Analysis of gene expression

For gene expression analysis samples were collected at ZT8. For apical samples (hereafter called ‘apices’), apex enriched tissue of 16 plants was combined. For leaf samples, leaves of 5

plants were pooled in case of *A. thaliana* and juvenile *A. alpina* plants, and the tip of the 10th true leaf was collected from 5 plants in case of adult *A. alpina* plants. RNA was extracted using the RNAeasy Plant Mini Kit (Qiagen) and samples were treated with RNAse-free DNAse (Ambion) according to the manufacturers' instructions. 5µg RNA were used for cDNA synthesis using the SuperscriptII DNA polymerase (Invitrogen). 3 µl of cDNA (diluted 1:10) were used for qPCR with my-budget Taq-DNA-Polymerase, Primers listed in Table A8 and EvaGreen Dye (Biotium) for detection in 10 µl reaction volume. qPCR was performed in three technical replicates for each biological replicate in a CFX384 Touch Real-Time PCR System (Biorad) using the *PP2A* as house-keeping gene. For expression analysis at single time points, mean and SEM of 4 independent biological replicates were calculated. For time course experiments, at least 2 biological replicates were performed and mean and SEM were calculated for normalized data (for details on individual experiments see figure legends).

Genome-wide expression data for *A. thaliana* were obtained from Mateos *et al*, 2015 (Mateos *et al*, 2015). Genes regulated by FLC were identified as genes differentially expressed between the tilling arrays performed in wild-type vs. *flc-3* mutant grown for 2 weeks under SD conditions followed by 2 LDs. Genome-wide expression data for *A. alpina* were generated by RNA-seq (analyzed data kindly provided by J. Mateos). RNA for RNA-seq was prepared in three independent biological replicates as described above. 4 µl of DNAse-treated RNA were used for library preparation using TruSeq RNA Sample Preparation (Illumina). After gel purification of 200-350 bp fragments, pair-end sequencing (read length 100 bp) was performed at the Cologne Center for Genomics, University of Cologne. Reads were mapped to the *A. alpina* reference assembly V3 (Willing *et al*, 2015) using TopHat with the parameters --min-anchor-length 10; --max-multihits 5; library-type fr-unstranded (Trapnell *et al*, 2009). High reproducibility between replicates was confirmed by principal component analysis that showed separation of tissues and genotypes by principal component 1 and 2 respectively (J. Mateos, data not shown). DEGs were defined as genes with adjusted p-value < 0.05 and $\log_2$ (fold change) > 1.5 using the R/Bioconductor package DESeq2 1.10.0 (Love *et al*, 2014). A binomial test was performed to test for significance of overlap between species.

Identification of enriched *cis*-elements

De novo motif enrichment analysis in the BSs was performed using MEME (Bailey & Elkan, 1994) with the 'zoops' (motif occurrence zero or one per sequence) or 'anr' (any number of motif occurrences per sequence) model. Parameters were set to identify the 10 most significantly enriched motifs of a length between 5 and 20 nucleotides. Background models for

A. thaliana and *A. alpina* were generated using the fasta-get-markov program which estimates a Markov model from a FASTA file. In addition, DREME (Bailey, 2011) with the standard settings was used to screen for shorter motifs. CArG-boxes shown here were identified by MEME with the 'zoops' model and the G-box was identified by DREME. In addition, BSs were screened for the G-box (CACRYG) and the TGGGCC motif to identify the position of the motif relative to the center of the BSs. Histograms were created using a bin size of 25 bp distance between the center of the BSs and the closest motif.

Identification of orthologous sequences in other species

BSs that were bound in *A. alpina* and *A. thaliana* (conserved BSs) were identified using BLAST. *A. alpina* BSs were aligned against *A. thaliana* BSs with BLAST (Word size for wordfinder algorithm 11, Penalty for a nucleotide mismatch -3, Penalty for a nucleotide match 2, Cost to open a gap 5, Cost to extend a gap 2, Discontiguous MegaBLAST template length 18, window size 40) (Data were kindly provided by J. Mateos). To test if BSs identified by the BLAST search are bound in regions with conserved synteny and to identify orthologous sequences of BSs in other species with varying evolutionary distances (Koch *et al*, 2001; Clauss & Koch, 2006; Couvreur *et al*, 2010; Hu *et al*, 2011; Karl *et al*, 2012; Willing *et al*, 2015), BSs were aligned to orthologous regions. Therefore, orthologs of genes associated with the BSs were identified by reciprocal BLAST and these genes, including 5 kb upstream of the transcriptional start site and 3 kb downstream of the transcriptional end site were extracted as orthologous loci. Finally, the best alignment of the BS in the orthologous locus was obtained by local Smith and Waterman alignments (Smith & Waterman, 1981) and percent identity between BS and orthologous sequence was calculated. Reciprocal BLAST and Smith Waterman alignments were performed by E. Severing.

Genome assemblies and annotation files of *A. lyrata* (A. lyrata v1.0) (Hu *et al*, 2011) were downloaded from Phytozome v11.0. Assemblies and annotations of the genomes of *A. arabicum* (Haudry *et al*, 2013) and *T. hassleriana* (Cheng *et al*, 2013), were downloaded from https://genomevolution.org/coge/. The *A. montbretiana* genome assembly and annotation was kindly provided by Wen-Biao Jiao and Korbinian Schneeberger prior to publication (personal communication).

All BSs that had at least one associated gene were included in the analysis (282 out of 297 for *A. thaliana* and 137 out of 156 for *A. alpina*). For the 282 *A. thaliana* FLC BSs with associated genes, orthologs were identified for 264 in *A. alpina*, 268 in *A. lyrata*, 251 in *A. montbretiana*, 227 in *A. arabicum* and 216 in *T. hassleriana* and SW alignments could be generated. Average

percent identity of the identified sequences with the BSs was 65.3 % for *A. alpina*, 79.3 % for *A. lyrata*, 66.6 % for *A. montbretiana*, 59.2 % for *A. arabicum* and 56.7 % for *T. hassleriana*. For the 137 *A. alpina* PEP1 BSs with associated genes, orthologs were identified for 124 in *A. thaliana*, 93 in *A. lyrata*, 126 in *A. montbretiana*, 110 in *A. arabicum* and 109 in *T. hassleriana* and SW alignments could be generated. Average percent identity of the identified sequences with the BSs was 67.3 % for *A. thaliana*, 65.5 % for *A. lyrata*, 86.7 % for *A. montbretiana*, 60 % for *A. arabicum* and 58.1 % for *T. hassleriana*.

Permutation test for enriched *cis*-elements

To test significance of enrichment of *de novo* identified motifs or candidate motifs in different subsets of BSs, a permutation test was performed. Therefore, the number of BSs that contain at least one motif was counted in the subset of interest. For the CArG-box variation was allowed and it was searched for MYHWAWWWRGWWW. For the G-box (CACGTG) and TGGGCC-motif no sequence variation was allowed. For the permutation test, 1000 samples of random genomic sequences of the same size as the subset of BSs of interest were generated. For these samples, mean number of sequences that contain at least one motif as well as standard deviation was calculated. Finally the Z-score, representing the number of standard deviations by which the number of BSs with motif in the subset of interest differs from the mean of random sequences was calculated. Z-scores above 3 were considered significant.

Permutation tests were also performed to test for significant enrichment of the 'TTT' trinucleotide at position 1-3 of CArG-boxes. Therefore, the number of $TTTN_{16}$ in the subsets of CArG-boxes that were identified by MEME was counted. In addition, 1000 random samples of the same size as the subset of interest were created out of all CArG-boxes identified in the all BSs of each species. Z-scores were calculated as described in the previous paragraph.

Alignments and visualization of synteny

For visualization of conserved synteny, orthologous sequences that were identified as described above, were aligned with mvista (Frazer *et al*, 2004) and GATA 1.0 (Nix & Eisen, 2005) using standard settings.

Identification of transposable elements within BSs

TE annotations were kindly provided by M. Piednoel. Therefore, assembled genomes were screened for any repeated sequences and the results clustered into repeat families. For each family, a consensus sequence was created. This consensus was compared to known TE reference sequences to annotate TEs. Consensus sequences with low similarity to reference sequences were termed 'confused'. Overlap between TEs and different subsets of BSs were

identified using the BEDtools intersect v2.16.2 function (Quinlan & Hall, 2010). The fraction of base pairs (bps) that correspond to a TE was calculated by dividing the number of bps that correspond to a TE by the number of bps that do not correspond to a TE. The enrichment ratio represents the fraction of bps of the BSs that correspond to a TE divided by the fraction of bps of the rest of the genome that correspond to a TE, and an enrichment ratio > 1 represents an enrichment of TEs in the BSs compared to the genomic background. A χ^2-test was performed to test for significance of enrichment. P-values > 0.05 were considered significant.

Analysis of overlapping binding sites of different TFs

Genome-wide BSs of *AP1*, *AP3*, *PI*, *AG*, *SOC1* and *SVP* were publically available as supplementary files (Kaufmann *et al*, 2010b; Deng *et al*, 2011; Tao *et al*, 2012; Wuest *et al*, 2012; ÓMaoiléidigh *et al*, 2013). *FLM* BSs (Posé *et al*, 2013b) were downloaded from GEO (GSE48082) and peaks with FDR <0.1 were selected. *SEP3* BSs and *A. lyrata SEP3* BSs in the *A. thaliana* genome (Muiño *et al*, 2016) were defined as 100 bp up- and downstream of the position with the maximum ChIP-seq score using peaks with FDR <0.01. Overlap of these BSs with the 200 central bps of *FLC* BSs or *PEP1* BSs in *A. thaliana* were identified using the BEDtools intersect v2.16.2 (Quinlan & Hall, 2010).

Functional category enrichment analysis

Significantly enriched GO categories among PEP1 and FLC direct and indirect target genes were identified using the BioMaps algorithm of the Virtual Plant software (Katari *et al*, 2010). Categories were considered as significantly enriched in a data set if P-value < 0.5. For selected categories that were significantly enriched in at least one of the data sets, the representation factor (RF) was calculated. RF is the observed frequency of genes in the GO-term category within the subset of target genes divided by the expected frequency based on the genomic background. RF >1 represents higher frequency than expected and RF >1 represents lower frequency than expected. Based on these results, two additional categories were created to specifically test enrichment of flowering time genes and genes encoding GA metabolic enzymes. The category "flowering time" includes all genes described at the website of the Coupland lab (http://www.mpipz.mpg.de/14637/Arabidopsis_flowering_genes). The category "GA metabolism" includes all genes encoding enzymes that function in GA biosynthesis or degradation pathway. The list of COR genes used to identify cold-related genes among PEP1 and FLC targets was the robust list defined by Park *et al*, 2015 which combined different experiments and growth conditions and contained 1279 COR genes (Park *et al*, 2015). Analysis of *A. alpina* PEP1 target genes was performed with *A. thaliana* orthologs.

Application of exogenous Gibberellins and Paclobutrazol

A. alpina plants were sprayed weekly with 20 µM GA4 (Sigma Aldrich, stock solution: 100 mM in EtOH, 0.1 % silwet L-77 Loveland industries) or mock (0.1 % EtOH, 0.1 % silwet). The GA biosynthesis inhibitor paclobutrazol (Sigma Aldrich) was dissolved in DMSO (stock concentration 100 mg/ml) and *A. alpina* plants were sprayed weekly with 1 mg/ml PAC, 0.1 % silwet or 1 % DMSO, 0.1 % silwet as mock treatment. *A. thaliana* plants were sprayed twice per week with the same solutions with the exception that the GA4 concentration applied on *A. thaliana* plants was 10 µM. Unless indicated otherwise, treatment was started after germination and continued throughout the experiment. For the GA-sensitivity test in *A. alpina,* plants were weekly sprayed with PAC as described above, to inhibit biosynthesis of endogenous GA. In addition, different concentrations of GA3 (Sigma Aldrich) were directly added to the soil with water once per week. Therefore, 200 µl of GA3 solution (different concentrations dissolved in EtOH) were added to 1 L of water to yield final concentrations of 0; 0.01; 0.1; 1 and 10 µM GA3. The experiment was performed in two biological replicates. Data of both replicates were combined to yield a total n of at least 23.

Quantification of Gibberellins

Plants were grown in LDs for 5.5w, then vernalized for 12 w and finally transferred back to LDs. Between 100 and 200 mg of fresh weight were harvested in liquid nitrogen per sample. Samples were harvested at ZT8 in three biological replicates and GAs were quantified at IBMCP, Valencia by Isabel Lopez Diaz as described by Seo *et. al* (Seo *et al*, 2011). GAs were purified from frozen material by extraction with 80 % methanol, 1 % acetic acid and successive passing through HLB (reverse phase), MCX (cationic exchange) and WAX (ionic exchange) columns (Oasis 30 mg, Waters). GAs were then dissolved in 5 % acetonitrile, 1 % acetic acid and separated by reverse phase UHPL chromatography (2.6 µm Accucore RP-MS column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific). Electrospray ionization (negative mode, spray voltage 3.0 kV, heater temperature 150ºC, sheath gas flow rate 40 µL/min, auxiliary gas flow rate 10 µL/min) and targeted-SIM (capillary temperature 300ºC, S-lens RF level 70, resolution 70000) using a Q-Exactive spectrometer (Orbitrap detector; ThermoFisher Scientific) was performed to analyze GAs. For quantification of GAs, [17,17-2H] GAs were added to the extracts as internal standards and concentrations of GAs in the extracts were determined using embedded calibration curves and the Xcalibur program 2.2 SP1 build 48. Data are represented as mean of three biological replicates +/- Stdev. Analysis of variance was performed using the SigmaStat 3.5 software.

6 References

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K & Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* **309:** 1052–1056

Achard P, Gong F, Cheminant S, Alioua M, Hedden P & Genschik P (2008) The Cold-Inducible CBF1 Factor–Dependent Signaling Pathway Modulates the Accumulation of the Growth-Repressing DELLA Proteins via Its Effect on Gibberellin Metabolism. *Plant Cell* **20:** 2117–2129

Achard P, Herr A, Baulcombe DC & Harberd NP (2004) Modulation of floral development by a gibberellin-regulated microRNA. *Development* **131:** 3357–3365

Aguilar-Martínez JA, Poza-Carrión C & Cubas P (2007) Arabidopsis BRANCHED1 Acts as an Integrator of Branching Signals within Axillary Buds. *Plant Cell* **19:** 458–472

Aikawa S, Kobayashi MJ, Satake A, Shimizu KK & Kudoh H (2010) Robust control of the seasonal expression of the Arabidopsis FLC gene in a fluctuating environment. *PNAS* **107:** 11632–11637

Albani MC, Castaings L, Wotzel S, Mateos JL, Wunder J, Wang R, Reymond M & Coupland G (2012) PEP1 of Arabis alpina Is Encoded by Two Overlapping Genes That Contribute to Natural Genetic Variation in Perennial Flowering. *PLoS Genet* **8:** e1003130

Albani MC & Coupland G (2010) Chapter Eleven - Comparative Analysis of Flowering in Annual and Perennial Plants. In *Current Topics in Developmental Biology*, Marja C.P. Timmermans (ed) pp 323–348.

Alonso-Blanco C, Andrade J, Becker C, Bemm F, Bergelson J, Borgwardt KM, Cao J, Chae E, Dezwaan TM, Ding W, Ecker JR, Exposito-Alonso M, Farlow A, Fitz J, Gan X, Grimm DG, Hancock AM, Henz SR, Holm S, Horton M, et al (2016) 1,135 Genomes Reveal the Global Pattern of Polymorphism in Arabidopsis thaliana. *Cell* **166:** 481–491

Alpert P (2006) Constraints of tolerance: why are desiccation-tolerant organisms so small or rare? *Journal of Experimental Biology* **209:** 1575–1584

Andrés F & Coupland G (2012) The genetic basis of flowering responses to seasonal cues. *Nat. Rev. Genet.* **13:** 627–639

Andrés F, Porri A, Torti S, Mateos J, Romera-Branchat M, García-Martínez JL, Fornara F, Gregis V, Kater MM & Coupland G (2014) SHORT VEGETATIVE PHASE reduces gibberellin biosynthesis at the Arabidopsis shoot apex to regulate the floral transition. *Proc. Natl. Acad. Sci. U.S.A.* **111:** E2760-2769

Aszódi A (2012) MULTOVL: fast multiple overlaps of genomic regions. *Bioinformatics* **28:** 3318–3319

Atkin OK, Loveys BR, Atkinson LJ & Pons TL (2006) Phenotypic plasticity and growth temperature: understanding interspecific variability. *J. Exp. Bot.* **57:** 267–281

Baduel P, Arnold B, Weisman CM, Hunter B & Bomblies K (2016) Habitat-Associated Life History and Stress-Tolerance Variation in Arabidopsis arenosa1[OPEN]. *Plant Physiol* **171:** 437–451

Bailey TL (2011) DREME: motif discovery in transcription factor ChIP-seq data. *Bioinformatics* **27:** 1653–1659

Bailey TL & Elkan C (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* **2:** 28–36

Ballester B, Medina-Rivera A, Schmidt D, Gonzàlez-Porta M, Carlucci M, Chen X, Chessman K, Faure AJ, Funnell AP, Goncalves A, Kutter C, Lukk M, Menon S, McLaren WM, Stefflova K, Watt S, Weirauch MT, Crossley M, Marioni JC, Odom DT, et al (2014) Multi-species, multi-transcription factor binding highlights conserved control of tissue-specific biological pathways. *eLife* **3:** e02626. DOI 10.7554/e.Life02626

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA & Dean C (2004) Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature* **427:** 164–167

Bennetzen JL, Ma J & Devos KM (2005) Mechanisms of recent genome size variation in flowering plants. *Ann. Bot.* **95:** 127–132

Bergonzi S, Albani MC, Themaat EVL van, Nordström KJV, Wang R, Schneeberger K, Moerland PD & Coupland G (2013) Mechanisms of Age-Dependent Response to Winter Temperature in Perennial Flowering of Arabis alpina. *Science* **340:** 1094–1097

Blázquez MA, Ahn JH & Weigel D (2003) A thermosensory pathway controlling flowering time in Arabidopsis thaliana. *Nat Genet* **33:** 168–171

Blázquez MA, Green R, Nilsson O, Sussman MR & Weigel D (1998) Gibberellins Promote Flowering of Arabidopsis by Activating the LEAFY Promoter. *Plant Cell* **10:** 791–800

Borneman AR, Gianoulis TA, Zhang ZD, Yu H, Rozowsky J, Seringhaus MR, Wang LY, Gerstein M & Snyder M (2007) Divergence of Transcription Factor Binding Sites Across Related Yeast Species. *Science* **317:** 815–819

Bradley RK, Li X-Y, Trapnell C, Davidson S, Pachter L, Chu HC, Tonkin LA, Biggin MD & Eisen MB (2010) Binding Site Turnover Produces Pervasive Quantitative Changes in Transcription Factor Binding between Closely Related Drosophila Species. *PLoS Biol* **8:** e1000343

Capovilla G, Schmid M & Posé D (2015) Control of flowering by ambient temperature. *J. Exp. Bot.* **66:** 59–69

Castillejo C & Pelaz S (2008) The balance between CONSTANS and TEMPRANILLO activities determines FT expression to trigger flowering. *Curr. Biol.* **18:** 1338–1343

Castillon A, Shen H & Huq E (2007) Phytochrome Interacting Factors: central players in phytochrome-mediated light signaling networks. *Trends Plant Sci.* **12:** 514–521

Chandler J, Martinez-Zapater JM & Dean C (2000) Mutations causing defects in the biosynthesis and response to gibberellins, abscisic acid and phytochrome B do not inhibit vernalization in Arabidopsis fca-1. *Planta* **210:** 677–682

Cheng H, Qin L, Lee S, Fu X, Richards DE, Cao D, Luo D, Harberd NP & Peng J (2004) Gibberellin regulates Arabidopsis floral development via suppression of DELLA protein function. *Development* **131:** 1055–1064

Cheng S, Bergh E van den, Zeng P, Zhong X, Xu J, Liu X, Hofberger J, Bruijn S de, Bhide AS, Kuelahoglu C, Bian C, Chen J, Fan G, Kaufmann K, Hall JC, Becker A, Bräutigam A, Weber APM, Shi C, Zheng Z, et al (2013) The Tarenaya hassleriana Genome Provides Insight into Reproductive Trait and Genome Evolution of Crucifers. *Plant Cell* **25:** 2813–2830

Chiang GCK, Barua D, Kramer EM, Amasino RM & Donohue K (2009) Major flowering time gene, FLOWERING LOCUS C, regulates seed germination in Arabidopsis thaliana. *PNAS* **106:** 11661–11666

Chiang HH, Hwang I & Goodman HM (1995) Isolation of the Arabidopsis GA4 locus. *Plant Cell* **7:** 195–201

Clauss MJ & Koch MA (2006) Poorly known relatives of Arabidopsis thaliana. *Trends in Plant Science* **11:** 449–459

Clough SJ & Bent AF (1998) Floral dip: a simplified method forAgrobacterium-mediated transformation ofArabidopsis thaliana. *The Plant Journal* **16:** 735–743

Conboy CM, Spyrou C, Thorne NP, Wade EJ, Barbosa-Morais NL, Wilson MD, Bhattacharjee A, Young RA, Tavaré S, Lees JA & Odom DT (2007) Cell Cycle Genes Are the Evolutionarily Conserved Targets of the E2F4 Transcription Factor. *PLoS ONE* **2:** e1061

Couvreur TLP, Franzke A, Al-Shehbaz IA, Bakker FT, Koch MA & Mummenhoff K (2010) Molecular Phylogenetics, Temporal Diversification, and Principles of Evolution in the Mustard Family (Brassicaceae). *Mol Biol Evol* **27:** 55–71

Degner JF, Pai AA, Pique-Regi R, Veyrieras J-B, Gaffney DJ, Pickrell JK, De Leon S, Michelini K, Lewellen N, Crawford GE, Stephens M, Gilad Y & Pritchard JK (2012) DNase I sensitivity QTLs are a major determinant of human expression variation. *Nature* **482:** 390–394

Dehal P & Boore JL (2005) Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol.* **3:** e314

Denas O, Sandstrom R, Cheng Y, Beal K, Herrero J, Hardison RC & Taylor J (2015) Genome-wide comparative analysis reveals human-mouse regulatory landscape and evolution. *BMC Genomics* **16:** 87. DOI 10.1186/s12864-015-1245-6

Deng W, Casao MC, Wang P, Sato K, Hayes PM, Finnegan EJ & Trevaskis B (2015) Direct links between the vernalization response and other key traits of cereal crops. *Nat Commun* **6:** 5882

Deng W, Ying H, Helliwell CA, Taylor JM, Peacock WJ & Dennis ES (2011) FLOWERING LOCUS C (FLC) regulates development pathways throughout the life cycle of Arabidopsis. *PNAS* **108:** 6680–6685

Deplancke B, Alpern D & Gardeux V (2016) The Genetics of Transcription Factor DNA Binding Variation. *Cell* **166:** 538–554

Di Stilio VS, Kramer EM & Baum DA (2005) Floral MADS box genes and homeotic gender dimorphism in Thalictrum dioicum (Ranunculaceae) - a new model for the study of dioecy. *Plant J.* **41:** 755–766

Domcke S, Bardet AF, Adrian Ginno P, Hartl D, Burger L & Schübeler D (2015) Competition between DNA methylation and transcription factors determines binding of NRF1. *Nature* **528:** 575–579

Edwards KD, Anderson PE, Hall A, Salathia NS, Locke JCW, Lynn JR, Straume M, Smith JQ & Millar AJ (2006) FLOWERING LOCUS C Mediates Natural Variation in the High-Temperature Response of the Arabidopsis Circadian Clock. *Plant Cell* **18:** 639–650

Egea-Cortines M, Saedler H & Sommer H (1999) Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. *EMBO J.* **18:** 5370–5379

Eriksson S, Böhlenius H, Moritz T & Nilsson O (2006) GA4 is the active gibberellin in the regulation of LEAFY transcription and Arabidopsis floral initiation. *Plant Cell* **18:** 2172–2181

Feng S, Martinez C, Gusmaroli G, Wang Y, Zhou J, Wang F, Chen L, Yu L, Iglesias-Pedraz JM, Kircher S, Schäfer E, Fu X, Fan L-M & Deng XW (2008) Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. *Nature* **451:** 475–479

Feng X, Grossman R & Stein L (2011) PeakRanger: a cloud-enabled peak caller for ChIP-seq data. *BMC Bioinformatics* **12:** 139

de Folter S & Angenent GC (2006) trans meets cis in MADS science. *Trends Plant Sci.* **11:** 224–231

Folter S de, Immink RGH, Kieffer M, Pařenicová L, Henz SR, Weigel D, Busscher M, Kooiker M, Colombo L, Kater MM, Davies B & Angenent GC (2005) Comprehensive Interaction Map of the Arabidopsis MADS Box Transcription Factors. *Plant Cell* **17:** 1424–1433

Fowler DB, Chauvin LP, Limin AE & Sarhan F (1996) The regulatory role of vernalization in the expression of low-temperature-induced genes in wheat and rye. *Theoret. Appl. Genetics* **93:** 554–559

Fowler SG, Cook D & Thomashow MF (2005) Low Temperature Induction of Arabidopsis CBF1, 2, and 3 Is Gated by the Circadian Clock. *Plant Physiol.* **137:** 961–968

Frazer KA, Pachter L, Poliakov A, Rubin EM & Dubchak I (2004) VISTA: computational tools for comparative genomics. *Nucl. Acids Res.* **32:** W273–W279

Fujiwara S, Oda A, Yoshida R, Niinuma K, Miyata K, Tomozoe Y, Tajima T, Nakagawa M, Hayashi K, Coupland G & Mizoguchi T (2008) Circadian clock proteins LHY and CCA1 regulate SVP protein accumulation to control flowering in Arabidopsis. *Plant Cell* **20:** 2960–2971

Galvão VC, Collani S, Horrer D & Schmid M (2015) Gibberellic acid signaling is required for ambient temperature-mediated induction of flowering in *Arabidopsis thaliana*. *The Plant Journal* **84:** 949–962

Galvão VC, Horrer D, Küttner F & Schmid M (2012) Spatial control of flowering by DELLA proteins in Arabidopsis thaliana. *Development* **139:** 4072–4082

Gendrel A-V, Lippman Z, Yordan C, Colot V & Martienssen RA (2002) Dependence of heterochromatic histone H3 methylation patterns on the Arabidopsis gene DDM1. *Science* **297:** 1871–1873

Geraldo N, Bäurle I, Kidou S, Hu X & Dean C (2009) FRIGIDA Delays Flowering in Arabidopsis via a Cotranscriptional Mechanism Involving Direct Interaction with the Nuclear Cap-Binding Complex. *Plant Physiol.* **150:** 1611–1618

Gocal GF, Sheldon CC, Gubler F, Moritz T, Bagnall DJ, MacMillan CP, Li SF, Parish RW, Dennis ES, Weigel D & King RW (2001) GAMYB-like genes, flowering, and gibberellin signaling in Arabidopsis. *Plant Physiol.* **127:** 1682–1693

González J & Petrov DA (2012) Evolution of genome content: population dynamics of transposable elements in flies and humans. *Methods Mol. Biol.* **855:** 361–383

Gregis V, Andres F, Sessa A, Guerra RF, Simonini S, Mateos JL, Torti S, Zambelli F, Prazzoli GM, Bjerkan KN, Grini PE, Pavesi G, Colombo L, Coupland G & Kater MM (2013) Identification of pathways directly regulated by SHORT VEGETATIVE PHASE during vegetative and reproductive development in Arabidopsis. *Genome Biol* **14:** R56

Griffiths J, Murase K, Rieu I, Zentella R, Zhang Z-L, Powers SJ, Gong F, Phillips AL, Hedden P, Sun T & Thomas SG (2006) Genetic Characterization and Functional Analysis of the GID1 Gibberellin Receptors in Arabidopsis. *Plant Cell* **18:** 3399–3414

Hartmann U, Höhmann S, Nettesheim K, Wisman E, Saedler H & Huijser P (2000) Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. *The Plant Journal* **21:** 351–360

Haudry A, Platts AE, Vello E, Hoen DR, Leclercq M, Williamson RJ, Forczek E, Joly-Lopez Z, Steffen JG, Hazzouri KM, Dewar K, Stinchcombe JR, Schoen DJ, Wang X, Schmutz J, Town CD, Edger PP, Pires JC, Schumaker KS, Jarvis DE, et al (2013) An atlas of over 90,000 conserved noncoding sequences provides insight into crucifer regulatory regions. *Nat Genet* **45:** 891–898

Hawkins JS, Proulx SR, Rapp RA & Wendel JF (2009) Rapid DNA loss as a counterbalance to genome expansion through retrotransposon proliferation in plants. *Proc. Natl. Acad. Sci. U.S.A.* **106:** 17811–17816

Hay A, Kaur H, Phillips A, Hedden P, Hake S & Tsiantis M (2002) The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. *Curr. Biol.* **12:** 1557–1565

Hazebroek JP & Metzger JD (1990) Thermoinductive Regulation of Gibberellin Metabolism in Thlaspi arvense L. I. Metabolism of [2H]-ent-Kaurenoic Acid and [14C]Gibberellin A12-Aldehyde. *Plant Physiol.* **94:** 157–165

Hazebroek JP, Metzger JD & Mansager ER (1993) Thermoinductive Regulation of Gibberellin Metabolism in Thlaspi arvense L. (II. Cold Induction of Enzymes in Gibberellin Biosynthesis). *Plant Physiol.* **102:** 547–552

He F, Zhang X, Hu J, Turck F, Dong X, Goebel U, Borevitz J & de Meaux J (2012) Genome-wide analysis of cis-regulatory divergence between species in the Arabidopsis genus. *Mol. Biol. Evol.* **29:** 3385–3395

He Q, Bardet AF, Patton B, Purvis J, Johnston J, Paulson A, Gogol M, Stark A & Zeitlinger J (2011) High conservation of transcription factor binding and evidence for combinatorial regulation across six Drosophila species. *Nat Genet* **43:** 414–420

Heinz S, Romanoski CE, Benner C, Allison KA, Kaikkonen MU, Orozco LD & Glass CK (2013) Effect of natural genetic variation on enhancer selection and function. *Nature* **503:** 487–492

Helliwell CA, Sheldon CC, Olive MR, Walker AR, Zeevaart JA, Peacock WJ & Dennis ES (1998) Cloning of the Arabidopsis ent-kaurene oxidase gene GA3. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 9019–9024

Helliwell CA, Wood CC, Robertson M, Peacock J, W & Dennis ES (2006) The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. *The Plant Journal* **46:** 183–192

Hepworth SR, Valverde F, Ravenscroft D, Mouradov A & Coupland G (2002) Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. *EMBO J.* **21:** 4327–4337

Herms DA & Mattson WJ (1992) The Dilemma of Plants: To Grow or Defend. *The Quarterly Review of Biology* **67:** 283–335

Heyndrickx KS, Velde JV de, Wang C, Weigel D & Vandepoele K (2014) A Functional and Evolutionary Perspective on Transcription Factor Binding in Arabidopsis thaliana. *Plant Cell***:** tpc.114.130591

Hisamatsu T & King RW (2008) The nature of floral signals in Arabidopsis. II. Roles for FLOWERING LOCUS T (FT) and gibberellin. *J. Exp. Bot.* **59:** 3821–3829

Hisamatsu T, Koshioka M & Mander LN (2004) Regulation of gibberellin biosynthesis and stem elongation by low temperature in Eustoma grandiflorum. *The Journal of Horticultural Science and Biotechnology* **79:** 354–359

Honma T & Goto K (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* **409:** 525–529

Horton MW, Willems G, Sasaki E, Koornneef M & Nordborg M (2016) The genetic architecture of freezing tolerance varies across the range of Arabidopsis thaliana. *Plant Cell Environ.*

Hu TT, Pattyn P, Bakker EG, Cao J, Cheng J-F, Clark RM, Fahlgren N, Fawcett JA, Grimwood J, Gundlach H, Haberer G, Hollister JD, Ossowski S, Ottilar RP, Salamov AA, Schneeberger K, Spannagl M, Wang X, Yang L, Nasrallah ME, et al (2011) The Arabidopsis lyrata genome sequence and the basis of rapid genome size change. *Nat. Genet.* **43:** 476–481

Huang X, Ding J, Effgen S, Turck F & Koornneef M (2013) Multiple loci and genetic interactions involving flowering time genes regulate stem branching among natural variants of Arabidopsis. *New Phytol* **199:** 843–857

Huijser P & Schmid M (2011) The control of developmental phase transitions in plants. *Development* **138:** 4117–4129

Hyun Y, Richter R & Coupland G (2017) Competence to Flower: Age-Controlled Sensitivity to Environmental Cues. *Plant Physiol.* **173:** 36–46

Hyun Y, Richter R, Vincent C, Martinez-Gallegos R, Porri A & Coupland G (2016) Multi-layered Regulation of SPL15 and Cooperation with SOC1 Integrate Endogenous Flowering Pathways at the Arabidopsis Shoot Meristem. *Developmental Cell* **37:** 254–266

Iglesias-Fernández R, Barrero-Sicilia C, Carrillo-Barral N, Oñate-Sánchez L & Carbonero P (2013) Arabidopsis thaliana bZIP44: a transcription factor affecting seed germination and expression of the mannanase-encoding gene AtMAN7. *Plant J.* **74:** 767–780

Immink RGH, Posé D, Ferrario S, Ott F, Kaufmann K, Valentim FL, Folter S de, Wal F van der, Dijk ADJ van, Schmid M & Angenent GC (2012) Characterization of SOC1's Central Role in Flowering by the Identification of Its Upstream and Downstream Regulators. *Plant Physiol.* **160:** 433–449

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P & Tsiantis M (2005) KNOX Action in Arabidopsis Is Mediated by Coordinate Regulation of Cytokinin and Gibberellin Activities. *Current Biology* **15:** 1560–1565

Johanson U, West J, Lister C, Michaels S, Amasino R & Dean C (2000) Molecular Analysis of FRIGIDA, a Major Determinant of Natural Variation in Arabidopsis Flowering Time. *Science* **290:** 344–347

Jordan ET, Hatfield PM, Hondred D, Talon M, Zeevaart JA & Vierstra RD (1995) Phytochrome A overexpression in transgenic tobacco. Correlation of dwarf phenotype with high concentrations of phytochrome in vascular tissue and attenuated gibberellin levels. *Plant Physiol.* **107:** 797–805

Jung J-H, Park J-H, Lee S, To TK, Kim J-M, Seki M & Park C-M (2013) The cold signaling attenuator HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 activates FLOWERING

LOCUS C transcription via chromatin remodeling under short-term cold stress in Arabidopsis. *Plant Cell* **25:** 4378–4390

Jung J-H, Seo PJ & Park C-M (2012) The E3 ubiquitin ligase HOS1 regulates Arabidopsis flowering by mediating CONSTANS degradation under cold stress. *J. Biol. Chem.* **287:** 43277–43287

Kanno A, Saeki H, Kameya T, Saedler H & Theissen G (2003) Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (Tulipa gesneriana). *Plant Mol. Biol.* **52:** 831–841

Karl R, Kiefer C, Ansell SW & Koch MA (2012) Systematics and evolution of arctic-alpine Arabis alpina (Brassicaceae) and its closest relatives in the eastern Mediterranean. *Am. J. Bot.* **99:** 778–794

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K & Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.* **17:** 287–291

Katari MS, Nowicki SD, Aceituno FF, Nero D, Kelfer J, Thompson LP, Cabello JM, Davidson RS, Goldberg AP, Shasha DE, Coruzzi GM & Gutiérrez RA (2010) VirtualPlant: a software platform to support systems biology research. *Plant Physiol.* **152:** 500–515

Kaufman PB, Ghosheh NS, Nakosteen L, Pharis RP, Durley RC & Morf W (1976) Analysis of Native Gibberellins in the Internode, Nodes, Leaves, and Inflorescence of Developing Avena Plants. *Plant Physiol.* **58:** 131–134

Kaufmann K, Muiño JM, Jauregui R, Airoldi CA, Smaczniak C, Krajewski P & Angenent GC (2009) Target Genes of the MADS Transcription Factor SEPALLATA3: Integration of Developmental and Hormonal Pathways in the Arabidopsis Flower. *PLoS Biol* **7:** e1000090

Kaufmann K, Pajoro A & Angenent GC (2010a) Regulation of transcription in plants: mechanisms controlling developmental switches. *Nat Rev Genet* **11:** 830–842

Kaufmann K, Wellmer F, Muiño JM, Ferrier T, Wuest SE, Kumar V, Serrano-Mislata A, Madueño F, Krajewski P, Meyerowitz EM, Angenent GC & Riechmann JL (2010b) Orchestration of Floral Initiation by APETALA1. *Science* **328:** 85–89

Kemi U, Niittyvuopio A, Toivainen T, Pasanen A, Quilot-Turion B, Holm K, Lagercrantz U, Savolainen O & Kuittinen H (2013) Role of vernalization and of duplicated FLOWERING LOCUS C in the perennial Arabidopsis lyrata. *New Phytologist* **197:** 323–335

Kim D-H, Doyle MR, Sung S & Amasino RM (2009) Vernalization: winter and the timing of flowering in plants. *Annu. Rev. Cell Dev. Biol.* **25:** 277–299

Kim H-J, Hyun Y, Park J-Y, Park M-J, Park M-K, Kim MD, Kim H-J, Lee MH, Moon J, Lee I & Kim J (2004) A genetic link between cold responses and flowering time through FVE in Arabidopsis thaliana. *Nature Genetics* **36:** 167–171

Kim S-Y, Park B-S, Kwon S-J, Kim J, Lim M-H, Park Y-D, Kim DY, Suh S-C, Jin Y-M, Ahn JH & Lee Y-H (2007) Delayed flowering time in Arabidopsis and Brassica rapa by the overexpression of FLOWERING LOCUS C (FLC) homologs isolated from Chinese cabbage (Brassica rapa L.: ssp. pekinensis). *Plant Cell Rep.* **26:** 327–336

King MC & Wilson AC (1975) Evolution at two levels in humans and chimpanzees. *Science* **188:** 107–116

King RW, Moritz T, Evans LT, Junttila O & Herlt AJ (2001) Long-Day Induction of Flowering in Lolium temulentumInvolves Sequential Increases in Specific Gibberellins at the Shoot Apex. *Plant Physiol.* **127:** 624–632

Klock H & Lesley S (2009) The Polymerase Incomplete Primer Extension (PIPE) Method Applied to High-Throughput Cloning and Site-Directed Mutagenesis. In *High Throughput Protein Expression and Purification*, Doyle S (ed) pp 91–103. Humana Press DOI 10.1007/978-1-59745-196-3

Kobayashi M, Yamaguchi I, Murofushi N, Ota Y & Takahashi N (1988) Fluctuation and Localization of Endogenous Gibberellins in Rice. *Agricultural and Biological Chemistry* **52:** 1189–1194

Koch M, Haubold B & Mitchell-Olds T (2001) Molecular systematics of the Brassicaceae: evidence from coding plastidic matK and nuclear Chs sequences. *Am. J. Bot.* **88:** 534–544

Koornneef M, Alonso-Blanco C & Vreugdenhil D (2004) Naturally Occurring Genetic Variation in Arabidopsis Thaliana. *Annual Review of Plant Biology* **55:** 141–172

Koornneef M, Blankestijn-de Vries H, Hanhart C, Soppe W & Peeters T (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg erecta wild-type. *The Plant Journal* **6:** 911–919

Koornneef M & van der Veen JH (1980) Induction and analysis of gibberellin sensitive mutants in Arabidopsis thaliana (L.) heynh. *Theor. Appl. Genet.* **58:** 257–263

Kumar SV, Lucyshyn D, Jaeger KE, Alós E, Alvey E, Harberd NP & Wigge PA (2012) Transcription factor PIF4 controls the thermosensory activation of flowering. *Nature* **484:** 242–245

Kunarso G, Chia N-Y, Jeyakani J, Hwang C, Lu X, Chan Y-S, Ng H-H & Bourque G (2010) Transposable elements have rewired the core regulatory network of human embryonic stem cells. *Nat Genet* **42:** 631–634

Lang A (1957) THE EFFECT OF GIBBERELLIN UPON FLOWER FORMATION*. *Proc Natl Acad Sci U S A* **43:** 709–717

Lange T, Kappler J, Fischer A, Frisse A, Padeffke T, Schmidtke S & Lange MJP (2005) Gibberellin biosynthesis in developing pumpkin seedlings. *Plant Physiol.* **139:** 213–223

Langmead B, Trapnell C, Pop M & Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* **10:** R25

Lee I, Michaels SD, Masshardt AS & Amasino RM (1994) The late-flowering phenotype of FRIGIDA and mutations in LUMINIDEPENDENS is suppressed in the Landsberg erecta strain of Arabidopsis. *The Plant Journal* **6:** 903–909

Lee JH, Ryu H-S, Chung KS, Posé D, Kim S, Schmid M & Ahn JH (2013) Regulation of temperature-responsive flowering by MADS-box transcription factor repressors. *Science* **342:** 628–632

Lee JH, Yoo SJ, Park SH, Hwang I, Lee JS & Ahn JH (2007) Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. *Genes Dev.* **21:** 397–402

Lee S, Cheng H, King KE, Wang W, He Y, Hussain A, Lo J, Harberd NP & Peng J (2002) Gibberellin regulates Arabidopsis seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition. *Genes Dev.* **16:** 646–658

Leivar P & Monte E (2014) PIFs: Systems Integrators in Plant Development. *Plant Cell* **26:** 56–78

Li C, Wang Y, Xu L, Nie S, Chen Y, Liang D, Sun X, Karanja BK, Luo X & Liu L (2016) Genome-Wide Characterization of the MADS-Box Gene Family in Radish (Raphanus sativus L.) and Assessment of Its Roles in Flowering and Floral Organogenesis. *Front Plant Sci* **7:** 1390

Li D, Liu C, Shen L, Wu Y, Chen H, Robertson M, Helliwell CA, Ito T, Meyerowitz E & Yu H (2008) A Repressor Complex Governs the Integration of Flowering Signals in Arabidopsis. *Developmental Cell* **15:** 110–120

Lincoln C, Long J, Yamaguchi J, Serikawa K & Hake S (1994) A knotted1-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell* **6:** 1859–1876

Long JA, Moan EI, Medford JI & Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. *Nature* **379:** 66–69

Love MI, Huber W & Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15:** 550

de Lucas M, Davière J-M, Rodríguez-Falcón M, Pontin M, Iglesias-Pedraz JM, Lorrain S, Fankhauser C, Blázquez MA, Titarenko E & Prat S (2008) A molecular framework for light and gibberellin control of cell elongation. *Nature* **451:** 480–484

Lutz U, Posé D, Pfeifer M, Gundlach H, Hagmann J, Wang C, Weigel D, Mayer KFX, Schmid M & Schwechheimer C (2015) Modulation of Ambient Temperature-Dependent Flowering in Arabidopsis thaliana by Natural Variation of FLOWERING LOCUS M. *PLoS Genet.* **11:** e1005588

MacArthur S, Li X-Y, Li J, Brown JB, Chu HC, Zeng L, Grondona BP, Hechmer A, Simirenko L, Keränen SVE, Knowles DW, Stapleton M, Bickel P, Biggin MD & Eisen MB (2009) Developmental roles of 21 Drosophila transcription factors are determined by quantitative differences in binding to an overlapping set of thousands of genomic regions. *Genome Biol.* **10:** R80

Magome H, Yamaguchi S, Hanada A, Kamiya Y & Oda K (2004) dwarf and delayed-flowering 1, a novel Arabidopsis mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor. *Plant J.* **37:** 720–729

Magome H, Yamaguchi S, Hanada A, Kamiya Y & Oda K (2008) The DDF1 transcriptional activator upregulates expression of a gibberellin-deactivating gene, GA2ox7, under high-salinity stress in Arabidopsis. *The Plant Journal* **56:** 613–626

Markwell J, Osterman JC & Mitchell JL (1995) Calibration of the Minolta SPAD-502 leaf chlorophyll meter. *Photosyn. Res.* **46:** 467–472

Mateos JL, Madrigal P, Tsuda K, Rawat V, Richter R, Romera-Branchat M, Fornara F, Schneeberger K, Krajewski P & Coupland G (2015) Combinatorial activities of SHORT VEGETATIVE PHASE and FLOWERING LOCUS C define distinct modes of flowering regulation in Arabidopsis. *Genome Biol* **16:** 31 DOI 10.1186/513059-015-0597-1

Melzer R & Theissen G (2009) Reconstitution of 'floral quartets' in vitro involving class B and class E floral homeotic proteins. *Nucleic Acids Res.* **37:** 2723–2736

Menkens AE, Schindler U & Cashmore AR (1995) The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. *Trends Biochem. Sci.* **20:** 506–510

Metzger JD (1985) Role of Gibberellins in the Environmental Control of Stem Growth in Thlaspi arvense L. *Plant Physiol.* **78:** 8–13

Metzger JD (1990) Comparison of Biological Activities of Gibberellins and Gibberellin-Precursors Native to Thlaspi arvense L. *Plant Physiol.* **94:** 151–156

Michael TP (2014) Plant genome size variation: bloating and purging DNA. *Brief Funct Genomics* **13:** 308–317

Michaels SD & Amasino RM (1999) FLOWERING LOCUS C Encodes a Novel MADS Domain Protein That Acts as a Repressor of Flowering. *Plant Cell* **11:** 949–956

Michaels SD, He Y, Scortecci KC & Amasino RM (2003) Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. *PNAS* **100:** 10102–10107

Middleton AM, Úbeda-Tomás S, Griffiths J, Holman T, Hedden P, Thomas SG, Phillips AL, Holdsworth MJ, Bennett MJ, King JR & Owen MR (2012) Mathematical modeling elucidates the role of transcriptional feedback in gibberellin signaling. *Proc. Natl. Acad. Sci. U.S.A.* **109:** 7571–7576

Mino M, Oka M, Tasaka Y & Iwabuchi M (2003) Thermoinduction of genes encoding the enzymes of gibberellin biosynthesis and a putative negative regulator of gibberellin signal transduction in Eustoma grandiflorum. *Plant Cell Rep.* **22:** 159–165

Mitchum MG, Yamaguchi S, Hanada A, Kuwahara A, Yoshioka Y, Kato T, Tabata S, Kamiya Y & Sun T (2006) Distinct and overlapping roles of two gibberellin 3-oxidases in Arabidopsis development. *The Plant Journal* **45:** 804–818

Moon J, Suh S-S, Lee H, Choi K-R, Hong CB, Paek N-C, Kim S-G & Lee I (2003) The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. *The Plant Journal* **35:** 613–623

Muiño JM, Bruijn S de, Pajoro A, Geuten K, Vingron M, Angenent GC & Kaufmann K (2016) Evolution of DNA-Binding Sites of a Floral Master Regulatory Transcription Factor. *Mol Biol Evol* **33:** 185–200

Muiño JM, Kaufmann K, van Ham RC, Angenent GC & Krajewski P (2011) ChIP-seq Analysis in R (CSAR): An R package for the statistical detection of protein-bound genomic regions. *Plant Methods* **7:** 11

Muiño JM, Smaczniak C, Angenent GC, Kaufmann K & van Dijk ADJ (2014) Structural determinants of DNA recognition by plant MADS-domain transcription factors. *Nucleic Acids Res* **42:** 2138–2146

Mutasa-Göttgens E & Hedden P (2009) Gibberellin as a factor in floral regulatory networks. *J. Exp. Bot.* **60:** 1979–1989

Nakajima M, Shimada A, Takashi Y, Kim Y-C, Park S-H, Ueguchi-Tanaka M, Suzuki H, Katoh E, Iuchi S, Kobayashi M, Maeda T, Matsuoka M & Yamaguchi I (2006) Identification and characterization of Arabidopsis gibberellin receptors. *Plant J.* **46:** 880–889

Nakayama M, Yamane H, Nojiri H, Yokota T, Yamaguchi I, Murofushi N, Takahashi N, Nishijima T, Koshioka M, Katsura N & Nonaka M (1995) Qualitative and Quantitative Analysis of Endogenous Gibberellins in Raphanus sativus L. during Cold Treatment and the Subsequent Growth. *Bioscience, Biotechnology, and Biochemistry* **59:** 1121–1125

Ni X, Zhang YE, Nègre N, Chen S, Long M & White KP (2012) Adaptive Evolution and the Birth of CTCF Binding Sites in the Drosophila Genome. *PLoS Biol* **10:** e1001420

Niwa M, Daimon Y, Kurotani K, Higo A, Pruneda-Paz JL, Breton G, Mitsuda N, Kay SA, Ohme-Takagi M, Endo M & Araki T (2013) BRANCHED1 Interacts with FLOWERING LOCUS T to Repress the Floral Transition of the Axillary Meristems in Arabidopsis. *Plant Cell* **25:** 1228–1242

Nix DA & Eisen MB (2005) GATA: a graphic alignment tool for comparative sequence analysis. *BMC Bioinformatics* **6:** 9

Nurrish SJ & Treisman R (1995) DNA binding specificity determinants in MADS-box transcription factors. *Mol. Cell. Biol.* **15:** 4076–4085

Odom DT, Dowell RD, Jacobsen ES, Gordon W, Danford TW, MacIsaac KD, Rolfe PA, Conboy CM, Gifford DK & Fraenkel E (2007) Tissue-specific transcriptional regulation has diverged significantly between human and mouse. *Nat. Genet.* **39:** 730–732

Oka M, Tasaka Y, Iwabuchi M & Mino M (2001) Elevated sensitivity to gibberellin by vernalization in the vegetative rosette plants of Eustoma grandiflorum and Arabidopsis thaliana. *Plant Science* **160:** 1237–1245

ÓMaoiléidigh DS, Wuest SE, Rae L, Raganelli A, Ryan PT, Kwasniewska K, Das P, Lohan AJ, Loftus B, Graciet E & Wellmer F (2013) Control of reproductive floral organ identity specification in Arabidopsis by the C function regulator AGAMOUS. *Plant Cell* **25:** 2482–2503

Osnato M, Castillejo C, Matías-Hernández L & Pelaz S (2012) TEMPRANILLO genes link photoperiod and gibberellin pathways to control flowering in Arabidopsis. *Nat Commun* **3:** 808

Pajoro A, Madrigal P, Muiño JM, Matus JT, Jin J, Mecchia MA, Debernardi JM, Palatnik JF, Balazadeh S, Arif M, Ó'Maoiléidigh DS, Wellmer F, Krajewski P, Riechmann J-L, Angenent GC & Kaufmann K (2014) Dynamics of chromatin accessibility and gene regulation by MADS-domain transcription factors in flower development. *Genome Biology* **15:** R41

Para A, Li Y, Marshall-Colón A, Varala K, Francoeur NJ, Moran TM, Edwards MB, Hackley C, Bargmann BOR, Birnbaum KD, McCombie WR, Krouk G & Coruzzi GM (2014) Hit-and-run transcriptional control by bZIP1 mediates rapid nutrient signaling in Arabidopsis. *PNAS* **111:** 10371–10376

Park S, Lee C-M, Doherty CJ, Gilmour SJ, Kim Y & Thomashow MF (2015) Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. *Plant J* **82:** 193–207

Pelaz S, Ditta GS, Baumann E, Wisman E & Yanofsky MF (2000) B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature* **405:** 200–203

Pellegrini L, Tan S & Richmond TJ (1995) Structure of serum response factor core bound to DNA. *Nature* **376:** 490–498

Phillips AL, Ward DA, Uknes S, Appleford N, Lange T, Huttly AK, Gaskin P, Graebe JE & Hedden P (1995) Isolation and Expression of Three Gibberellin 20-Oxidase cDNA Clones from Arabidopsis. *Plant Physiol.* **108:** 1049–1057

Porri A, Torti S, Romera-Branchat M & Coupland G (2012) Spatially distinct regulatory roles for gibberellins in the promotion of flowering of Arabidopsis under long photoperiods. *Development* **139:** 2198–2209

Posé D, Verhage L, Ott F, Yant L, Mathieu J, Angenent GC, Immink RGH & Schmid M (2013a) Temperature-dependent regulation of flowering by antagonistic FLM variants. *Nature* **503:** 414–417

Posé D, Verhage L, Ott F, Yant L, Mathieu J, Angenent GC, Immink RGH & Schmid M (2013b) Temperature-dependent regulation of flowering by antagonistic FLM variants. *Nature* **503:** 414–417

Quinlan AR & Hall IM (2010) BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26:** 841–842

Ramsay L, Comadran J, Druka A, Marshall DF, Thomas WTB, Macaulay M, MacKenzie K, Simpson C, Fuller J, Bonar N, Hayes PM, Lundqvist U, Franckowiak JD, Close TJ, Muehlbauer GJ & Waugh R (2011) INTERMEDIUM-C, a modifier of lateral spikelet fertility in barley, is an ortholog of the maize domestication gene TEOSINTE BRANCHED 1. *Nat. Genet.* **43:** 169–172

Ravasi T, Suzuki H, Cannistraci CV, Katayama S, Bajic VB, Tan K, Akalin A, Schmeier S, Kanamori-Katayama M, Bertin N, Carninci P, Daub CO, Forrest ARR, Gough J, Grimmond S, Han J-H, Hashimoto T, Hide W, Hofmann O, Kamburov A, et al (2010) An atlas of combinatorial transcriptional regulation in mouse and man. *Cell* **140:** 744–752

Reeves PA, He Y, Schmitz RJ, Amasino RM, Panella LW & Richards CM (2007) Evolutionary Conservation of the FLOWERING LOCUS C-Mediated Vernalization Response: Evidence From the Sugar Beet (Beta vulgaris). *Genetics* **176:** 295–307

Reeves PH & Coupland G (2001) Analysis of Flowering Time Control in Arabidopsis by Comparison of Double and Triple Mutants. *Plant Physiol.* **126:** 1085–1091

Regnault T, Davière J-M, Wild M, Sakvarelidze-Achard L, Heintz D, Carrera Bergua E, Lopez Diaz I, Gong F, Hedden P & Achard P (2015) The gibberellin precursor GA12 acts as a long-distance growth signal in Arabidopsis. *Nat Plants* **1:** 15073

Richter R, Behringer C, Müller IK & Schwechheimer C (2010) The GATA-type transcription factors GNC and GNL/CGA1 repress gibberellin signaling downstream from DELLA proteins and PHYTOCHROME-INTERACTING FACTORS. *Genes Dev.* **24:** 2093–2104

Ridge S, Brown PH, Hecht V, Driessen RG & Weller JL (2014) The role of BoFLC2 in cauliflower (Brassica oleracea var. botrytis L.) reproductive development. *J. Exp. Bot.***:** eru408

Riechmann JL, Wang M & Meyerowitz EM (1996) DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. *Nucleic Acids Res* **24:** 3134–3141

Rieu I, Eriksson S, Powers SJ, Gong F, Griffiths J, Woolley L, Benlloch R, Nilsson O, Thomas SG, Hedden P & Phillips AL (2008a) Genetic Analysis Reveals That C19-GA 2-Oxidation Is a Major Gibberellin Inactivation Pathway in Arabidopsis. *Plant Cell* **20:** 2420–2436

Rieu I, Ruiz-Rivero O, Fernandez-Garcia N, Griffiths J, Powers SJ, Gong F, Linhartova T, Eriksson S, Nilsson O, Thomas SG, Phillips AL & Hedden P (2008b) The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *Plant J.* **53:** 488–504

Romero IG, Ruvinsky I & Gilad Y (2012) Comparative studies of gene expression and the evolution of gene regulation. *Nat Rev Genet* **13:** 505–516

Ruelens P, de Maagd RA, Proost S, Theißen G, Geuten K & Kaufmann K (2013) FLOWERING LOCUS C in monocots and the tandem origin of angiosperm-specific MADS-box genes. *Nat Commun* **4:** 2280 DOI 10.1038-ncomms3280

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF & Coupland G (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. *Science* **288:** 1613–1616

Sather DN, Jovanovic M & Golenberg EM (2010) Functional analysis of B and C class floral organ genes in spinach demonstrates their role in sexual dimorphism. *BMC Plant Biol.* **10:** 46

Scheres B & van der Putten WH (2017) The plant perceptron connects environment to development. *Nature* **543:** 337–345

Schmid M, Uhlenhaut NH, Godard F, Demar M, Bressan R, Weigel D & Lohmann JU (2003) Dissection of floral induction pathways using global expression analysis. *Development* **130:** 6001–6012

Schmidt D, Schwalie PC, Wilson MD, Ballester B, Gonçalves Â, Kutter C, Brown GD, Marshall A, Flicek P & Odom DT (2012) Waves of Retrotransposon Expansion Remodel Genome Organization and CTCF Binding in Multiple Mammalian Lineages. *Cell* **148:** 335–348

Schmidt D, Wilson MD, Ballester B, Schwalie PC, Brown GD, Marshall A, Kutter C, Watt S, Martinez-Jimenez CP, Mackay S, Talianidis I, Flicek P & Odom DT (2010) Five-Vertebrate ChIP-seq Reveals the Evolutionary Dynamics of Transcription Factor Binding. *Science* **328:** 1036–1040

Schomburg FM, Bizzell CM, Lee DJ, Zeevaart JAD & Amasino RM (2003) Overexpression of a Novel Class of Gibberellin 2-Oxidases Decreases Gibberellin Levels and Creates Dwarf Plants. *Plant Cell* **15:** 151–163

Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M & Weigel D (2005) Specific Effects of MicroRNAs on the Plant Transcriptome. *Developmental Cell* **8:** 517–527

Schwarz S, Grande AV, Bujdoso N, Saedler H & Huijser P (2008) The microRNA regulated SBP-box genes SPL9 and SPL15 control shoot maturation in Arabidopsis. *Plant Mol Biol* **67:** 183–195

Schwarz-Sommer Z, Hue I, Huijser P, Flor PJ, Hansen R, Tetens F, Lönnig WE, Saedler H & Sommer H (1992) Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.* **11:** 251–263

Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H & Sommer H (1990) Genetic Control of Flower Development by Homeotic Genes in Antirrhinum majus. *Science* **250:** 931–936

Searle I, He Y, Turck F, Vincent C, Fornara F, Kröber S, Amasino RA & Coupland G (2006) The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. *Genes Dev.* **20:** 898–912

Seo E, Lee H, Jeon J, Park H, Kim J, Noh Y-S & Lee I (2009) Crosstalk between Cold Response and Flowering in Arabidopsis Is Mediated through the Flowering-Time Gene SOC1 and Its Upstream Negative Regulator FLC. *Plant Cell* **21:** 3185–3197

Seo M, Jikumaru Y & Kamiya Y (2011) Profiling of hormones and related metabolites in seed dormancy and germination studies. *Methods Mol. Biol.* **773:** 99–111

Shan D-P, Huang J-G, Yang Y-T, Guo Y-H, Wu C-A, Yang G-D, Gao Z & Zheng C-C (2007) Cotton GhDREB1 increases plant tolerance to low temperature and is negatively regulated by gibberellic acid. *New Phytologist* **176:** 70–81

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ & Dennis ES (1999) The FLF MADS Box Gene: A Repressor of Flowering in Arabidopsis Regulated by Vernalization and Methylation. *Plant Cell* **11:** 445–458

Sheldon CC, Hills MJ, Lister C, Dean C, Dennis ES & Peacock WJ (2008) Resetting of FLOWERING LOCUS C expression after epigenetic repression by vernalization. *PNAS* **105:** 2214–2219

Sheldon CC, Rouse DT, Finnegan EJ, Peacock WJ & Dennis ES (2000) The molecular basis of vernalization: The central role of FLOWERING LOCUS C (FLC). *PNAS* **97:** 3753–3758

Shibata Y, Sheffield NC, Fedrigo O, Babbitt CC, Wortham M, Tewari AK, London D, Song L, Lee B-K, Iyer VR, Parker SCJ, Margulies EH, Wray GA, Furey TS & Crawford GE (2012) Extensive evolutionary changes in regulatory element activity during human origins are associated with altered gene expression and positive selection. *PLoS Genet.* **8:** e1002789

Shore P & Sharrocks AD (1995) The MADS-Box Family of Transcription Factors. *European Journal of Biochemistry* **229:** 1–13

Simpson GG (2004) The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. *Current Opinion in Plant Biology* **7:** 570–574

Smaczniak C, Immink RGH, Angenent GC & Kaufmann K (2012a) Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies. *Development* **139:** 3081–3098

Smaczniak C, Immink RGH, Muino JM, Blanvillain R, Busscher M, Busscher-Lange J, Dinh QD (Peter), Liu S, Westphal AH, Boeren S, Parcy F, Xu L, Carles CC, Angenent GC & Kaufmann K (2012b) Characterization of MADS-domain transcription factor complexes in Arabidopsis flower development. *Proc Natl Acad Sci U S A* **109:** 1560–1565

Smith TF & Waterman MS (1981) Identification of common molecular subsequences. *J. Mol. Biol.* **147:** 195–197

Smith VA, Knatt CJ, Gaskin P & Reid JB (1992) The Distribution of Gibberellins in Vegetative Tissues of Pisum sativum L. I. Biological and Biochemical Consequences of the le Mutation. *Plant Physiol.* **99:** 368–371

Stefflova K, Thybert D, Wilson MD, Streeter I, Aleksic J, Karagianni P, Brazma A, Adams DJ, Talianidis I, Marioni JC, Flicek P & Odom DT (2013) Cooperativity and Rapid Evolution of Cobound Transcription Factors in Closely Related Mammals. *Cell* **154:** 530–540

Stinchcombe JR, Weinig C, Ungerer M, Olsen KM, Mays C, Halldorsdottir SS, Purugganan MD & Schmitt J (2004) A latitudinal cline in flowering time in Arabidopsis thaliana modulated by the flowering time gene FRIGIDA. *PNAS* **101:** 4712–4717

Stone JR & Wray GA (2001) Rapid Evolution of cis-Regulatory Sequences via Local Point Mutations. *Mol Biol Evol* **18:** 1764–1770

Suge H & Rappaport L (1968) Role of gibberellins in stem elongation and flowering in radish. *Plant Physiol.* **43:** 1208–1214

Sung S & Amasino RM (2004) Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. *Nature* **427:** 159–164

Suzuki H, Park S-H, Okubo K, Kitamura J, Ueguchi-Tanaka M, Iuchi S, Katoh E, Kobayashi M, Yamaguchi I, Matsuoka M, Asami T & Nakajima M (2009) Differential expression and affinities of Arabidopsis gibberellin receptors can explain variation in phenotypes of multiple knock-out mutants. *The Plant Journal* **60:** 48–55

Tadege M, Sheldon CC, Helliwell CA, Stoutjesdijk P, Dennis ES & Peacock WJ (2001) Control of flowering time by FLC orthologues in Brassica napus. *Plant J.* **28:** 545–553

Talon M & Zeevaart JAD (1990) Gibberellins and Stem Growth as Related to Photoperiod in Silene armeria L. *Plant Physiol.* **92:** 1094–1100

Tao Z, Shen L, Liu C, Liu L, Yan Y & Yu H (2012) Genome-wide identification of SOC1 and SVP targets during the floral transition in Arabidopsis. *The Plant Journal* **70:** 549–561

Theissen G (2001) Development of floral organ identity: stories from the MADS house. *Curr. Opin. Plant Biol.* **4:** 75–85

Theissen G & Saedler H (2001) Plant biology. Floral quartets. *Nature* **409:** 469–471

Thomashow MF (1999) PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology* **50:** 571–599

Thorvaldsdóttir H, Robinson JT & Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief. Bioinformatics* **14:** 178–192

Tirosh I, Reikhav S, Levy AA & Barkai N (2009) A Yeast Hybrid Provides Insight into the Evolution of Gene Expression Regulation. *Science* **324:** 659–662

Toräng P, Wunder J, Obeso JR, Herzog M, Coupland G & Ågren J (2014) Large-scale adaptive differentiation in the alpine perennial herb Arabis alpina. *New Phytol***:** DOI 10.111/nph.13176

Trapnell C, Pachter L & Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25:** 1105–1111

Tuch BB, Galgoczy DJ, Hernday AD, Li H & Johnson AD (2008) The Evolution of Combinatorial Gene Regulation in Fungi. *PLOS Biol* **6:** e38

Turck F, Fornara F & Coupland G (2008) Regulation and Identity of Florigen: FLOWERING LOCUS T Moves Center Stage. *Annual Review of Plant Biology* **59:** 573–594

Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR & Sun T-P (2004) Della proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. *Plant Physiol.* **135:** 1008–1019

Vidal AM, Gisbert C, Talón M, Primo-Millo E, López-Díaz I & García-Martínez JL (2001) The ectopic overexpression of a citrus gibberellin 20-oxidase enhances the non-13-hydroxylation pathway of gibberellin biosynthesis and induces an extremely elongated phenotype in tobacco. *Physiol Plant* **112:** 251–260

Villar D, Flicek P & Odom DT (2014) Evolution of transcription factor binding in metazoans — mechanisms and functional implications. *Nat Rev Genet* **15:** 221–233

Wang J-W, Czech B & Weigel D (2009a) miR156-Regulated SPL Transcription Factors Define an Endogenous Flowering Pathway in Arabidopsis thaliana. *Cell* **138:** 738–749

Wang R, Albani MC, Vincent C, Bergonzi S, Luan M, Bai Y, Kiefer C, Castillo R & Coupland G (2011) Aa TFL1 Confers an Age-Dependent Response to Vernalization in Perennial Arabis alpina. *Plant Cell* **23:** 1307–1321

Wang R, Farrona S, Vincent C, Joecker A, Schoof H, Turck F, Alonso-Blanco C, Coupland G & Albani MC (2009b) PEP1 regulates perennial flowering in Arabis alpina. *Nature* **459:** 423–427

Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU & Weigel D (2005) Integration of Spatial and Temporal Information During Floral Induction in Arabidopsis. *Science* **309:** 1056–1059

Wilkie JD, Sedgley M & Olesen T (2008) Regulation of floral initiation in horticultural trees. *J. Exp. Bot.* **59:** 3215–3228

Willing E-M, Rawat V, Mandáková T, Maumus F, James GV, Nordström KJV, Becker C, Warthmann N, Chica C, Szarzynska B, Zytnicki M, Albani MC, Kiefer C, Bergonzi S, Castaings L, Mateos JL, Berns MC, Bujdoso N, Piofczyk T, Lorenzo L de, et al (2015) Genome expansion of Arabis alpina linked with retrotransposition and reduced symmetric DNA methylation. *Nature Plants* **1:** 14023 DOI 10.1038/nplants.2014.23

Willmann MR & Poethig RS (2011) The effect of the floral repressor FLC on the timing and progression of vegetative phase change in Arabidopsis. *Development* **138:** 677–685

Wilson MD, Barbosa-Morais NL, Schmidt D, Conboy CM, Vanes L, Tybulewicz VLJ, Fisher EMC, Tavaré S & Odom DT (2008) Species-Specific Transcription in Mice Carrying Human Chromosome 21. *Science* **322:** 434–438

Wilson RN, Heckman JW & Somerville CR (1992) Gibberellin Is Required for Flowering in Arabidopsis thaliana under Short Days. *Plant Physiol.* **100:** 403–408

Wittkopp PJ, Haerum BK & Clark AG (2008) Regulatory changes underlying expression differences within and between Drosophila species. *Nat. Genet.* **40:** 346–350

Wu G, Park MY, Conway SR, Wang J-W, Weigel D & Poethig RS (2009) The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. *Cell* **138:** 750–759

Wuest SE, O'Maoileidigh DS, Rae L, Kwasniewska K, Raganelli A, Hanczaryk K, Lohan AJ, Loftus B, Graciet E & Wellmer F (2012) Molecular basis for the specification of floral organs by APETALA3 and PISTILLATA. *PNAS* **109:** 13452–13457

Xu YL, Gage DA & Zeevaart J (1997) Gibberellins and Stem Growth in Arabidopsis thaliana (Effects of Photoperiod on Expression of the GA4 and GA5 Loci). *Plant Physiol.* **114:** 1471–1476

Yamaguchi A, Kobayashi Y, Goto K, Abe M & Araki T (2005) TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. *Plant Cell Physiol.* **46:** 1175–1189

Yamaguchi N, Winter CM, Wu M-F, Kanno Y, Yamaguchi A, Seo M & Wagner D (2014) Gibberellin acts positively then negatively to control onset of flower formation in Arabidopsis. *Science* **344:** 638–641

Yamauchi Y, Ogawa M, Kuwahara A, Hanada A, Kamiya Y & Yamaguchi S (2004) Activation of Gibberellin Biosynthesis and Response Pathways by Low Temperature during Imbibition of Arabidopsis thaliana Seeds. *Plant Cell* **16:** 367–378

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V & Dubcovsky J (2004) The Wheat VRN2 Gene Is a Flowering Repressor Down-Regulated by Vernalization. *Science* **303:** 1640–1644

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T & Dubcovsky J (2003) Positional cloning of the wheat vernalization gene VRN1. *PNAS* **100:** 6263–6268

Yu S, Galvão VC, Zhang Y-C, Horrer D, Zhang T-Q, Hao Y-H, Feng Y-Q, Wang S, Schmid M & Wang J-W (2012) Gibberellin Regulates the Arabidopsis Floral Transition through miR156-Targeted SQUAMOSA PROMOTER BINDING–LIKE Transcription Factors. *Plant Cell* **24:** 3320–3332

Zanewich KP & Rood SB (1995) Vernalization and Gibberellin Physiology of Winter Canola (Endogenous Gibberellin (GA) Content and Metabolism of [3H]GA1 and [3H]GA20. *Plant Physiol.* **108:** 615–621

Zarka DG, Vogel JT, Cook D & Thomashow MF (2003) Cold Induction of Arabidopsis CBF Genes Involves Multiple ICE (Inducer of CBF Expression) Promoter Elements and a Cold-Regulatory Circuit That Is Desensitized by Low Temperature. *Plant Physiol.* **133:** 910–918

Zeevaart JA, Gage DA & Talon M (1993) Gibberellin A1 is required for stem elongation in spinach. *PNAS* **90:** 7401–7405

Zhang Y, Schwarz S, Saedler H & Huijser P (2007) SPL8, a local regulator in a subset of gibberellin-mediated developmental processes in Arabidopsis. *Plant Mol Biol* **63:** 429–439

Zhou C-M, Zhang T-Q, Wang X, Yu S, Lian H, Tang H, Feng Z-Y, Zozomova-Lihová J & Wang J-W (2013a) Molecular basis of age-dependent vernalization in Cardamine flexuosa. *Science* **340:** 1097–1100

Zhou M, Xu M, Wu L, Shen C, Ma H & Lin J (2014) CbCBF from Capsella bursa-pastoris enhances cold tolerance and restrains growth in Nicotiana tabacum by antagonizing with gibberellin and affecting cell cycle signaling. *Plant Mol Biol* **85:** 259–275

Zhou Q, Su X, Wang A, Xu J & Ning K (2013b) QC-Chain: fast and holistic quality control method for next-generation sequencing data. *PLoS ONE* **8:** e60234

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, Zhu X, Mander LN, Kamiya Y, Yamaguchi S & He Z (2006) ELONGATED UPPERMOST INTERNODE Encodes a Cytochrome P450 Monooxygenase That Epoxidizes Gibberellins in a Novel Deactivation Reaction in Rice. *Plant Cell* **18:** 442–456

Appendix

A. alpina PEP1 BSs and associated target genes

Chromosome	Start	End	Associated genes
Aa.chr1	674691	675297	Aa_G106130;Aa_G106140;Aa_G106150
Aa.chr1	2254396	2254902	Aa_G286330;Aa_G286340
Aa.chr1	3908566	3909100	Aa_G375870
Aa.chr1	4934037	4934502	Aa_G394740;Aa_G394730
Aa.chr1	4939752	4940313	Aa_G394720
Aa.chr1	5156811	5157315	Aa_G73490;
Aa.chr1	6133077	6133599	Aa_G535900;Aa_G535910
Aa.chr1	8689639	8690062	Aa_G164300;Aa_G164290
Aa.chr1	8716365	8716945	Aa_G164280;Aa_G164270
Aa.chr1	8767101	8767678	Aa_G630450
Aa.chr1	9727993	9728558	Aa_G93030;;Aa_G93040;
Aa.chr1	10524929	10525418	Aa_G242480
Aa.chr1	12906947	12907520	NA
Aa.chr1	14693952	14694435	Aa_G323710;Aa_G323720
Aa.chr1	17222423	17222989	Aa_G198680
Aa.chr1	18415794	18416434	Aa_G613120
Aa.chr1	19015919	19016513	Aa_G443010
Aa.chr1	21275920	21276444	Aa_G248830;Aa_G248820;Aa_G655020
Aa.chr1	22046074	22046585	Aa_G299120;Aa_G299110
Aa.chr1	22395479	22395950	Aa_G489220;Aa_G489230;Aa_G489240
Aa.chr2	1711420	1711874	NA
Aa.chr2	10985739	10986190	Aa_G30840;
Aa.chr2	14167771	14168305	Aa_G248630
Aa.chr2	16510978	16511415	Aa_G169760
Aa.chr2	16743335	16743888	NA
Aa.chr2	16748501	16749051	NA
Aa.chr2	24452859	24453425	Aa_G198410
Aa.chr2	24994573	24995080	Aa_G315340;Aa_G315330
Aa.chr2	25025571	25026027	Aa_G785460;Aa_G111220;Aa_G111210
Aa.chr2	26606691	26607185	Aa_G656410;Aa_G656400;Aa_G656390
Aa.chr2	27056653	27057333	Aa_G557400;Aa_G557410;Aa_G557420
Aa.chr2	27298308	27298890	Aa_G144150
Aa.chr2	27758252	27758788	Aa_G239010;Aa_G239000;Aa_G238990;Aa_G238980
Aa.chr2	27954316	27954787	Aa_G312140
Aa.chr2	28191828	28192225	Aa_G410440;Aa_G410430
Aa.chr2	28251119	28251553	Aa_G462530;Aa_G462540
Aa.chr3	814272	814757	Aa_G42340;;Aa_G42330;
Aa.chr3	2515586	2516096	Aa_G51110;;Aa_G51120;
Aa.chr3	2539119	2539573	Aa_G51140;
Aa.chr3	3448321	3448892	Aa_G49840;
Aa.chr3	3758219	3758680	Aa_G47030;;Aa_G47020;
Aa.chr3	5420849	5421370	NA

Aa.chr3	6517480	6517917	Aa_G102840;Aa_G102850
Aa.chr3	6802615	6803099	Aa_G5290;N;Aa_G5280;N;Aa_G5270;N
Aa.chr3	7178054	7178592	Aa_G39940;
Aa.chr3	7180368	7180860	NA
Aa.chr3	7589495	7590014	Aa_G176060
Aa.chr3	8954542	8955081	Aa_G56170;
Aa.chr3	9760908	9761409	NA
Aa.chr3	10048490	10049051	Aa_G32190;;Aa_G32200;
Aa.chr3	10555624	10556144	Aa_G367470;Aa_G367480
Aa.chr3	11136131	11136605	Aa_G20350;;Aa_G20340;
Aa.chr3	12885968	12886440	Aa_G104740;Aa_G104730
Aa.chr3	13753422	13753962	NA
Aa.chr3	15602143	15602552	NA
Aa.chr3	16464103	16464602	Aa_G198870;Aa_G198880
Aa.chr3	19233124	19233575	Aa_G568410;Aa_G568400
Aa.chr3	20908612	20910036	NA
Aa.chr3	22825576	22826119	Aa_G105330;Aa_G105320
Aa.chr3	24883050	24883590	Aa_G199520
Aa.chr3	27147243	27147668	Aa_G64190;;Aa_G64200;
Aa.chr3	27149991	27150424	Aa_G64200;
Aa.chr4	181876	182365	Aa_G667120
Aa.chr4	221921	222417	Aa_G229970;Aa_G229980;Aa_G229990
Aa.chr4	2613456	2614092	Aa_G504530;Aa_G504520;Aa_G504510
Aa.chr4	4238947	4239503	Aa_G431640
Aa.chr4	4261801	4262766	Aa_G431680;Aa_G561960;Aa_G561950
Aa.chr4	5945151	5945598	Aa_G95960;;Aa_G95970;
Aa.chr4	8099262	8099728	Aa_G27720;
Aa.chr4	12349961	12350340	Aa_G11260;
Aa.chr4	15756361	15756812	Aa_G471880
Aa.chr4	16750110	16750640	Aa_G216820;Aa_G216810
Aa.chr4	18441404	18441842	Aa_G281350;Aa_G281360;Aa_G281370
Aa.chr4	19377617	19378117	Aa_G36620;;Aa_G36610;
Aa.chr4	21729555	21730031	Aa_G16230;;Aa_G16220;
Aa.chr4	21859790	21860338	Aa_G15970;
Aa.chr4	22133200	22133682	Aa_G536070;Aa_G536060
Aa.chr4	22789259	22789695	Aa_G226210
Aa.chr5	2003003	2003517	NA
Aa.chr5	2094269	2094753	Aa_G25680;;Aa_G25670;
Aa.chr5	3027585	3028083	Aa_G570800
Aa.chr5	6067212	6067713	Aa_G207710;Aa_G207720
Aa.chr5	7418746	7419326	Aa_G377260;Aa_G377250;Aa_G377240
Aa.chr5	9498691	9499181	Aa_G404300
Aa.chr5	12394247	12394674	Aa_G114560;Aa_G114570
Aa.chr5	13874770	13875326	Aa_G14180;
Aa.chr5	13987485	13987969	Aa_G13980;
Aa.chr5	14086192	14086569	Aa_G402280
Aa.chr5	15874511	15875062	NA

Aa.chr5	17134871	17135273	Aa_G287230;Aa_G287220;Aa_G287210;Aa_G287200
Aa.chr5	19144053	19144496	Aa_G345830;Aa_G345840;Aa_G345850;Aa_G345860
Aa.chr5	21096142	21096652	Aa_G48840;;Aa_G48850;
Aa.chr5	21418269	21418809	Aa_G57870;;Aa_G57860;
Aa.chr5	21422163	21422723	Aa_G57870;;Aa_G57860;;Aa_G57850;;Aa_G57840;
Aa.chr5	22633795	22634161	Aa_G163570
Aa.chr6	1277192	1277759	Aa_G46820;;Aa_G46830;;Aa_G46840;
Aa.chr6	1954639	1955077	Aa_G99750;;Aa_G99740;
Aa.chr6	4845818	4846209	Aa_G243730;Aa_G243740
Aa.chr6	12143513	12144030	Aa_G518710;Aa_G518700;Aa_G518690
Aa.chr6	16170421	16170842	Aa_G558790
Aa.chr6	22581148	22581649	Aa_G142720
Aa.chr6	27088662	27089439	Aa_G35730;;Aa_G35740;
Aa.chr6	28951336	28951837	Aa_G531970
Aa.chr6	30266632	30267467	Aa_G311490
Aa.chr6	32450369	32450906	Aa_G375680
Aa.chr6	34113904	34114430	Aa_G495350;Aa_G495360
Aa.chr6	35386966	35387468	NA
Aa.chr7	45077	45615	Aa_G152250
Aa.chr7	87744	88470	Aa_G152180;Aa_G152170
Aa.chr7	2902282	2902795	Aa_G37650;
Aa.chr7	3492705	3493186	Aa_G504650
Aa.chr7	6322829	6323303	Aa_G537940
Aa.chr7	7268621	7269081	Aa_G331990
Aa.chr7	7788420	7789354	Aa_G29820;
Aa.chr7	7877168	7877690	Aa_G29660;;Aa_G29650;;Aa_G29640;
Aa.chr7	17694145	17694682	Aa_G301350
Aa.chr7	21341825	21342359	Aa_G74800;
Aa.chr7	24513189	24513560	Aa_G909220;Aa_G483230
Aa.chr7	26819261	26819798	NA
Aa.chr7	27072513	27073020	Aa_G214270
Aa.chr7	28756637	28757227	Aa_G374870;Aa_G374860
Aa.chr8	1912554	1913005	Aa_G556560;Aa_G556550
Aa.chr8	2615098	2615672	Aa_G331850;Aa_G331840;Aa_G331830
Aa.chr8	4044295	4044765	NA
Aa.chr8	4544144	4544667	Aa_G697580;Aa_G697570
Aa.chr8	6155588	6156150	Aa_G168630
Aa.chr8	6211142	6211661	Aa_G168710;Aa_G168720;Aa_G168730;Aa_G168740
Aa.chr8	6694235	6694722	Aa_G158480
Aa.chr8	10130827	10131344	Aa_G320890;Aa_G320900;Aa_G320910;Aa_G320920
Aa.chr8	10236779	10237333	Aa_G321090;Aa_G321100
Aa.chr8	10296852	10297348	Aa_G431400
Aa.chr8	10387724	10388271	Aa_G236960;Aa_G236950
Aa.chr8	10849112	10849627	Aa_G356690;Aa_G356680;Aa_G356670;Aa_G356660
Aa.chr8	16628630	16629148	Aa_G15550;;Aa_G15540;;Aa_G15530;
Aa.chr8	17178895	17179438	Aa_G8920;N;Aa_G8930;N
Aa.chr8	24130920	24131441	Aa_G503910;Aa_G503920

Aa.chr8	25541727	25542175	Aa_G396870;Aa_G396880
Aa.chr8	26833317	26833820	Aa_G94130;;Aa_G94140;;Aa_G94150;
Aa.chr8	31346521	31346974	Aa_G307430
Aa.chr8	31625095	31625600	Aa_G227750
Aa.chr8	32128611	32129203	Aa_G93810;;Aa_G93820;;Aa_G93830;
Aa.chr8	32424941	32425524	Aa_G297090;Aa_G297080
Aa.chr8	34756096	34756847	Aa_G630810;Aa_G630820
Aa.chr8	35577506	35577981	Aa_G398090;Aa_G398080
Aa.chr8	35630879	35631412	Aa_G70560;;Aa_G70570;
Aa.chr8	36377200	36377769	Aa_G165590
Aa.chr8	37542747	37543250	Aa_G13410;;Aa_G13420;;Aa_G13430;;Aa_G13440;
Aa.chr8	38743169	38743632	Aa_G306090;Aa_G306100
Aa.chr8	40247010	40247480	Aa_G147100;Aa_G147110
Aa.chr8	40258843	40259335	Aa_G147130;Aa_G147140
Aa.chr8	40471897	40472658	Aa_G383040;Aa_G383050;Aa_G383060
scaff_45200_1	8160	9338	NA
scaff_46051_1	3281	4748	NA
scaff_47772_1	3394	4554	NA
scaff_65081_1	3781	4712	NA
scaff_72209_1	32963	33505	Aa_G470530;Aa_G470540

Table A1 List of PEP1 BSs and associated target genes

A. thaliana FLC BSs and associated target genes

Chromosome	Start	End	Associated genes
Chr1	63522	64948	AT1G01130;AT1G01140
Chr1	71966	72563	AT1G01150;AT1G01160
Chr1	1143554	1144204	AT1G04260;AT1G04270;AT1G04280
Chr1	1587829	1588502	AT1G05410;AT1G05420
Chr1	2189989	2190925	AT1G07128;AT1G07135
Chr1	2203569	2204328	AT1G07180
Chr1	2520508	2520957	AT1G08080
Chr1	3076782	3077497	AT1G09530
Chr1	3663934	3664495	AT1G10950;AT1G10960;AT1G10970
Chr1	4292000	4292483	AT1G12610
Chr1	4366993	4367493	AT1G12800;AT1G12805;AT1G12810
Chr1	4548486	4549060	AT1G13280
Chr1	5067277	5067783	AT1G14710;AT1G14720
Chr1	5137887	5138472	AT1G14890;AT1G14900
Chr1	5945043	5945923	AT1G17360
Chr1	5967880	5968516	AT1G17410
Chr1	6174311	6175058	AT1G17940;AT1G17950
Chr1	6335496	6336190	AT1G18410
Chr1	6443279	6443847	AT1G18700
Chr1	6862870	6863512	AT1G19835

Chr1	7065142	7065781	AT1G20380
Chr1	7075035	7075694	AT1G20400;AT1G20405
Chr1	7236018	7236600	AT1G20823
Chr1	7251546	7252027	AT1G20850
Chr1	7267326	7267852	AT1G20880;AT1G20890
Chr1	7518154	7518683	AT1G21470;AT1G21480
Chr1	7851196	7851680	AT1G22230;AT1G22240
Chr1	7930219	7930845	AT1G22460
Chr1	7989184	7989779	AT1G22600
Chr1	8105055	8105559	AT1G22900;AT1G22910
Chr1	8431799	8432363	AT1G23860;AT1G23870
Chr1	8598504	8599213	AT1G24260;AT1G24265
Chr1	8983464	8984199	AT1G25560
Chr1	9777795	9778379	AT1G28040;AT1G28050;AT1G28060
Chr1	10477891	10478912	AT1G29920;AT1G29930;AT1G29940
Chr1	11023839	11024486	NA
Chr1	11454727	11455213	AT1G31910
Chr1	16713342	16714043	AT1G44010
Chr1	16836968	16837535	AT1G44350
Chr1	19512982	19513461	AT1G52390;AT1G52400
Chr1	19699182	19699765	AT1G52890
Chr1	20450834	20451388	AT1G54820;AT1G54830
Chr1	23734405	23735006	AT1G63940;AT1G63950
Chr1	24331365	24332055	AT1G65480
Chr1	25041552	25042057	AT1G67070
Chr1	25048333	25049374	AT1G67080;AT1G67090
Chr1	25320090	25320620	AT1G67560
Chr1	25743808	25744300	AT1G68570
Chr1	26038092	26038695	AT1G69252;AT1G69260
Chr1	26097360	26097948	AT1G69420;AT1G69430
Chr1	26105585	26106077	AT1G69440
Chr1	26141313	26141762	AT1G69530
Chr1	26313323	26313761	AT1G69870
Chr1	26317193	26317724	AT1G69870
Chr1	26476081	26476652	AT1G70290
Chr1	26655470	26656408	AT1G70700
Chr1	26970874	26971502	AT1G71696;AT1G71697;AT1G71700
Chr1	27264357	27265026	AT1G72420;AT1G72430
Chr1	27276831	27277424	AT1G72450;AT1G72460
Chr1	27294226	27294774	AT1G72500
Chr1	27360961	27361494	AT1G72670;AT1G72680;AT1G72690
Chr1	27714648	27715191	AT1G73687;AT1G73690
Chr1	27992498	27992871	AT1G74460;AT1G74470;AT1G74480
Chr1	28292731	28293325	AT1G75388;AT1G75390
Chr1	28467403	28467915	AT1G75820
Chr1	29099512	29100040	AT1G77450
Chr1	29120811	29121386	AT1G77490;AT1G77500

Chr1	29233493	29234016	AT1G77750
Chr1	29399097	29399719	AT1G78130
Chr1	29406595	29407126	AT1G78140;AT1G78150;AT1G78160
Chr1	29563248	29563786	AT1G78580;AT1G78590
Chr1	29743194	29744117	AT1G79060;AT1G79075
Chr1	29763297	29763769	AT1G79120
Chr1	30083634	30084233	AT1G79970;AT1G79980;AT1G79990
Chr1	30108799	30109326	AT1G80030;AT1G80040
Chr2	368575	369111	AT2G01830
Chr2	382920	383573	AT2G01850
Chr2	884107	884673	AT2G03020
Chr2	7190734	7191633	AT2G16586
Chr2	8057125	8057771	AT2G18560
Chr2	8138861	8139343	AT2G18780;AT2G18790
Chr2	8534591	8535151	AT2G19800
Chr2	8813371	8814004	AT2G20440;AT2G20450;AT2G20453
Chr2	9466208	9466707	AT2G22270;AT2G22280;AT2G22290
Chr2	9579517	9580489	AT2G22530;AT2G22540
Chr2	10628143	10628675	AT2G24990;AT2G25000
Chr2	10836048	10836759	AT2G25460;AT2G25470
Chr2	11214329	11214940	AT2G26330;AT2G26340
Chr2	11951962	11952504	AT2G28060
Chr2	12829792	12830124	NA
Chr2	12849328	12849847	AT2G30100;AT2G30105
Chr2	13288418	13288975	AT2G31180
Chr2	13716860	13717405	AT2G32290;AT2G32295
Chr2	13965391	13965971	AT2G32920;AT2G32930
Chr2	14489440	14489926	AT2G34340
Chr2	15540898	15541572	AT2G36990;AT2G37000;AT2G37010
Chr2	15883890	15884404	AT2G37950
Chr2	15941152	15941690	NA
Chr2	16110088	16111556	AT2G38470;AT2G38480
Chr2	17086992	17087641	AT2G40940;AT2G40950
Chr2	17360142	17360620	AT2G41630;AT2G41640
Chr2	17819556	17820090	AT2G42820;AT2G42830
Chr2	17926154	17926691	AT2G43120
Chr2	18810845	18811457	AT2G45660
Chr2	18891361	18891999	AT2G45910
Chr2	18945252	18945690	AT2G46060;AT2G46070
Chr2	19198018	19198562	AT2G46720
Chr2	19467226	19467812	AT2G47440
Chr3	611026	611553	AT3G02810;AT3G02820;AT3G02830
Chr3	822530	823085	AT3G03450;AT3G03456;AT3G03460
Chr3	826292	826742	AT3G03460;AT3G03470
Chr3	1608691	1609274	AT3G05540;AT3G05545
Chr3	1652658	1653308	AT3G05660;AT3G05670
Chr3	1880324	1880822	AT3G06210

Chr3	1959598	1960374	AT3G06430
Chr3	1975806	1976411	AT3G06440;AT3G06450
Chr3	2131791	2132566	AT3G06750;AT3G06760
Chr3	2413359	2413767	AT3G07560;AT3G07565
Chr3	2620406	2620913	AT3G08620;AT3G08630;AT3G08636;AT3G08640
Chr3	3138852	3139464	AT3G10150;AT3G10160
Chr3	3502408	3502979	AT3G11170;AT3G11180
Chr3	3718046	3718579	AT3G11750;AT3G11760
Chr3	3884917	3885463	AT3G12180;AT3G12190
Chr3	4119247	4119833	NA
Chr3	4571551	4572081	AT3G13870;AT3G13880;AT3G13882
Chr3	4827225	4827632	AT3G14430
Chr3	5085664	5086235	AT3G15110;AT3G15111;AT3G15115
Chr3	5216122	5216737	AT3G15460;AT3G15470
Chr3	5233919	5234357	AT3G15500
Chr3	5236494	5237151	AT3G15500
Chr3	5292960	5293822	AT3G15610;AT3G15620
Chr3	5470178	5470747	AT3G16140;AT3G16150
Chr3	5517383	5518466	AT3G16270;AT3G16280
Chr3	6380545	6381417	AT3G18550
Chr3	6617445	6618023	AT3G19140;AT3G19150
Chr3	7275323	7275875	AT3G20800;AT3G20810
Chr3	7880105	7880598	AT3G22275;AT3G22290
Chr3	8308746	8309608	AT3G23250;AT3G23255
Chr3	8500370	8500891	AT3G23635;AT3G23637;AT3G23640
Chr3	8511539	8512157	AT3G23650
Chr3	8601220	8601635	AT3G23820
Chr3	8941135	8942496	AT3G24518;AT3G24520
Chr3	9300648	9301276	AT3G25590
Chr3	10094636	10095245	AT3G27325
Chr3	11386800	11387374	NA
Chr3	13586781	13587991	NA
Chr3	13709371	13710207	NA
Chr3	14316404	14316842	AT3G42160
Chr3	15951707	15953137	AT3G44250;AT3G44260
Chr3	15986488	15987084	AT3G44310
Chr3	16755353	16755758	AT3G45630;AT3G45638;AT3G45640
Chr3	17221287	17221794	AT3G46740;AT3G46750;AT3G46760;AT3G46770
Chr3	17223566	17224144	AT3G46760;AT3G46770
Chr3	18051951	18052465	AT3G48730;AT3G48740
Chr3	18092863	18093399	AT3G48780;AT3G48790
Chr3	18763180	18763671	AT3G50560
Chr3	18782051	18782915	AT3G50610
Chr3	19453087	19453608	AT3G52480
Chr3	19722697	19723239	AT3G53220;AT3G53230
Chr3	20120933	20121541	AT3G54340;AT3G54350
Chr3	20478078	20478605	AT3G55250

Chr3	20583810	20584426	NA
Chr3	20785944	20786468	AT3G56000;AT3G56010
Chr3	20987840	20988528	AT3G56660;AT3G56670
Chr3	21153430	21154037	AT3G57140;AT3G57150
Chr3	21177134	21177753	AT3G57220;AT3G57230
Chr3	21412413	21413121	AT3G57800
Chr3	21445673	21446233	AT3G57920
Chr3	21678506	21679077	AT3G58620
Chr3	22141010	22141509	AT3G59926;AT3G59930;AT3G59940
Chr3	22197350	22197905	AT3G60110
Chr3	22291228	22291742	AT3G60310;AT3G60318;AT3G60320
Chr3	22320364	22320885	AT3G60380;AT3G60390
Chr3	22328090	22328579	AT3G60400
Chr3	22378784	22379228	AT3G60550
Chr3	22933749	22934322	AT3G61920;AT3G61930
Chr3	22990784	22991571	AT3G62090
Chr3	23242138	23242585	AT3G62860;AT3G62870
Chr3	23281410	23282121	AT3G62988;AT3G62990;AT3G63000
Chr4	271965	272547	AT4G00650;AT4G00651;AT4G00660
Chr4	459401	459936	AT4G01060
Chr4	725064	725548	NA
Chr4	1278045	1279491	AT4G02880;AT4G02890
Chr4	1494672	1495306	AT4G03390
Chr4	5498252	5498771	AT4G08620
Chr4	5793135	5793660	AT4G09030
Chr4	6771691	6772272	AT4G11110
Chr4	6794369	6795063	AT4G11140
Chr4	7585543	7586166	AT4G12970
Chr4	8100284	8100891	AT4G14030;AT4G14040
Chr4	8435241	8435839	AT4G14720
Chr4	8606792	8607300	AT4G15070;AT4G15075
Chr4	9415155	9415668	AT4G16745
Chr4	9599755	9600195	AT4G17070;AT4G17080
Chr4	9929340	9929845	AT4G17870
Chr4	10469139	10469780	AT4G19130;AT4G19140
Chr4	10716794	10717484	AT4G19700
Chr4	11097812	11098301	AT4G20690;AT4G20700
Chr4	11303463	11303916	AT4G21200
Chr4	11604184	11604891	AT4G21860;AT4G21870;AT4G21880
Chr4	11745015	11745679	AT4G22190
Chr4	12379821	12380513	AT4G23760
Chr4	12393475	12394021	AT4G23810
Chr4	12622572	12623121	AT4G24410;AT4G24415
Chr4	13021081	13022120	AT4G25480;AT4G25490;AT4G25500
Chr4	13023252	13023985	AT4G25490;AT4G25500
Chr4	13094188	13094711	AT4G25690;AT4G25692;AT4G25700
Chr4	13674436	13675142	AT4G27300;AT4G27310

Chr4	13708623	13709804	AT4G27410;AT4G27415
Chr4	13749299	13749974	AT4G27510;AT4G27520;AT4G27530
Chr4	13809974	13811046	AT4G27650;AT4G27652;AT4G27654;AT4G27657
Chr4	13854025	13854600	AT4G27800;AT4G27810
Chr4	14374097	14374626	NA
Chr4	14551615	14552129	NA
Chr4	14578326	14578897	AT4G29770;AT4G29780
Chr4	14624718	14625238	NA
Chr4	14768291	14768725	AT4G30180
Chr4	14790086	14790558	AT4G30200
Chr4	15072412	15072912	AT4G30970;AT4G30972;AT4G30975
Chr4	15591368	15591857	AT4G32290;AT4G32295
Chr4	15811695	15812239	AT4G32785;AT4G32790
Chr4	16270921	16271426	AT4G33945;AT4G33950
Chr4	16448054	16448752	AT4G34400
Chr4	16594084	16594757	AT4G34780;AT4G34790;AT4G34800
Chr4	16612825	16613395	AT4G34860;AT4G34870;AT4G34880
Chr4	17048694	17049996	AT4G36030;AT4G36040
Chr4	17395524	17396179	AT4G36910
Chr4	17397782	17398168	AT4G36920
Chr4	17618360	17619041	AT4G37460;AT4G37470;AT4G37480
Chr4	17798472	17799034	AT4G37850
Chr5	26738	27222	AT5G01070;AT5G01075
Chr5	74977	75529	AT5G01190;AT5G01200
Chr5	259693	260360	AT5G01690;AT5G01700
Chr5	516346	516902	AT5G02400;AT5G02410
Chr5	973893	974397	AT5G03720;AT5G03730
Chr5	1375687	1376250	AT5G04760
Chr5	1855541	1856221	AT5G06125;AT5G06130;AT5G06140
Chr5	1932219	1932574	AT5G06320
Chr5	2176167	2176724	AT5G07010
Chr5	2561090	2561580	AT5G07990;AT5G08000
Chr5	3395909	3396509	AT5G10740;AT5G10745;AT5G10750
Chr5	3457495	3458090	AT5G10945;AT5G10946
Chr5	4423602	4424055	AT5G13700;AT5G13710
Chr5	4450817	4451346	AT5G13780;AT5G13790;AT5G13800
Chr5	4554105	4554593	AT5G14100;AT5G14105;AT5G14110;AT5G14120
Chr5	4787552	4788148	AT5G14800
Chr5	4800543	4801024	NA
Chr5	5177680	5178139	AT5G15845;AT5G15850;AT5G15853;AT5G15860
Chr5	5227287	5227797	AT5G16000;AT5G16010;AT5G16020
Chr5	5759562	5760044	AT5G17460;AT5G17480
Chr5	5771617	5772645	AT5G17510;AT5G17520
Chr5	6095466	6095880	AT5G18400;AT5G18403;AT5G18404;AT5G18407
Chr5	6832463	6833346	AT5G20250
Chr5	7824893	7825492	AT5G23230
Chr5	7833112	7833681	AT5G23250;AT5G23260

Chr5	8122446	8122971	AT5G24030
Chr5	8376423	8376935	AT5G24530
Chr5	8455297	8455840	AT5G24680;AT5G24690
Chr5	8686443	8687203	AT5G25160
Chr5	9628865	9629599	AT5G27320
Chr5	10356473	10357039	AT5G28410
Chr5	14755448	14755958	AT5G37260
Chr5	15460398	15460979	AT5G38600;AT5G38610
Chr5	16237111	16237748	AT5G40540
Chr5	16450685	16451329	AT5G41100;AT5G41110
Chr5	16817061	16817989	AT5G42040;AT5G42050;AT5G42053;AT5G42060
Chr5	16911377	16911795	AT5G42290;AT5G42300
Chr5	18378649	18379280	NA
Chr5	18983698	18984257	AT5G46790
Chr5	19620077	19620540	AT5G48410
Chr5	20014646	20015208	AT5G49360
Chr5	20020497	20021163	AT5G49370
Chr5	20327605	20328199	AT5G49960;AT5G49970
Chr5	21240333	21240890	AT5G52310
Chr5	21437365	21437982	AT5G52882
Chr5	21557933	21558608	AT5G53150;AT5G53160
Chr5	21692442	21693059	AT5G53450;AT5G53451
Chr5	22312656	22313233	AT5G54950;AT5G54960;AT5G54970;AT5G54980
Chr5	22368836	22369464	AT5G55110;AT5G55120;AT5G55125
Chr5	22665669	22666116	AT5G55960;AT5G55970
Chr5	22666462	22666894	AT5G55970
Chr5	22691511	22692269	AT5G56040
Chr5	22740965	22741465	AT5G56180;AT5G56190
Chr5	23350378	23350852	AT5G57655
Chr5	23388275	23388838	AT5G57710
Chr5	23391560	23392538	AT5G57720;AT5G57735
Chr5	23456495	23456881	AT5G57930;AT5G57940
Chr5	23762371	23762915	AT5G58840;AT5G58850
Chr5	23840442	23841058	AT5G59050
Chr5	24010669	24011306	AT5G59590;AT5G59600;AT5G59610
Chr5	24239234	24239975	AT5G60200
Chr5	24471626	24472055	AT5G60820;AT5G60830
Chr5	24476743	24477407	AT5G60840
Chr5	24908129	24908632	AT5G61997;AT5G62000
Chr5	25086727	25087232	NA
Chr5	25321956	25322549	AT5G63120;AT5G63130
Chr5	26669070	26669559	AT5G66800

Table A2 List of FLC BSs and associated target genes

***A. alpina* PEP1 BSs and associated target genes from a second experiment**

Chromosome	Start	End	Associated genes
Aa.chr1	35196	35759	Aa_G211780;Aa_G211790
Aa.chr1	674549	675333	Aa_G106130;Aa_G106140;Aa_G106150
Aa.chr1	2254284	2254909	Aa_G286330;Aa_G286340
Aa.chr1	3908435	3909123	Aa_G375870
Aa.chr1	4933904	4934481	Aa_G394740;Aa_G394730
Aa.chr1	4939565	4940116	Aa_G394720
Aa.chr1	5156630	5157322	Aa_G73490
Aa.chr1	6132946	6133612	Aa_G535900;Aa_G535910
Aa.chr1	8689183	8690077	Aa_G164300;Aa_G164290
Aa.chr1	8716236	8716939	Aa_G164280;Aa_G164270
Aa.chr1	8766945	8767669	Aa_G630450
Aa.chr1	9727869	9728554	Aa_G93030Aa_G93040
Aa.chr1	10524785	10525441	Aa_G242480
Aa.chr1	12906864	12907526	NA
Aa.chr1	15021945	15022801	Aa_G266560;Aa_G266550
Aa.chr1	17222285	17222980	Aa_G198680
Aa.chr1	18230764	18231329	Aa_G652270;Aa_G652280
Aa.chr1	18415678	18416407	Aa_G613120
Aa.chr1	18552449	18553038	Aa_G61720Aa_G61710
Aa.chr1	18897428	18898008	NA
Aa.chr1	19015802	19016513	Aa_G443010
Aa.chr1	22045943	22046757	Aa_G299120;Aa_G299110
Aa.chr1	22395325	22395960	Aa_G489220;Aa_G489230;Aa_G489240
Aa.chr1	23379898	23380434	Aa_G107860
Aa.chr2	1711283	1712132	NA
Aa.chr2	9534021	9534552	Aa_G560570
Aa.chr2	10860656	10861102	Aa_G30770
Aa.chr2	10985597	10986232	Aa_G30840
Aa.chr2	11518229	11518824	NA
Aa.chr2	14167643	14168412	Aa_G248630
Aa.chr2	16510852	16511415	Aa_G169760
Aa.chr2	16743205	16743876	NA
Aa.chr2	16748376	16749035	NA
Aa.chr2	20317233	20317828	Aa_G413020
Aa.chr2	24452749	24453435	Aa_G198410
Aa.chr2	24994426	24995111	Aa_G315340;Aa_G315330
Aa.chr2	25438116	25438783	Aa_G164950
Aa.chr2	25716596	25717170	Aa_G129290;Aa_G129300
Aa.chr2	26606578	26607215	Aa_G656410;Aa_G656400;Aa_G656390
Aa.chr2	27056530	27057309	Aa_G557400;Aa_G557410;Aa_G557420
Aa.chr2	27298173	27298883	Aa_G144150
Aa.chr2	27758115	27758865	Aa_G239010;Aa_G239000;Aa_G238990;Aa_G238980
Aa.chr2	27954148	27954831	Aa_G312140
Aa.chr2	27970782	27971669	Aa_G312120;Aa_G312110

Aa.chr2	28250934	28251561	Aa_G462530;Aa_G462540
Aa.chr3	20043	21335	Aa_G14540Aa_G14550
Aa.chr3	767117	767706	Aa_G42480Aa_G42470Aa_G42460
Aa.chr3	2515430	2516115	Aa_G51110Aa_G51120
Aa.chr3	2538981	2539607	Aa_G51140
Aa.chr3	3031835	3032369	Aa_G65740
Aa.chr3	3426575	3427218	Aa_G49810
Aa.chr3	3448176	3448893	Aa_G49840
Aa.chr3	3758119	3758715	Aa_G47030Aa_G47020
Aa.chr3	4118749	4119829	Aa_G174660;Aa_G174650
Aa.chr3	5036701	5037222	NA
Aa.chr3	6517319	6517902	Aa_G102840;Aa_G102850
Aa.chr3	7046817	7047357	Aa_G40170Aa_G40160
Aa.chr3	7177427	7178544	Aa_G39940
Aa.chr3	7180252	7181477	NA
Aa.chr3	7589334	7589980	Aa_G176060
Aa.chr3	8954393	8955060	Aa_G56170
Aa.chr3	9760781	9761409	NA
Aa.chr3	9767498	9767937	NA
Aa.chr3	10555441	10556142	Aa_G367470;Aa_G367480
Aa.chr3	11136011	11136608	Aa_G20350Aa_G20340
Aa.chr3	13753310	13753960	NA
Aa.chr3	16463948	16464636	Aa_G198870;Aa_G198880
Aa.chr3	18907191	18907771	Aa_G178940;Aa_G178950;Aa_G178960
Aa.chr3	22825438	22826102	Aa_G105330;Aa_G105320
Aa.chr3	24882928	24883582	Aa_G199520
Aa.chr3	27069366	27069936	Aa_G331510
Aa.chr3	27147033	27147684	Aa_G64190Aa_G64200
Aa.chr3	27149832	27150449	Aa_G64200
Aa.chr4	181747	182377	Aa_G667120
Aa.chr4	221809	222401	Aa_G229970;Aa_G229980
Aa.chr4	2613362	2614102	Aa_G504530;Aa_G504520;Aa_G504510
Aa.chr4	4238800	4239500	Aa_G431640
Aa.chr4	4261684	4262773	Aa_G431680;Aa_G561960;Aa_G561950
Aa.chr4	8099121	8099735	Aa_G27720
Aa.chr4	12349668	12350375	Aa_G11260
Aa.chr4	13117200	13118227	NA
Aa.chr4	13416944	13417556	Aa_G282200;Aa_G282210
Aa.chr4	15756212	15756807	Aa_G471890
Aa.chr4	16748270	16748927	Aa_G216820
Aa.chr4	16749955	16750716	Aa_G216820;Aa_G216810
Aa.chr4	18441268	18441882	Aa_G281350;Aa_G281360;Aa_G281370
Aa.chr4	19377490	19378123	Aa_G36620Aa_G36610
Aa.chr4	20099595	20100037	Aa_G199960;Aa_G199970;Aa_G199980
Aa.chr4	21669365	21669964	Aa_G289680;Aa_G289690;Aa_G289700
Aa.chr4	21729395	21730043	Aa_G16230Aa_G16220
Aa.chr4	21751740	21752422	Aa_G16180Aa_G16170Aa_G16160

Aa.chr4	21859653	21860329	Aa_G15970
Aa.chr4	22133061	22133707	Aa_G536070;Aa_G536060
Aa.chr5	860357	860925	Aa_G484270;Aa_G484260;Aa_G344960
Aa.chr5	2093969	2094761	Aa_G25680Aa_G25670
Aa.chr5	2986880	2987460	Aa_G264230;Aa_G264220;Aa_G264210
Aa.chr5	3027455	3028052	Aa_G570800
Aa.chr5	6067092	6067713	Aa_G207710;Aa_G207720
Aa.chr5	7418633	7419328	Aa_G377260;Aa_G377250;Aa_G377240
Aa.chr5	9216517	9217058	Aa_G690750
Aa.chr5	9498612	9499343	Aa_G404300
Aa.chr5	12394102	12395043	Aa_G114560;Aa_G114570
Aa.chr5	13874663	13875294	Aa_G14180
Aa.chr5	14086045	14086568	Aa_G402280
Aa.chr5	14969864	14970496	Aa_G300500;Aa_G300510
Aa.chr5	15874393	15875165	NA
Aa.chr5	17017776	17018309	Aa_G302210
Aa.chr5	17134434	17135303	Aa_G287230;Aa_G287220;Aa_G287210;Aa_G287200
Aa.chr5	17635904	17636701	Aa_G174990;Aa_G174980
Aa.chr5	18471103	18471862	Aa_G494450
Aa.chr5	19143890	19144523	Aa_G345830;Aa_G345840;Aa_G345850;Aa_G345860
Aa.chr5	21096032	21096657	Aa_G48840
Aa.chr5	21418114	21419051	Aa_G57870Aa_G57860
Aa.chr5	21422013	21422719	Aa_G57870Aa_G57860Aa_G57850;Aa_G57840
Aa.chr5	21945765	21946928	Aa_G2230N;Aa_G2240N
Aa.chr5	22633619	22634666	Aa_G163570
Aa.chr6	1277063	1277756	Aa_G46820Aa_G46830Aa_G46840
Aa.chr6	1657645	1658488	Aa_G191620;Aa_G191630;Aa_G191640;Aa_G191650
Aa.chr6	1954466	1955062	Aa_G99750Aa_G99740
Aa.chr6	2397149	2397768	Aa_G188890;Aa_G188900;Aa_G188910
Aa.chr6	4845602	4846117	Aa_G243730;Aa_G243740
Aa.chr6	7792407	7793065	Aa_G63400Aa_G63390Aa_G63380
Aa.chr6	9129593	9130116	Aa_G26280
Aa.chr6	12143440	12144039	Aa_G518710;Aa_G518700;Aa_G518690
Aa.chr6	12863421	12864035	Aa_G519830;Aa_G519820;Aa_G519810
Aa.chr6	16170331	16170831	Aa_G558790
Aa.chr6	16709405	16710003	Aa_G273610
Aa.chr6	17477452	17478104	Aa_G446030;Aa_G446020
Aa.chr6	21785172	21785900	Aa_G158280
Aa.chr6	22581023	22581652	Aa_G142720
Aa.chr6	27088515	27089308	Aa_G35730Aa_G35740
Aa.chr6	27909533	27910350	NA
Aa.chr6	28406512	28407062	NA
Aa.chr6	30266490	30267311	Aa_G311490
Aa.chr6	31160366	31160962	Aa_G666750;Aa_G666740
Aa.chr6	32450259	32450896	Aa_G375680
Aa.chr6	34113760	34114432	Aa_G495350;Aa_G495360
Aa.chr6	35386860	35387469	NA

Aa.chr7	44916	45616	Aa_G152250
Aa.chr7	2142189	2142876	NA
Aa.chr7	2902136	2902780	Aa_G37650
Aa.chr7	3492581	3493191	Aa_G504650
Aa.chr7	5097894	5098342	Aa_G6230N
Aa.chr7	6322647	6323279	Aa_G537940
Aa.chr7	7788197	7789405	Aa_G29820
Aa.chr7	7877048	7877704	Aa_G29660Aa_G29650Aa_G29640
Aa.chr7	11455442	11456139	Aa_G107310
Aa.chr7	17694042	17694664	Aa_G301350
Aa.chr7	21341547	21342347	Aa_G74800
Aa.chr7	24513004	24513563	Aa_G909220;Aa_G483230
Aa.chr7	26819123	26819799	NA
Aa.chr7	27072388	27073020	Aa_G214270
Aa.chr7	28756521	28757226	Aa_G374870;Aa_G374860
Aa.chr8	1912248	1913061	Aa_G556560;Aa_G556550
Aa.chr8	2614979	2615658	Aa_G331850;Aa_G331840;Aa_G331830
Aa.chr8	4044097	4044802	NA
Aa.chr8	4067029	4067608	Aa_G244390;Aa_G244380;Aa_G244370
Aa.chr8	4544031	4544689	Aa_G697580;Aa_G697570
Aa.chr8	6155459	6156143	Aa_G168630
Aa.chr8	6210828	6211676	Aa_G168710;Aa_G168720;Aa_G168730;Aa_G168740
Aa.chr8	6636777	6637707	Aa_G84670Aa_G84680
Aa.chr8	6693614	6694707	Aa_G158490;Aa_G158480
Aa.chr8	6854584	6855257	Aa_G681990
Aa.chr8	10130696	10131338	Aa_G320890;Aa_G320900;Aa_G320910;Aa_G320920
Aa.chr8	10236656	10237323	Aa_G321090;Aa_G321100
Aa.chr8	10296706	10297356	Aa_G431400
Aa.chr8	10387605	10388274	Aa_G236960;Aa_G236950
Aa.chr8	10848968	10849963	Aa_G356680;Aa_G356670;Aa_G356660
Aa.chr8	16628500	16629154	Aa_G15550Aa_G15540Aa_G15530
Aa.chr8	24130770	24131477	Aa_G503910;Aa_G503920
Aa.chr8	24625624	24626336	Aa_G348270
Aa.chr8	25541571	25542182	Aa_G396870;Aa_G396880
Aa.chr8	26833214	26833821	Aa_G94130Aa_G94140Aa_G94150
Aa.chr8	29846987	29847580	Aa_G441210
Aa.chr8	30583014	30583617	Aa_G232870
Aa.chr8	31346398	31346978	Aa_G307430
Aa.chr8	31624981	31625602	Aa_G227750
Aa.chr8	32128468	32129140	Aa_G93810Aa_G93820Aa_G93830
Aa.chr8	32424792	32425490	Aa_G297090
Aa.chr8	33798130	33798632	Aa_G457010;Aa_G457000;Aa_G456990
Aa.chr8	34755922	34756847	Aa_G630810;Aa_G630820
Aa.chr8	34939087	34939687	Aa_G46190Aa_G46180Aa_G46170
Aa.chr8	35475054	35475626	Aa_G169010;Aa_G169000
Aa.chr8	35577329	35578002	Aa_G398090;Aa_G398080
Aa.chr8	35630756	35631379	Aa_G70560Aa_G70570

Aa.chr8	36376886	36377778	Aa_G165590
Aa.chr8	37542648	37543269	Aa_G13410Aa_G13420Aa_G13430;Aa_G13440
Aa.chr8	38742996	38743933	Aa_G306090;Aa_G306100
Aa.chr8	39049623	39050457	Aa_G585290;Aa_G585300
Aa.chr8	40246882	40247455	Aa_G147100;Aa_G147110
Aa.chr8	40471748	40472718	Aa_G383040;Aa_G383050;Aa_G383060
scaff_46979_1	17217	18465	Aa_G646530
scaff_48794_1	35862	36910	Aa_G475960
scaff_51395_1	28402	29041	NA
scaff_52007_1	30497	31636	Aa_G510580
scaff_55656_1	19203	19843	Aa_G538770
scaff_55697_1	25844	26961	Aa_G565270;Aa_G565280
scaff_57616_1	11304	12346	NA
scaff_57989_1	31934	32868	Aa_G674060;Aa_G674070
scaff_57989_1	33857	34729	Aa_G674060;Aa_G674070
scaff_58716_1	12804	13873	NA
scaff_59833_1	11809	12814	NA
scaff_72209_1	32838	33478	Aa_G470530;Aa_G470540
scaff_73299_1	67556	68154	NA

Table A3 List of PEP1 BSs and associated target genes from a second experiment

This table lists PEP1 BSs and associated genes that were obtained in a second experiment. In this experiment, two replicates from the experiment presented in table A1 were re-sequenced together with one new replicate. All analyses presented here were performed with the first replicate shown in table A1.

Differentially expressed genes in *pep1-1*

apices	
ID	log_2(fold change)
Aa_G579940.t1	-2,111375492
Aa_G111060.t1	-1,972178966
Aa_G680400.t2	-1,61749689
Aa_G437560.t1	-1,593768654
Aa_G680400.t1	-1,516628284
Aa_G365540.t3	-1,506820383
Aa_G426950.t2	-1,476565334
Aa_G365540.t1	-1,471313637
Aa_G103800.t1	-1,457410085
Aa_G437570.t1	-1,401901804
Aa_G426950.t1	-1,400031441
Aa_G901190.t1	-1,356184365
Aa_G365540.t2	-1,286161043
Aa_G544180.t1	-1,232156316
Aa_G188220.t1	-1,165319823
Aa_G530690.t1	-1,160271318
Aa_G672840.t1	-1,114351941

leaves	
ID	log_2(fold change)
Aa_G111060.t1	-2,210566267
Aa_G579940.t1	-1,750722322
Aa_G365540.t1	-1,640871415
Aa_G365540.t3	-1,583832205
Aa_G365540.t2	-1,229876914
Aa_G437560.t1	-1,226850879
Aa_G103800.t1	-1,07434422
Aa_G437570.t1	-1,042801945
Aa_G454880.t1	-0,923404997
Aa_G680400.t2	-0,834349214
Aa_G680400.t1	-0,828987106
Aa_G356820.t1	-0,807004504
Aa_G544180.t1	-0,802469874
Aa_G90120.t1	-0,790647198
Aa_G901190.t1	-0,766963656
Aa_G103790.t1	-0,698255744
Aa_G795460.t1	-0,689630668

Aa_G463090.t1	-1,095733328
Aa_G530680.t1	-1,0320138
Aa_G614230.t1	-1,031884852
Aa_G90120.t1	-1,012448286
Aa_G268240.t1	-1,010549458
Aa_G680410.t1	-0,990826217
Aa_G103790.t1	-0,955671625
Aa_G117060.t1	-0,85510796
Aa_G348790.t1	-0,821478624
Aa_G448760.t1	-0,814250779
Aa_G680390.t1	-0,791026991
Aa_G76600.t1	-0,786804385
Aa_G66070.t1	-0,779892047
Aa_G943760.t1	-0,750484468
Aa_G29520.t1	-0,735988682
Aa_G29520.t2	-0,735988682
Aa_G287210.t1	-0,733880715
Aa_G223140.t1	-0,728492224
Aa_G126710.t1	-0,726185837
Aa_G451920.t2	-0,711695605
Aa_G451920.t1	-0,707276477
Aa_G11780.t1	-0,703582132
Aa_G206250.t1	-0,69981096
Aa_G356820.t1	-0,696968259
Aa_G600530.t2	-0,694364412
Aa_G600530.t1	-0,686873295
Aa_G586660.t1	-0,683746816
Aa_G206560.t1	-0,68150133
Aa_G997470.t1	-0,678524015
Aa_G214040.t1	-0,676801597
Aa_G594410.t1	-0,674133403
Aa_G566620.t1	-0,664454168
Aa_G126740.t1	-0,662050711
Aa_G307220.t1	-0,654302532
Aa_G274530.t1	-0,652833462
Aa_G43670.t1	-0,651351721
Aa_G356830.t1	-0,647715094
Aa_G43660.t1	-0,638809356
Aa_G389740.t3	-0,632274151
Aa_G206190.t1	-0,628380731
Aa_G240820.t1	-0,627927695
Aa_G389740.t2	-0,627814494
Aa_G240810.t1	-0,623667455
Aa_G831180.t1	-0,616710244
Aa_G110840.t1	-0,59907542
Aa_G103410.t1	-0,589831361
Aa_G339050.t1	-0,587262838

Aa_G426950.t2	-0,678809925
Aa_G287630.t1	-0,674494368
Aa_G188220.t1	-0,67257234
Aa_G763160.t1	-0,667511692
Aa_G463090.t1	-0,655650196
Aa_G763160.t2	-0,645325603
Aa_G426950.t1	-0,644206239
Aa_G126720.t1	-0,634857468
Aa_G600530.t2	-0,629566756
Aa_G600530.t1	-0,61763833
Aa_G356830.t1	-0,581237554
Aa_G206250.t1	-0,573087322
Aa_G79880.t1	-0,539565505
Aa_G50810.t3	-0,539348188
Aa_G389740.t5	-0,53375016
Aa_G682710.t1	-0,533351981
Aa_G389740.t4	-0,527268244
Aa_G126740.t1	-0,515908044
Aa_G680410.t1	-0,512957017
Aa_G50810.t1	-0,507152661
Aa_G50810.t2	-0,50534924
Aa_G322190.t1	-0,50215529
Aa_G298520.t1	-0,500008419
Aa_G37860.t1	0,500136713
Aa_G114570.t1	0,501371272
Aa_G455340.t1	0,502265876
Aa_G213390.t1	0,505894567
Aa_G91520.t1	0,509213027
Aa_G62420.t1	0,512262399
Aa_G346420.t1	0,512407672
Aa_G154150.t1	0,516118982
Aa_G303380.t1	0,522412774
Aa_G329800.t1	0,523107277
Aa_G47580.t2	0,524369471
Aa_G47580.t1	0,52901586
Aa_G306940.t1	0,531419492
Aa_G1090420.t1	0,534178739
Aa_G247670.t1	0,534400204
Aa_G292260.t1	0,534877332
Aa_G303380.t2	0,536216983
Aa_G259180.t1	0,537624818
Aa_G169570.t1	0,538419745
Aa_G303380.t3	0,539976887
Aa_G18710.t1	0,540325053
Aa_G69730.t1	0,540929324
Aa_G154140.t1	0,542576924
Aa_G96280.t1	0,545300728

Aa_G367250.t1	-0,58634557
Aa_G135940.t3	-0,583929636
Aa_G339920.t1	-0,575492273
Aa_G216840.t1	-0,570419133
Aa_G610350.t1	-0,569251416
Aa_G105030.t1	-0,568351141
Aa_G222150.t1	-0,566677716
Aa_G135940.t2	-0,561028798
Aa_G280410.t1	-0,557805884
Aa_G135940.t1	-0,557343328
Aa_G546740.t1	-0,545491755
Aa_G104670.t1	-0,544266961
Aa_G104670.t2	-0,544266961
Aa_G320570.t1	-0,543819508
Aa_G53710.t1	-0,543327326
Aa_G193670.t2	-0,541557712
Aa_G546740.t2	-0,540610471
Aa_G206090.t1	-0,534612255
Aa_G99560.t1	-0,531765269
Aa_G80170.t1	-0,531646571
Aa_G275570.t1	-0,528685487
Aa_G80170.t2	-0,525469008
Aa_G685480.t1	-0,520751524
Aa_G659680.t1	-0,518437545
Aa_G553700.t1	-0,515924772
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Aa_G36410.t1	-0,513020377
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Aa_G18400.t1	-0,502249403
Aa_G858190.t1	0,508177939
Aa_G484960.t1	0,509543778
Aa_G457340.t1	0,509967221
Aa_G502280.t1	0,511747959
Aa_G288950.t1	0,517582963
Aa_G53030.t1	0,521220635
Aa_G46710.t1	0,522175806
Aa_G446940.t1	0,524553233
Aa_G169010.t1	0,525275374
Aa_G10970.t1	0,526042589
Aa_G147540.t1	0,527822831
Aa_G193760.t1	0,527834377
Aa_G164300.t1	0,529635921
Aa_G491200.t1	0,529860997
Aa_G91520.t1	0,530056818
Aa_G32210.t2	0,531058688
Aa_G743030.t1	0,532817658
Aa_G31730.t1	0,534686744
Aa_G708450.t1	0,545940643
Aa_G97310.t1	0,55068038
Aa_G39360.t1	0,5509033
Aa_G14810.t1	0,55406837
Aa_G493700.t1	0,555588544
Aa_G98330.t1	0,556125678
Aa_G437270.t1	0,557992885
Aa_G169800.t1	0,558899646
Aa_G550040.t1	0,571018595
Aa_G481200.t1	0,578730374
Aa_G216820.t1	0,582354887
Aa_G443940.t1	0,584124199
Aa_G479240.t1	0,603895511
Aa_G657190.t2	0,606144225
Aa_G458160.t1	0,607017309
Aa_G657190.t1	0,608320097
Aa_G107860.t1	0,612166273
Aa_G600900.t1	0,612263204
Aa_G325820.t1	0,625106657
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Aa_G1019580.t1	0,640105147
Aa_G518690.t1	0,654823165
Aa_G128890.t1	0,657582579
Aa_G105830.t1	0,659247561
Aa_G189550.t1	0,671758496
Aa_G290950.t1	0,678361364
Aa_G189550.t2	0,711359375
Aa_G593160.t1	0,725224312
Aa_G571830.t1	0,739603685
Aa_G600910.t1	0,766607199
Aa_G508370.t1	0,785682453
Aa_G508370.t2	0,785682453
Aa_G246250.t1	0,800155882
Aa_G430660.t1	0,814446837
Aa_G430660.t2	0,833799007
Aa_G297760.t1	0,8540617
Aa_G361210.t1	0,884225317
Aa_G7910.t1	0,888113229
Aa_G110450.t1	0,899758794
Aa_G479080.t1	0,902716733
Aa_G238060.t1	0,923722572
Aa_G479080.t2	0,935524011
Aa_G110390.t1	0,953493384
Aa_G345840.t1	1,116971965
Aa_G157420.t1	1,167275237
Aa_G526200.t1	1,244659171
Aa_G356640.t1	1,310242198

Aa_G32210.t1	0,534851699
Aa_G80640.t1	0,535605905
Aa_G212200.t1	0,535639635
Aa_G4790.t1	0,536067017
Aa_G193020.t1	0,540263989
Aa_G140280.t1	0,540456425
Aa_G81690.t1	0,544084519
Aa_G422640.t1	0,544098584
Aa_G125430.t1	0,544506594
Aa_G31720.t1	0,545521756
Aa_G264210.t1	0,546886782
Aa_G247670.t1	0,547108263
Aa_G189750.t1	0,547144355
Aa_G99880.t1	0,552641234
Aa_G1010560.t1	0,553219302
Aa_G432310.t1	0,554840498
Aa_G170110.t1	0,555343836
Aa_G177460.t1	0,555586354
Aa_G391630.t1	0,556886528
Aa_G332640.t1	0,558716905
Aa_G766140.t1	0,559669051
Aa_G546850.t1	0,559794734
Aa_G464590.t1	0,559979347
Aa_G30760.t1	0,560707605
Aa_G204290.t1	0,561337724
Aa_G170110.t2	0,561349517
Aa_G358190.t1	0,56198803
Aa_G90410.t1	0,563646277
Aa_G508500.t1	0,56553152
Aa_G560370.t2	0,56743546
Aa_G368610.t1	0,568263492
Aa_G524710.t1	0,569050842
Aa_G560370.t1	0,569078689
Aa_G523390.t1	0,572322674
Aa_G125250.t1	0,573931997
Aa_G32210.t3	0,576771786
Aa_G333240.t1	0,580065372
Aa_G39360.t1	0,580669106
Aa_G204720.t1	0,581215375
Aa_G269490.t1	0,584414268
Aa_G67160.t1	0,585016779
Aa_G103940.t1	0,588103283
Aa_G589090.t1	0,592619491
Aa_G127200.t1	0,592736845
Aa_G216820.t1	0,594318716
Aa_G695140.t1	0,594610387
Aa_G96280.t1	0,595254163
Aa_G15970.t1	1,324844995
Aa_G633320.t1	1,331186976

Aa_G497410.t1	0,5962388
Aa_G757820.t1	0,596384555
Aa_G56170.t1	0,596818183
Aa_G214640.t1	0,597360878
Aa_G237640.t1	0,599247617
Aa_G382290.t1	0,601933552
Aa_G185520.t1	0,603393039
Aa_G168080.t1	0,605084563
Aa_G441210.t1	0,605300869
Aa_G489230.t1	0,605652429
Aa_G739680.t1	0,610426589
Aa_G417000.t1	0,611723098
Aa_G32200.t1	0,614062946
Aa_G246250.t1	0,615158944
Aa_G71670.t1	0,615424396
Aa_G214640.t2	0,615679035
Aa_G271510.t1	0,615725173
Aa_G12850.t1	0,617016284
Aa_G43090.t1	0,61722857
Aa_G110610.t1	0,61764383
Aa_G489080.t1	0,618975455
Aa_G238700.t1	0,619997389
Aa_G657190.t2	0,623288963
Aa_G290210.t1	0,625847321
Aa_G471220.t1	0,626869032
Aa_G448450.t1	0,627039814
Aa_G27380.t1	0,627823923
Aa_G657190.t1	0,628641661
Aa_G398700.t1	0,628816721
Aa_G137390.t1	0,629416608
Aa_G211420.t1	0,630162394
Aa_G59780.t1	0,631760959
Aa_G549800.t1	0,633149835
Aa_G480920.t1	0,634440152
Aa_G94360.t1	0,634707651
Aa_G190620.t1	0,638648224
Aa_G142490.t1	0,639075085
Aa_G110390.t1	0,639292018
Aa_G304200.t1	0,641472426
Aa_G325770.t1	0,641651313
Aa_G409770.t1	0,645369197
Aa_G254990.t1	0,646597362
Aa_G132990.t1	0,646730957
Aa_G174680.t1	0,646960514
Aa_G30840.t1	0,647501748
Aa_G229990.t1	0,647825676
Aa_G229990.t2	0,648164262

Aa_G287450.t1	0,650397313
Aa_G102840.t1	0,650911009
Aa_G16580.t1	0,651498482
Aa_G182230.t1	0,651678438
Aa_G437810.t1	0,651887143
Aa_G554000.t1	0,656894943
Aa_G39370.t1	0,658355258
Aa_G554000.t2	0,658906169
Aa_G41550.t1	0,661135493
Aa_G455340.t1	0,66221582
Aa_G317040.t1	0,664039766
Aa_G159540.t1	0,665747188
Aa_G613120.t1	0,667023511
Aa_G534430.t1	0,667181208
Aa_G250360.t1	0,668829126
Aa_G316810.t1	0,671538991
Aa_G218600.t1	0,673093504
Aa_G356670.t1	0,67312823
Aa_G25780.t1	0,673864304
Aa_G285140.t1	0,67477929
Aa_G189550.t1	0,676007883
Aa_G458420.t1	0,67727429
Aa_G821380.t1	0,678002478
Aa_G821380.t3	0,67818132
Aa_G821380.t2	0,679087526
Aa_G53230.t1	0,681406954
Aa_G647150.t1	0,681413417
Aa_G535570.t1	0,681767707
Aa_G14870.t1	0,681900602
Aa_G484790.t1	0,681969574
Aa_G53230.t2	0,682461211
Aa_G169800.t1	0,682664205
Aa_G189550.t2	0,68280716
Aa_G290220.t1	0,685557509
Aa_G50700.t1	0,689026298
Aa_G107860.t1	0,69384843
Aa_G170160.t1	0,69484244
Aa_G214780.t1	0,697954368
Aa_G203350.t1	0,703331131
Aa_G137430.t1	0,707684388
Aa_G173150.t1	0,710084419
Aa_G298350.t1	0,711462493
Aa_G52810.t1	0,711744449
Aa_G513240.t1	0,712248566
Aa_G72890.t1	0,712678406
Aa_G340190.t1	0,715670554
Aa_G442220.t1	0,716470369

Aa_G385690.t1	0,72659596
Aa_G553050.t1	0,728212365
Aa_G93990.t1	0,734408369
Aa_G105830.t1	0,736262233
Aa_G306090.t1	0,736726204
Aa_G288440.t1	0,736731562
Aa_G105780.t1	0,739601005
Aa_G477450.t1	0,740714898
Aa_G28500.t1	0,742957635
Aa_G766100.t1	0,747933094
Aa_G133550.t1	0,749722957
Aa_G112850.t1	0,751735581
Aa_G431410.t1	0,75322363
Aa_G153260.t1	0,757540995
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Aa_G518690.t1	0,762813523
Aa_G233520.t1	0,763909864
Aa_G117120.t1	0,764571251
Aa_G110450.t1	0,772432782
Aa_G828630.t1	0,773277891
Aa_G625050.t1	0,773896771
Aa_G81360.t1	0,77701861
Aa_G408350.t1	0,778382364
Aa_G438760.t1	0,779103074
Aa_G95150.t1	0,77989989
Aa_G163820.t1	0,781947426
Aa_G243730.t1	0,783951937
Aa_G661230.t1	0,789269641
Aa_G219270.t1	0,791071368
Aa_G420200.t1	0,792686014
Aa_G293600.t1	0,7947444
Aa_G331850.t1	0,794806401
Aa_G64160.t1	0,795716084
Aa_G596330.t1	0,800840605
Aa_G39380.t1	0,80697116
Aa_G420180.t1	0,811549998
Aa_G155410.t1	0,818169033
Aa_G128880.t1	0,820126817
Aa_G28060.t1	0,820770011
Aa_G186260.t1	0,82748743
Aa_G346420.t1	0,82865413
Aa_G627450.t1	0,832044284
Aa_G46220.t1	0,833805047
Aa_G312050.t1	0,834693917
Aa_G214090.t1	0,838592497
Aa_G168630.t1	0,841938778

Aa_G287220.t1	0,847813804
Aa_G51460.t1	0,851212998
Aa_G70560.t1	0,854883929
Aa_G765980.t1	0,864813733
Aa_G238060.t1	0,869466321
Aa_G259180.t1	0,890888822
Aa_G297070.t1	0,895196517
Aa_G27360.t1	0,898491757
Aa_G89330.t1	0,900138082
Aa_G320850.t1	0,910571366
Aa_G64190.t1	0,913644607
Aa_G797470.t1	0,91391575
Aa_G108760.t1	0,927713336
Aa_G16530.t1	0,931004087
Aa_G329430.t1	0,932140054
Aa_G200890.t1	0,933181565
Aa_G16530.t2	0,936728109
Aa_G471390.t1	0,948208663
Aa_G535590.t1	0,949893388
Aa_G114570.t1	0,950259747
Aa_G562070.t1	0,950479547
Aa_G361210.t1	0,951710848
Aa_G311160.t1	0,958840965
Aa_G200880.t1	0,965107189
Aa_G306110.t1	0,965791781
Aa_G332710.t1	0,983293625
Aa_G430660.t2	1,009085781
Aa_G430660.t1	1,010169217
Aa_G396640.t1	1,023417119
Aa_G426590.t1	1,03293463
Aa_G107220.t1	1,043221572
Aa_G12840.t1	1,047188809
Aa_G73490.t1	1,061995967
Aa_G129290.t1	1,077215297
Aa_G64180.t1	1,077335734
Aa_G18710.t1	1,081454058
Aa_G111000.t1	1,100683597
Aa_G550040.t1	1,107399994
Aa_G121580.t1	1,120876071
Aa_G437270.t1	1,16159137
Aa_G325820.t1	1,162275757
Aa_G552210.t1	1,202387185
Aa_G345840.t1	1,392928584
Aa_G25290.t1	1,394931531
Aa_G320630.t1	1,425120887
Aa_G561960.t1	1,430052589
Aa_G6500.t1	1,493769398

Aa_G633320.t1	1,56026525
Aa_G526200.t1	1,596847476
Aa_G157420.t1	1,6475988
Aa_G15970.t1	2,653863821
Aa_G263810.t1	2,729526598
Aa_G874400.t1	2,888302829

Table A4 List of DEG in *pep1-1* in leaves and apices

Common target genes of PEP1 and FLC

Common target genes among PEP1 and FLC			
***A. alpina* ID**	***A. thaliana* ID**	**BS**	**Description**
Aa_G73490	AT1G09530	conserved	phytochrome interacting factor 3 (PIF3)
Aa_G164280	AT1G13280	conserved	allene oxide cyclase 4 (AOC4)
Aa_G242480	AT1G14720	different	xyloglucan endotransglucosylase/hydrolase 28 (XTH28)
Aa_G557400	AT1G17940	conserved	Endosomal targeting BRO1-like domain-containing protein
Aa_G198680	AT1G22460	conserved	O-fucosyltransferase family protein
Aa_G613120	AT1G24260	conserved	SEPALLATA3 (SEP3)
Aa_G27720	AT1G44350	conserved	IAA-leucine resistant (ILR)-like gene 6 (ILL6)
Aa_G198410	AT1G75390	conserved	basic leucine-zipper 44 (bZIP44)
Aa_G239000	AT1G79970	conserved	unknown protein
Aa_G238990	AT1G79990	conserved	structural molecules
Aa_G311490	AT2G22540	conserved	SHORT VEGETATIVE PHASE (SVP)
Aa_G243730	AT2G32290	conserved	beta-amylase 6 (BAM6)
Aa_G15970	AT2G45660	conserved	AGAMOUS-like 20 (AGL20)
Aa_G226210	AT2G47440	conserved	Tetratricopeptide repeat (TPR)-like superfamily protein
Aa_G49840	AT3G06450	conserved	HCO3- transporter family
Aa_G104730	AT3G18550	conserved	BRANCHED 1 (BRC1)
Aa_G198880	AT3G22275	conserved	unknown protein
Aa_G345840	AT3G57920	conserved	squamosa promoter binding protein-like 15 (SPL15)
Aa_G152250	AT4G17870	different	PYRABACTIN RESISTANCE 1 (PYR1)
Aa_G37650	AT4G21200	different	gibberellin 2-oxidase 8 (GA2OX8)
Aa_G503910	AT4G23760	conserved	Cox19-like CHCH family protein
Aa_G29820	AT4G25490	different	C-repeat/DRE binding factor 1 (CBF1)
Aa_G301350	AT4G29780	different	unknown protein/nuclease
Aa_G443010	AT5G01190	conserved	laccase 10 (LAC10)
Aa_G168630	AT5G10740	different	Protein phosphatase 2C family protein
Aa_G356670	AT5G15850	conserved	CONSTANS-like 1 (COL1)
Aa_G356660	AT5G15860	conserved	prenylcysteine methylesterase (PCME)
Aa_G396870	AT5G52310	different	LOW-TEMPERATURE-INDUCED 78 (LTI78)
Aa_G297090	AT5G59050	conserved	unknown protein
Aa_G165590	AT5G60840	conserved	hypothetical protein
Aa_G320910	AT5G63120	conserved	P-loop containing nucleoside triphosphate hydrolases superfamily protein
Aa_G320900	AT5G63130	conserved	Octicosapeptide/Phox/Bem1p family protein
Aa_G147140	AT5G66800	conserved	unknown protein

Table A5 List of common targets of PEP1 and FLC

Flowering genes that were bound by PEP1 and FLC

PEP1 direct targets involved in flowering			
***A. alpina* ID**	***A. thaliana* ID**	**Gene name**	**pathway**
Aa_G73490	AT1G09530	PIF3 (PHYTOCHROME INTERACTING FACTOR 3)	photoperiod
Aa_G613120	AT1G24260	SEP3 (SEPALLATA 3)	Flower development
Aa_G655020	AT1G30040	GA2ox2 (GIBBERELLIN 2-OXIDASE 2)	GA
Aa_G312140	AT1G80340	GA3ox2 (GIBBERELLIN 3-OXIDASE 2)	GA
Aa_G311490	AT2G22540	SVP (SHORT VEGETATIVE PHASE)	Floral repressor
Aa_G15970	AT2G45660	SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS)	Floral integrator
Aa_G345840	AT3G57920	SPL15 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15)	age
Aa_G163570	AT3G63010	GID1B (GA INSENSITIVE DWARF1B)	GA
Aa_G37650	AT4G21200	GA2ox8 (GIBBERELLIN 2-OXIDASE 8)	GA
Aa_G356690	AT5G15840	CO (CONSTANS)	photoperiod
Aa_G307430	AT5G57380	VIN3 (VERNALIZATION INSENSITIVE 3)	Vernalization
Aa_G227750	AT5G57660	COL5 (CONSTANS LIKE 5)	Photoperiod
Aa_G207710	AT5G60120	TOE2 (TARGET OF EAT 2)	photoperiod
FLC direct targets involved in flowering			
	AT1G09530	PIF3 (PHYTOCHROME INTERACTING FACTOR 3)	photoperiod
	AT1G24260	SEP3 (SEPALLATA 3)	Flower development
	AT1G25560	TEM1 (TEMPRANILLO 1)	Floral repressor
	AT1G54830	NFYC9 (NUCLEAR TRANSCRIPTION FACTOR Y SUBUNIT C-9)	photoperiod
	AT1G65480	FT (FLOWERING LOCUS T)	photoperiod
	AT1G73687	miR159a (micro RNA 159a)	GA
	AT2G18790	PHYB (PHYTOCHROME B)	Light perception
	AT2G22540	SVP (SHORT VEGETATIVE PHASE)	Floral repressor
	AT2G45660	SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS)	Floral integrator
	AT3G57920	SPL15 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15)	age
	AT4G00650	FRI (FRIGIDA)	Vernalization
	AT4G11110	SPA2 (SPA1-RELATED 2)	photoperiod
	AT4G21200	GA2ox8 (GIBBERELLIN 2-OXIDASE 8)	GA
	AT4G30972	miR156b (micro RNA 156b)	age
	AT5G13790	AGL15 (AGAMOUS-LIKE 15)	Floral repressor
	AT5G27320	GID1C (GA INSENSITIVE DWARF1C)	GA
	AT5G37260	CIR1 (CIRCADIAN 1)	photoperiod

Table A6 List of PEP1 and FLC target genes that are involved in flowering

Table lists all direct targets of PEP1 and FLC that are involved in flowering according to the list list on the website of the Coupland lab (http://www.mpipz.mpg.de/14637/Arabidopsis_flowering_genes). Conserved targets of PEP1 and FLC are marked in dark green, common targets with different BSs in light green.

COR genes that were bound and regulated by PEP1 and FLC

PEP1 direct targets		
***A. alpina* ID**	***A. thaliana* ID**	**Gene name**
Aa_G394720	AT1G09350	galactinol synthase 3 (GolS3)
Aa_G396870	AT5G52310	LOW-TEMPERATURE-INDUCED 78 (LTI78)
Aa_G561960	AT1G51090	Heavy metal transport/detoxification superfamily protein
Aa_G13430	AT5G62360	Plant invertase/pectin methylesterase inhibitor superfamily protein
Aa_G320910	AT5G63120	P-loop containing nucleoside triphosphate hydrolases superfamily protein
Aa_G164300	AT1G13260	related to ABI3/VP1 1 (RAV1)
Aa_G15970	AT2G45660	AGAMOUS-like 20 (AGL20)
Aa_G56170	AT3G14440	nine-cis-epoxycarotenoid dioxygenase 3 (NCED3)
Aa_G656410	AT1G78610	mechanosensitive channel of small conductance-like 6 (MSL6)
Aa_G29660	AT4G25640	detoxifying efflux carrier 35 (DTX35)
Aa_G99750	AT4G02330	ATPMEPCRB
Aa_G495360	AT5G40390	seed imbibition 1-like (SIP1)
Aa_G163570	AT3G63010	GA INSENSITIVE DWARF1B (GID1B)
Aa_G287230	AT3G56070	rotamase cyclophilin 2 (ROC2)
Aa_G227750	AT5G57660	CONSTANS-like 5 (COL5)
Aa_G64190	AT2G05540	Glycine-rich protein family
Aa_G94130	AT4G12420	SKU5
Aa_G518700	AT1G22890	unknown protein
Aa_G70560	AT5G25280	serine-rich protein-related
Aa_G462540	AT1G80850	DNA glycosylase superfamily protein
Aa_G536060	AT2G46330	arabinogalactan protein 16 (AGP16)
Aa_G229990	AT1G56220	Dormancy/auxin associated family protein
Aa_G114570	AT3G52360	unknown protein
Aa_G57850	AT3G61210	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Aa_G281350	AT2G34620	Mitochondrial transcription termination factor family protein
Aa_G198410	AT1G75390	basic leucine-zipper 44 (bZIP44)
FLC direct targets		
	AT5G52310	LOW-TEMPERATURE-INDUCED 78 (LTI78)
	AT4G25480	dehydration response element B1A (DREB1A)
	AT3G05660	receptor like protein 33 (RLP33)
	AT1G69870	nitrate transporter 1.7 (NRT1.7)
	AT5G63120	P-loop containing nucleoside triphosphate hydrolases superfamily protein
	AT4G25500	arginine/serine-rich splicing factor 35 (RSP35)
	AT2G45660	AGAMOUS-like 20 (AGL20)
	AT1G69260	ABI five binding protein (AFP1)
	AT4G27410	RESPONSIVE TO DESICCATION 26 (RD26)
	AT1G68570	Major facilitator superfamily protein
	AT4G27520	early nodulin-like protein 2 (ENODL2)
	AT3G15460	Ribosomal RNA processing Brix domain protein
	AT1G75388	conserved peptide upstream open reading frame 5 (CPuORF5)
	AT5G18400	Cytokine-induced anti-apoptosis inhibitor 1, Fe-S biogenesis
	AT5G07990	TRANSPARENT TESTA 7 (TT7)
	AT3G53230	ATPase, AAA-type, CDC48 protein

	AT4G02880	unknown protein
	AT1G29940	nuclear RNA polymerase A2 (NRPA2)
	AT3G57150	homologue of NAP57 (NAP57)
	AT3G08640	Protein of unknown function (DUF3411)
	AT1G28060	Pre-mRNA-splicing factor 3
	AT5G17460	unknown protein
	AT1G52890	NAC domain containing protein 19 (NAC019)
	AT3G50560	NAD(P)-binding Rossmann-fold superfamily protein
	AT4G17070	peptidyl-prolyl cis-trans isomerases
	AT5G54980	Uncharacterised protein family (UPF0497)
	AT3G62860	alpha/beta-Hydrolases superfamily protein
	AT3G07560	peroxin 13 (PEX13)
	AT1G23870	trehalose-phosphatase/synthase 9 (TPS9)
	AT2G19800	myo-inositol oxygenase 2 (MIOX2)
	AT1G54820	Protein kinase superfamily protein
	AT1G10960	ferredoxin 1 (FD1)
	AT1G14900	high mobility group A (HMGA)
	AT3G06750	hydroxyproline-rich glycoprotein family protein
	AT1G67080	abscisic acid (ABA)-deficient 4 (ABA4)
	AT1G72430	SAUR-like auxin-responsive protein family
	AT5G20250	DARK INDUCIBLE 10 (DIN10)
	AT1G23860	RS-containing zinc finger protein 21 (RSZP21)
	AT5G49360	beta-xylosidase 1 (BXL1)
	AT5G14120	Major facilitator superfamily protein
	AT1G69530	expansin A1 (EXPA1)
	AT1G70290	trehalose-6-phosphatase synthase S8 (TPS8)
	AT4G21870	HSP20-like chaperones superfamily protein
	AT1G75390	basic leucine-zipper 44 (bZIP44)
genes regulated by PEP1		
Aa_G561960	AT1G51090	Heavy metal transport/detoxification superfamily protein
Aa_G706910	AT1G61800	glucose-6-phosphate/phosphate translocator 2 (GPT2)
Aa_G181570	AT5G04340	zinc finger of Arabidopsis thaliana 6 (ZAT6)
Aa_G111000	AT1G76590	PLATZ transcription factor family protein
Aa_G105830	AT1G01470	LATE EMBRYOGENESIS ABUNDANT 14 (LEA14)
Aa_G164300	AT1G13260	related to ABI3/VP1 1 (RAV1)
Aa_G15970	AT2G45660	AGAMOUS-like 20 (AGL20)
Aa_G28500	AT5G48540	receptor-like protein kinase-related family protein
Aa_G107220	AT4G27410	RESPONSIVE TO DESICCATION 26 (RD26)
Aa_G554000	AT2G24100	unknown protein
Aa_G56170	AT3G14440	nine-cis-epoxycarotenoid dioxygenase 3 (NCED3)
Aa_G332710	AT2G46680	homeobox 7 (HB-7)
Aa_G110450	AT1G68570	Major facilitator superfamily protein
Aa_G103940	AT1G16510	SAUR-like auxin-responsive protein family
Aa_G522030	AT4G23990	cellulose synthase like G3 (CSLG3)
Aa_G43090	AT4G26080	ABA INSENSITIVE 1 (ABI1)
Aa_G168080	AT5G57050	ABA INSENSITIVE 2 (ABI2)

Aa_G206190	AT5G01290	mRNA capping enzyme family protein
Aa_G409770	AT3G25730	ethylene response DNA binding factor 3 (EDF3)
Aa_G12850	AT2G47780	Rubber elongation factor protein (REF)
Aa_G430660	AT1G28330	dormancy-associated protein-like 1 (DYL1)
Aa_G69730	AT4G26530	Aldolase superfamily protein
Aa_G52810	AT3G49790	Carbohydrate-binding protein
Aa_G16580	AT1G32700	PLATZ transcription factor family protein
Aa_G290220	AT3G13062	Polyketide cyclase/dehydrase and lipid transport superfamily protein
Aa_G455340	AT4G19420	Pectinacetylesterase family protein
Aa_G39360	AT4G11360	RING-H2 finger A1B (RHA1B)
Aa_G718210	AT2G05540	Glycine-rich protein family
Aa_G41550	AT2G24550	unknown protein
Aa_G110390	AT1G68520	B-box type zinc finger protein with CCT domain
Aa_G298350	AT5G41080	PLC-like phosphodiesterases superfamily protein
Aa_G426590	AT2G18700	trehalose phosphatase/synthase 11 (TPS11)
Aa_G562070	AT1G23870	trehalose-phosphatase/synthase 9 (TPS9)
Aa_G51460	AT5G64410	oligopeptide transporter 4 (OPT4)
Aa_G70560	AT5G25280	serine-rich protein-related
Aa_G312050	AT1G80440	Galactose oxidase/kelch repeat superfamily protein
Aa_G304410	AT5G15120	Protein of unknown function (DUF1637)
Aa_G81690	AT2G15960	unknown protein
Aa_G480920	AT2G25900	ATCTH
Aa_G471390	AT3G15450	Aluminium induced protein with YGL and LRDR motifs
Aa_G304200	AT1G68840	related to ABI3/VP1 2 (RAV2)
Aa_G193020	AT1G56220	Dormancy/auxin associated family protein
Aa_G346420	AT2G32150	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein
Aa_G368610	AT5G28770	BZO2H3
Aa_G385690	AT1G80180	unknown protein
Aa_G62420	AT1G23390	Kelch repeat-containing F-box family protein
Aa_G42390	AT3G02550	LOB domain-containing protein 41 (LBD41)
Aa_G247670	AT3G29240	Protein of unknown function (DUF179)
Aa_G333240	AT3G11090	LOB domain-containing protein 21 (LBD21)
Aa_G7910	AT5G14740	carbonic anhydrase 2 (CA2)
Aa_G99880	AT2G18300	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
Aa_G658400	AT3G23880	F-box and associated interaction domains-containing protein
Aa_G96280	AT4G17245	RING/U-box superfamily protein
Aa_G374640	AT2G42690	alpha/beta-Hydrolases superfamily protein
Aa_G53230	AT4G01330	Protein kinase superfamily protein
Aa_G68090	AT5G20250	DARK INDUCIBLE 10 (DIN10)
Aa_G325820	AT3G48360	BTB and TAZ domain protein 2 (BT2)
Aa_G114570	AT3G52360	unknown protein
Aa_G361210	AT5G49360	beta-xylosidase 1 (BXL1)
Aa_G33740	AT5G18670	beta-amylase 3 (BMY3)
Aa_G128880	AT5G19120	Eukaryotic aspartyl protease family protein
Aa_G206580	AT4G13830	DNAJ-like 20 (J20)
Aa_G223450	AT3G16240	delta tonoplast integral protein (DELTA-TIP)
Aa_G297070	AT5G59080	unknown protein

Aa_G293600	AT1G80920	J8
Aa_G213390	AT4G21870	HSP20-like chaperones superfamily protein
Aa_G25780	AT3G26510	Octicosapeptide/Phox/Bem1p family protein
Aa_G214780	AT4G33666	unknown protein
Aa_G552210	AT5G22920	CHY-type/CTCHY-type/RING-type Zinc finger protein
genes regulated by FLC		
	AT4G33666	unknown protein
	AT1G13260	related to ABI3/VP1 1 (RAV1)
	AT2G45660	AGAMOUS-like 20 (AGL20)
	AT4G27410	RESPONSIVE TO DESICCATION 26 (RD26)
	AT1G28330	dormancy-associated protein-like 1 (DYL1)
	AT3G49790	Carbohydrate-binding protein
	AT1G32700	PLATZ transcription factor family protein
	AT2G24550	unknown protein
	AT1G68520	B-box type zinc finger protein with CCT domain
	AT5G41080	PLC-like phosphodiesterases superfamily protein
	AT2G18700	trehalose phosphatase/synthase 11 (TPS11)
	AT1G23870	trehalose-phosphatase/synthase 9 (TPS9)
	AT5G25280	serine-rich protein-related
	AT1G80440	Galactose oxidase/kelch repeat superfamily protein
	AT2G25900	ATCTH
	AT3G15450	Aluminium induced protein with YGL and LRDR motifs
	AT1G68840	related to ABI3/VP1 2 (RAV2)
	AT2G32150	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein
	AT5G28770	BZO2H3
	AT1G23390	Kelch repeat-containing F-box family protein
	AT5G20250	DARK INDUCIBLE 10 (DIN10)
	AT3G48360	BTB and TAZ domain protein 2 (BT2)
	AT5G49360	beta-xylosidase 1 (BXL1)
	AT5G18670	beta-amylase 3 (BMY3)
	AT5G19120	Eukaryotic aspartyl protease family protein
	AT1G80920	J8
	AT3G26510	Octicosapeptide/Phox/Bem1p family protein
	AT5G22920	CHY-type/CTCHY-type/RING-type Zinc finger protein
	AT5G46710	PLATZ transcription factor family protein
	AT2G39710	Eukaryotic aspartyl protease family protein
	AT5G20790	unknown protein
	AT2G19810	CCCH-type zinc finger family protein
	AT5G06860	polygalacturonase inhibiting protein 1 (PGIP1)
	AT3G44450	unknown protein
	AT2G29670	Tetratricopeptide repeat (TPR)-like superfamily protein
	AT3G50260	cooperatively regulated by ethylene and jasmonate 1 (CEJ1)
	AT3G47500	cycling DOF factor 3 (CDF3)
	AT2G44940	Integrase-type DNA-binding superfamily protein
	AT5G20230	blue-copper-binding protein (BCB)
	AT1G19530	unknown protein

AT3G61060	phloem protein 2-A13 (PP2-A13)
AT3G56260	unknown protein
AT4G35770	SENESCENCE 1 (SEN1)
AT2G28630	3-ketoacyl-CoA synthase 12 (KCS12)
AT1G72060	serine-type endopeptidase inhibitors
AT1G13700	6-phosphogluconolactonase 1 (PGL1)
AT1G27290	unknown protein
AT3G15630	unknown protein
AT5G11070	unknown protein
AT2G25200	Plant protein of unknown function (DUF868)
AT1G12780	UDP-D-glucose/UDP-D-galactose 4-epimerase 1 (UGE1)
AT1G22890	unknown protein
AT4G23180	cysteine-rich RLK (RECEPTOR-like protein kinase) 10 (CRK10)
AT5G56870	beta-galactosidase 4 (BGAL4)
AT4G17460	HAT1
AT1G54740	Protein of unknown function (DUF3049)
AT4G37610	BTB and TAZ domain protein 5 (BT5)
AT1G49200	RING/U-box superfamily protein
AT3G52060	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein
AT1G19770	purine permease 14 (PUP14)
AT1G03090	MCCA
AT2G45170	AUTOPHAGY 8E (ATG8E)
AT5G67420	LOB domain-containing protein 37 (LBD37)
AT5G61440	atypical CYS HIS rich thioredoxin 5 (ACHT5)
AT5G21170	AKINBETA1
AT1G68190	B-box zinc finger family protein
AT5G14120	Major facilitator superfamily protein
AT4G28270	RING membrane-anchor 2 (RMA2)
AT1G70290	trehalose-6-phosphatase synthase S8 (TPS8)
AT4G27450	Aluminium induced protein with YGL and LRDR motifs

Table A7 List of PEP1 and FLC target genes that are cold-regulated

Table lists all direct targets of PEP1 and FLC and genes that are regulated by the two TFs which are regulated by cold according to the robust list of 1279 COR genes defined by Park *et al*, 2015.

Table of Primers

Primers for ChIP-qPCR				
Species	**Gene**	**Primer**	**Sequence**	**Reference**
A. alpina	*AaBRC1*	V045	f AGGTGAAGAAGACGGCATGT	
		V046	r ACGTGCAAAGATGAAACACTCT	
A. alpina	*AaBRC1(-)*	V047	f TCCCTAAATTCAATCGTGTTTCCA	
		V048	r TCAAGTTTGCAAACATTGGGT	
A. alpina	*AaCBF1*	V093	f TGACCTGTTCTACTAGATCCTTCT	
		V094	r ACAATTTATCATCATCACCCGTCT	
A. alpina	*AaCBF1(-)*	V095	f AGATTCAGGCACTTAGGTATTGGTT	

		V096	r	CCTTTCCTTTCTTAAGATGCGGTTT
A. alpina	*AaCOL1*	V101	f	CTCTGGAGAAAATAACCTGAACATT
		V102	r	TGACACACCATGATGTCGCA
A. alpina	*AaCOL1(-)*	V103	f	TCCTTTCTTAGTGACCATTGCTACA
		V104	r	ACATGTTTACAATTGGACTACGCAA
A. alpina	*AaCOL5*	V037	f	AGTCTGAAGCAAAGATATGTGCAATA
		V038	r	GGTCGGCATGATTTTTCATTTATCG
A. alpina	*AaCOL5(-)*	V039	f	GTTACTCTAACCAGCCACCTCT
		V040	r	ACACCATACTCCATAGACGAAG
A. alpina	*AaG51090*	V089	f	TAATTCGTTTGTGATTCGAGACTCC
		V090	r	GAAATTAGAGTAAAGAACCTTCTCAGC
A. alpina	*AaG51090(-)*	V091	f	AGGGTTCTTACTAAAAGTGAGACCC
		V092	r	TATGAGCATGGTAAAATCAGACCCA
A. alpina	*AaGA2OX2*	V065	f	AGAGCGTGGAGTTGAACAGG
		V066	r	GGAGCAAGGATGGTGAGGTT
A. alpina	*AaGA2OX2(-)*	V067	f	ACCCGGTTAGAGCAAGAAGC
		V068	r	TCAATCCAACCAACGTCACCA
A. alpina	*AaGA2OX8*	V077	f	ACCAAAGTGCCTTCCTAAGCT
		V078	r	CCAACAGCTACGGGACTACC
A. alpina	*AaGA2OX8(-)*	V079	f	TATTTCTGACAACAAGGACTTCACG
		V080	r	TCAAAATTAACATGAAAGGTGTGACA
A. alpina	*AaGA3OX2*	V069	f	ACCAAAAAGATGATATGTGGACGT
		V070	r	TCTAGAGTGGGTCCATGAGCT
A. alpina	*AaGA3OX2(-)*	V071	f	TGTCACCAACACGACGATTCA
		V072	r	GCCCAACAAAAGGAACGCAA
A. alpina	*AaGID1B*	V073	f	CCAACGTTGAAAGGGAGGGA
		V074	r	GAAACGCCCTTGTCCGATCT
A. alpina	*AaGID1B(-)*	V075	f	GCAGTTGGCTTATGTTGATGGG
		V076	r	CCGAGTGAACAAACTTCTTCAACT
A. alpina	*AaGRP2B*	V053	f	ACTATCACGAACATTTGGCTGG
		V054	r	TCTACACAATTTCTTTTCATATTCCCA
A. alpina	*AaGRP2B(-)*	V055	f	CGAGAACCGGAGTGCTATCC
		V056	r	TGCAAGAACAAAGCAAAAGGA
A. alpina	*AaGolS3*	V105	f	AGTAGGTCGGTCATGTGTAGG
		V106	r	TGGGATCCACTACCGCAATAG
A. alpina	*AaGolS3(-)*	V107	f	AACAGTTTGATATGATTGTCACCTT
		V108	r	CTATACTCTACGAACTGCATCCACA
A. alpina	*AaLOS1*	V085	f	TCATCCATGCGAAAGTACAGTCT
		V086	r	AGTTTATGTTTCTATCCGTGACAAAA
A. alpina	*AaLOS1(-)*	V087	f	GAAAGAACAGATGACTCCACTCTCT
		V088	r	TAACCATCAACGACCCTAAAAGGAA
A. alpina	*AaLTI78*	V097	f	TGGTTATTCTTGGGACCCTCC
		V098	r	CGGCAATGATCTTTTATCCAGGAC
A. alpina	*AaLTI78(-)*	V099	f	GACGACAAAGATGAATCAGCTCTTG
		V100	r	TGACGAAGAAGAAAATGTCTCCATT
A. alpina	*AaPIF3*	V061	f	TGCATATTGACAAAGACTTCGATT
		V062	r	GCTCAAACAGAGGCATGTTGT

A. alpina	*AaPIF3(-)*	V063	f TGCATGTCAACGTGTACAAGT	
		V064	r TGGCCACGTGGATAAAGTAGA	
A. alpina	*AaSEP3*	V033	f GTGAGGATGGAATCGGACGG	
		V034	r GTCTTGACGTGGACCCTGTC	
A. alpina	*AaSEP3(-)*	V035	f TGAGAGAGAAGTGTCTTGTTTGTGA	
		V036	r TCTCTCTCCCCAACTCCACA	
A. alpina	*AaSPL8*	V081	f AACGTCGATGGCAGCCTTTA	
		V082	r GGTGCGAGTGAGGGTGTAAT	
A. alpina	*AaSPL8(-)*	V083	f ATGTTTGTTTGTCCGTACACTTCTC	
		V084	r TGATTAAGCAAAGGTCATTTCAGTCA	
A. alpina	*AaSPL15*	V041	f ACCAAAACGCAAACGCTAAAGT	
		V042	r AGAAAGGACCAAGAAGAAGAAAGGA	
A. alpina	*AaSPL15(-)*	V043	f AGGTTCTGAGGCAATACATGGA	
		V044	r AGCTCTTGGTGCAGATGAAA	
A. alpina	*AaSOC1*	J174	f GAAGGTGGAAAAAGATGTGT	provided by J. Mateos
		J175	r GTTGTAGTAATGGTGTTGGAACC	provided by J. Mateos
A. alpina	*AaSOC1(-)*	J176	f GGATGTTTGTGGATTCACATC	provided by J. Mateos
		J177	r ATCATAATTATTTCCGAAACGA	provided by J. Mateos
A. alpina	*AaSVP*	V029	f TCCACAAGATTGTTATCATTTCTTTCA	
		V030	r AGAGAGTGACAAGACGACTGAAAT	
A. alpina	*AaSVP(-)*	V031	f TGTTATGTGTAGAGATTTGAGCTTAGA	
		V032	r GGGCGTGATCAGTGTTCTCT	
A. alpina	*AaTOE2*	V057	f ACCATCCCACGCATGATACT	
		V058	r TGAAAGCATTGAACAAGAAGTGGT	
A. alpina	*AaTOE2(-)*	V059	f TTCAGCTTAACCATGCCCCT	
		V060	r TGAGATATCGAGAAGGCAAGTCT	
A. alpina	*AaVIN3*	V049	f TCCAATGGTTACCAGTCACATCA	
		V050	r CTCTGAGAGCTTCTTTCCTTCT	
A. alpina	*AaVIN3(-)*	V051	f TGCTGGTATCTGCCTCAAGC	
		V052	r CATCAGAAGCAAAGCCGTGT	
A. thaliana	*PIF3*	V151	f TGCATATTGATGATTGAGACATTGA	
		V152	r TGCCAGAAACAAAAATTGTACACT	
A. thaliana	*PIF3(-)*	V153	f TGGACCTGGTTAAACAAGCGT	
		V154	r TCGCGTGGGTATATATTCAGCT	
A. thaliana	*GA2OX8*	J253	f TCCCCATATCTCATGCGTTTCT	provided by J. Mateos
		J254	r ACATGCCAACTTGCTATCCCA	provided by J. Mateos
A. thaliana	*GA2OX8(-)*	J255	f AGACTGACCGGATTGTGGTA	provided by J. Mateos
		J256	r ATCCGGTTGGATTAGCTCGG	provided by J. Mateos

Primers for RT-qPCR

Species	Gene	Primer	Sequence	Reference
A. alpina	*AaAP1*	SDP186	f CAG TGG GAT CAG CAG AAT CA	provided by S. della Pina
		SDP187	r CTC CTC ATT GCC ATT GGA TC	provided by S. della Pina
A. alpina	*AaBRC1*	V003	f ACTCACCAGTGCAGCTTCAG	
		V004	r GTTGCTACCTTTGTCGTCCG	
A. alpina	*AaCBF1*	V021	f CGATTGTGTTATGTCCAGGTAATCAT	
		V022	r GTTTACAGAGCTCGGTACTTTCC	
A. alpina	*AaCOL1*	V025	f TGGTGACTCTGGCACAACTC	

		V026	r AGGGTCACATGCACCATGAG	
A. alpina	*AaCOL5*	V001	f GCTCTCTGCTTTCACTTACCCA	
		V002	r ACCTCATTACCCTAGCTTCCCT	
A. alpina	*AaFUL*	SDP196	f GGA TAC TTG AAC GCT ATG ATC G	provided by S. della Pina
		SDP197	r TCA ACG AAT CAA GAT CTT CCC C	provided by S. della Pina
A. alpina	*AaG51090*	V019	f GCTTTGGTGGTTCGAGAATGAC	
		V020	r TCTCGATTGTTCCAAATGCTACA	
A. alpina	*AaGA2OX1*	V198	f TCCGGTGCAAATCCTCTCTT	
		V199	r TGTGTTCCTCGGTTTGATTCCT	
A. alpina	*AaGA2OX2*	J150	f CGGTTAGAGCAAGAAGCTATG	provided by J. Mateos
		J151	r ACTCCTCCATGTACTCCTCTACTG	provided by J. Mateos
A. alpina	*AaGA2OX6*	J166	f CTTCATGCAAACCCAACTTC	provided by J. Mateos
		J167	r CGTTAGATCAACGATCTCACATG	provided by J. Mateos
A. alpina	*AaGA3*	V190	f CCTCAATTCGAACGATACTGCC	
		V191	r GTCGCAAGTCAGGACTGTCA	
A. alpina	*AaGA3ox1*	V188	f TTCCGGTTACCTGTCCAACG	
		V189	r GCCTGAGATGGTGAAGCCTT	
A. alpina	*AaGA3OX2*	V11	f GCCAACCACATCAACTTCGC	
		V12	r TGGCCCAACCATCACGTAAA	
A. alpina	*AaGA20OX1*	V202	f CCGCTCAAAAACCGTCCAAG	
		V203	r ACAGAGAACTCATTGCCTCACA	
A. alpina	*AaGA20ox2*	J134	f CAATCAATGGCGATCCATTCTAC	provided by J. Mateos
		J135	r ATCTTCTTGTTGTCACCTTCTGC	provided by J. Mateos
A. alpina	*AaGID1B*	V13	f GTCTGGACGACGGAGAATGT	
		V14	r GGTAACCAAGTCAACCTCAACG	
A. alpina	*AaGolS3*	V027	f AACGAAGCCAAGGTTGTTCATT	
		V028	r TTCTTGTCCACAATGCACATGA	
A. alpina	*AaGRP2B*	V007	f GGAGAGAGACGCAAGGGTTC	
		V008	r ACCGTCGTTTGGTGTGATGA	
A. alpina	*AaLFY*	SDP105	f ACG CCG TCA TTT GCT ACT CT	Bergonzi et al, 2013
		SDP106	r TTT GCG TCA TCG TCT GTC TC	Bergonzi et al, 2013
A. alpina	*AaLOS1*	V017	f TGCAGAGGAGCAACGGAAAA	
		V018	r TCTCCAGCGGATTCTTGAGC	
A. alpina	*AaLTI78*	V023	f GCACGATCAAGATGTGGACG	
		V024	r CCAGTTACTCCTCTCGCAGC	
A. alpina	*AaPEP1*	PEP1-RT-fwd	f CTTGTCGTCTCCTCCTCTGG	Wang et al. 2011
		PEP1-RT-rev	r ACTACGGCGAGAGCAGTTTC	Wang et al. 2011
A. alpina	*AaPIF3*	J117/118	f ACAACATGCCTCTGTTTGAGC	
		J118	r TGGTGGTGGAATGTTCCTTG	
A. alpina	*AaPP2A*	V125	f AGTATCGCTTCTCGCTCCAG	Bergonzi et al, 2013
		V126	r AACCGGTTGGTCGACTATTG	Bergonzi et al, 2013
A. alpina	*AaSEP3*	J044	f GGTATCAGATGCCACTCCAAC	provided by J. Mateos
		J045	r ACACTTGGTCCTGCTCCCATTC	
A. alpina	*AaSOC1*	W268	f GCT TTC AGT GCT TTG TGA TGC	Wang et al. 2011
		W269	r GGA TGC TTC GAG TTG TTC GAT	Wang et al. 2011
A. alpina	*AaSPL8*	V15/16	f AAAGCATCGACGGTTGTTGC	
		V16	r GGTCAGCCAGTCTCTTACGG	

A. alpina	*AaSPL15*	YH13	f ACG CTA GAA CTG CTC CAT CTC	provided by Y. Hyun
		YH14	r GAG TCT GTG CCA TTG TTG TTC	provided by Y. Hyun
A. alpina	*AaSTM*	V110	f TCCTCAGATGACCCATTGTTGT	
		V111	r TCAACGCGTCGAGTGTCAAT	
A. alpina	*AaSVP*	SBR SW01	f CGTTCCATCTCTAACCACCA	provided by S. Wötzel
		SSF SW01	r CAGCAAGGATCGCAACTTAC	provided by S. Wötzel
A. alpina	*AaTEM1*	V355	f ACCACGGAACTTACGAGCAG	
		V356	r CAAACGGAAGATGGGGAGCT	
A. alpina	*AaTEM2*	V357	f CGTGACGGGTAAAGTGTGGA	
		V358	r ACGTAACAACATCACCGGCT	
A. alpina	*AaTOE2*	V009	f GACCACCATCGGCTTGATCT	
		V010	r CCAGGATGACTTGCCTACTCC	
A. alpina	*AaVIN3*	V005	f ACGCCTCCTCGGAGTCTAAT	
		V006	r GTCGCACCATCGGAATCTGA	
A. thaliana	*AT1G51090*	V121	f GGTCAAGAAAGCTATCCGCAAAT	
		V122	r AGCCTCTCAGGATCGTAACA	
A. thaliana	*BRC1*	V129	f TTCCCAGTGATTAACCACCAT	
		V130	r TCCGTAAACTGATGCTGCTC	
A. thaliana	*CBF1*	V113	f CCTTATCCAGTTTCTTGAAACAGAG	Deng et al, 2011
		V114	r GCGAAGTTGAGACATGCTGA	Deng et al, 2012
A. thaliana	*COL1*	142	f CACCTTACCCTCCAGCTCAG	Simon et al, 2015
		145	r GTGGAGAAAGCTTGGTTTGC	Simon et al, 2015
A. thaliana	*COL5*	V127	f GAAAACAGAGCCTCTCCCGT	
		V128	r TCCGTCAGGAACTACACCGT	
A. thaliana	*DDF1*	V319	f GATCCGCATGTTTGAATTTCG	Magome et al, 2004
		V320	r ATCATTGGATTCCGGCACC	Magome et al, 2004
A. thaliana	*GA2OX1*	V218	f CTTCGCTGGACCTTCATTGAC	Achard et al, 2008
		V219	r ACAACCTCTCGTCCTCATTGTCT	Achard et al, 2008
A. thaliana	*GA2OX2*	V135	f GCAGGAGGCTATTGGCTTCTTCG	
		V136	r CTGAGGATTAGCATTGAGGAGGAGATAC	
A. thaliana	*GA2OX6*	k308	f CCC ATC TGA CCC TAC ATG CT	provided by F. Andrés
		k309	r GAC ACC ATT TTT GGC AAC G	provided by F. Andrés
A. thaliana	*GA2OX8*	J251	f TAGCTTCTCAGTCACAGCCG	provided by J. Mateos
		J252	r CCTCCTCGGCTCCATCAATC	provided by J. Mateos
A. thaliana	*GA3*	V208	f GTCTCCACTCTCCCCTCTGT	
		V209	r AGTTGCAGCAAGTTCCCAAT	
A. thaliana	*GA3OX1*	J196	f CATCCCATTCACCTCCCACACTCTCACATAC	Osnato et al, 2012
		J197	r AGGAGAAGGAGCAGCGGAGAAGAGGAG	Osnato et al, 2012
A. thaliana	*GA3OX2*	J198	f GACTTGCTCCACATTTTAACCAACGGAATCTTC	Nakajima et al, 2006
		J199	r CCACAGGTAAGCCATTGAGAACCGAGATC	Nakajima et al, 2006
A. thaliana	*GA20OX1*	V192	f TTTCACCGGACGCTTCTCC	Achard et al, 2008
		V193	r CGCAAAACCGGAAAGAAAGG	Achard et al, 2008
A. thaliana	*GA20OX2*	k211	f ATG GCG TTT TTC TTG TGT CC	provided by F. Andrés
		k212	r CCA ATT CGA AAA GGA ATC GA	provided by F. Andrés
A. thaliana	*GID1B*	V137	f GAACCCTCGAGCTAACCAAACCTCTC	
		V138	r GGAGTAAGAAGCACAGGACTTGACTTGC	
A. thaliana	*GolS3*	V117	f GGCTTACTACGTCCTCAACTACT	

		V118	r TGCCGTCAGGAAGATCAAACA	
A. thaliana	*GRP2B*	V123	f GACCTGGTTCACCGCACTTA	
		V124	r AGGAAGCTACGGAGGAGGTT	
A. thaliana	*LTI78*	V115	f GCAATGAGCATGAGCAAGATCA	
		V116	r TCCTCCGATGCTGGAACATTAG	
A. thaliana	*PIF3*	V141	f ACCATGCCTCTGTTTGAGCT	
		V142	r CCACACCAGCTCCACAACTT	
A. thaliana	*PP2A*	PP2AA3-F	f CAGCAACGAATTGTGTTTGG	Czechowski et al. 2005
		PP2AA3-R	r AAATACGCCCAACGAACAAA	Czechowski et al. 2005
A. thaliana	*RGL2*	J278	f CCAAAACCACTACCAGCTTCTC	
		J279	r CAGCCATCTCAGAAGATCGAAC	
A. thaliana	*SEP3*	Q21	f GGCTGGTATCGAACAGAGGT	provided by R. Richter
		Q22	r TTGAAGGCACATTGGGTTCT	provided by R. Richter
A. thaliana	*SOC1*	k288	f TGATGAAGAGAGTAGCCCAAG	provided by F. Andrés
		k289	r TGAGAGAGAGAGAGTGAGAGAGAAA	provided by F. Andrés
A. thaliana	*SPL8*	V139	f ACTTGCTGACCATAACCGCC	
		V140	r GACGCTTTAACACCCGAATCG	
A. thaliana	*SPL15*	Q023	f CAAAGTTTGTTGCATTCACTCTAAA	provided by R. Richter
		Q024	r CAAACTCAGAAAGCTGGTGAAA	provided by R. Richter
A. thaliana	*SVP*	Q059	f CCGGAAAACTGTTCGAGTTC	provided by R. Richter
		Q060	r TGACTGCAAGTTATGCCTCTCT	provided by R. Richter
A. thaliana	*TEM1*	V315	f ATCCACTGGAAAGTCCGGTCTA	Osnato et al, 2012
		V316	r GAATAGCCTAACCACAGTCTGAACC	Osnato et al, 2012
A. thaliana	*TEM2*	V317	f TGGTCCGAGAGAAAACCCG	Osnato et al, 2012
		V318	r TCAACTCCGAAAAGCCGAAC	Osnato et al, 2012
A. thaliana	*TOE2*	V133	f TGTCAAATTCCGGGGTCTGG	
		V134	r AGAAACCAGAGCTCTGTCGC	
A. thaliana	*VIN3*	V131	f CCATGTTCTCTGGACCCACA	
		V132	r AGAACACGAAGAACGACAAACA	

Primers for cloning			
Gene	**Primer**	**Sequence**	**Usage**
AtGA2OX2	GW_gAtGA2OX2_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATATAAA TAAATTCTGTTTGTTGGAAAAAACAAAAAAAATTGT	amplify genomic fragment
	gAtGA2OX2_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATTTTGT TCTTTTGATTCTTTCTCTGGCTTTT	
	9A_AtGA2OX2_F	GGCGCTGCGGCTGCTGCCGCTGCGGCAGCGGTGGTTTT GCCACAGCCAGTC	add N-terminal 9A-venus
	AtGA2OX2_9A_R	GCCCGCTGCCGCAGCGGCAGCAGCCGCAGCCATTACAA GGGTTTTATGATTGAGAAGAGGTTGTTT	add C-terminal 9A-venus
	pAtGA2OX2_1Ven_R	CCTCGCCCTTGCTCACCATGTTTGGTTTTTGTAGGTTTAC ACTTAACTTGCAAA	add N-terminal 9A-venus
	pAtGA2OX2_1Ven_F	TTTGCAAGTTAAGTGTAAACCTACAAAAACCAAACATGG TGAGCAAGGGCGAGG	add N-terminal 9A-venus
	3pAtGA2OX2_1Ven_F	GGCATGGACGAGCTGTACAAGTAAGAGTAGTCATGATG ATCTTTATCATCCTTTGTACG	add C-terminal 9A-venus
	1Ven_3pGA2OX2R	CGTACAAAGGATGATAAAGATCATCATGACTACTCTTAC TTGTACAGCTCGTCCATGCC	add C-terminal 9A-venus

AtGA3OX1	ORF_At2ox2_Poverl_F	TACAAAAACCAAACATGGTGGTTTTGC	add C-terminal 9A-venus
	PAt2ox2_ORF_overl_R	GCAAAACCACCATGTTTGGTTTTTGTA	add C-terminal 9A-venus
	GW_gAtGA3OX1_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTTTACTTTGGTTTGTTTGCTTAGTCAAAATTTAAGTTT	amplify genomic fragment
	gAtGA3OX1_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAACCTGATCATTTTCTTGATCTTATTATAGGAAAAAATGTC	
	9A_AtGA3OX1_F	GGCGCTGCGGCTGCTGCCGCTGCGGCAGCGCCTGCTATGTTAACAGATGTGTTTAGAGGCC	add N-terminal 9A-venus
	AtGA3OX1_9A_R	GCCCGCTGCCGCAGCGGCAGCAGCCGCAGCCATTTCTTCTCTGTGATTTCTAATCATTGAAAGAGCTT	add C-terminal 9A-venus
	pAtGA3OX1_1Ven_R	CCTCGCCCTTGCTCACCATCTTGCTCTTTTTTAATTAGTTTTAAAACTTTGTAAATTTGATAGATGT	add N-terminal 9A-venus
	pAtGA3OX1_1Ven_F	ACATCTATCAAATTTACAAAGTTTTAAAACTAATTAAAAAAGAGCAAGATGGTGAGCAAGGGCGAGG	add N-terminal 9A-venus
	3pAtGA3OX1_1Ven_F	GGCATGGACGAGCTGTACAAGTAATTAGATAATAATAGTTGTGATCTACTAGTTAGTTTGATTAATAAATTGTTGTAAATGATT	add C-terminal 9A-venus
	1Ven_3pAtGA3OX1R	AATCATTTACAACAATTTATTAATCAAACTAACTAGTAGATCACAACTATTATTATCTAATTACTTGTACAGCTCGTCCATGCC	add C-terminal 9A-venus
9A-venus	ORF_At3ox1_Poverl_F	CTAATTAAAAAAGAGCAAGATGCCTGCTATGTTAAC	add C-terminal 9A-venus
	PAt3ox1_ORF_overl_R	GTTAACATAGCAGGCATCTTGCTCTTTTTTAATTAG	add C-terminal 9A-venus
	FA21-R	CGCTGCCGCAGCGGCAGCAGCCGCAGCGCCCTTGTACAGCTCGTCCATGCCG	add 9A-venus N-terminal (kindly provided by R. Martinez)
	Q058-F	ATGGCTGCGGCTGCTGCCGCTGCGGCAGCGGGCATGGTGAGCAAGGGCGAGG	add 9A-venus C-terminal (kindly provided by R. Martinez)
AtmSPL15	V174	TTTCTTTCTTTCTCTCTCTTCTCTTCTCTCTGATTCCCCGGGAGATAGCAACATCTAAAATCTGCAAAACCACAT	mutate CArG-box in *AtSPL15*
	V175	ATGTGGTTTTGCAGATTTTAGATGTTGCTATCTCCCGGGGAATCAGAGAGAGAGAAGAGAGAGAAAGAAAGAAA	
AamSPL15	V163	GTCAGCTTCTCTCTTTCTCTTTCTCTCTCTCATCTCCCGGGAGATAGCAAAAACCTCATTTTATTTCCTTTCTTCTTCTT	mutate CArG-box in *AaSPL15*
	V164	AAGAAGAAGAAAGGAAATAAAATGAGGTTTTTGCTATCTCCCGGGAGATGAGAGAGAGAAAGAGAAAGAGAGAAGCTGAC	
PEP1	HY88	GACAAAATATAGTTTCAGTTATAACCAATATTAA	genotype for *pep1-1* mutation
	HY89	CCGAATGATCCTATATATCTGAGA	

Table A8 Primers

Generation of transgenic *A. alpina* and *A. thaliana* lines expressing mutant *SPL15*

Cloning of the complete genomic region of *A. thaliana SPL15*, the N-terminal fusion to *9A-Venus* (9A-V) as well as the mutation of the miR156 BS (*rSPL15*) was previously described (Hyun *et al*, 2016). In addition, a CArG-box with the sequence CTTTAAAAG in the FLC BS was mutated to CCCCGGGAG (*mSPL15*) using the polymerase incomplete primer extension cloning method (Klock & Lesley, 2009). The primers to introduce the CArG-box mutation were

V174 and V175 (Table A8). The resulting *9A-V-mSPL15* and *9-AV-mrSPL15* were completely sequenced and cloned into destination vector pEarlyGate301 (kindly provided by R. Martinez-Gallegos). Plasmids were transformed into *A. tumefaciens* and the different versions of *AtSPL15* (*9A-V-WtSPL15, 9A-V-rSPL15, 9A-V-mSPL15 and 9A-V-mrSPL15*) were transformed into *ColFRI*$^{+}$ as well as *flc-3* mutant plants by floral dip (Clough & Bent, 1998) according to the common lab protocol for 'floral massage'. T3 Transgenic lines are available for analysis of *SPL15* expression and the influence of FLC, vernalization and miR156 on *SPL15* expression.

The same procedure was used to generate different versions of *9A-V-SPL15* in *A. alpina*. The complete genomic region of *A. alpina SPL15* with the N-terminal fusion to *9A-Venus* (*9A-V-AaSPL15*) as well as the mutation of the miR156 BS (*rAaSPL15*) was kindly provided by R. Martinez-Gallegos. A CArG-box with the sequence CTTTTAAAAG in the PEP1 BS was mutated to CTCCCGGGAG (*mAaSPL15*) using the polymerase incomplete primer extension cloning method (Klock & Lesley, 2009). The primers to introduce the CArG-box mutation were V163 and V164 (Table A8). The resulting *9A-V-mAaSPL15* and *9-AV-mrAaSPL15* were completely sequenced and cloned into destination vector pEarlyGate301 (kindly provided by R. Martinez-Gallegos). Plasmids were transformed into *A. tumefaciens* and *pep1-1* mutant plants were transformed by floral dip (Clough & Bent, 1998) according to the common lab protocol for *A. alpina* floral dipping. T1 transformants were crossed to Pajares and F1 hybrids as well as T2 transgenic lines are available for analysis of Aa*SPL15* expression and the influence of PEP1, vernalization and miR156 on Aa*SPL15* expression.

Generation of *A. thaliana* lines expressing *GA3OX1::GA3OX1* and *GA2OX2::GA2OX2* fused to Venus

The complete genomic regions (genes and up- and downstream sequence until the neighboring genes) of *A. thaliana GA3OX1* and *GA2OX2* were amplified by PCR and cloned into entry vector stb205 (R. Martinez-Gallegos, unpublished). N-terminal and C-terminal fusions to 9A-Venus (cloned genes were kindly provided by R. Martinez-Gallegos) were created using the polymerase incomplete primer extension cloning method (Klock & Lesley, 2009). After complete sequencing of the insert, inserts were cloned into destination vector pAlligator2only. Plasmids were transformed into *A. tumefaciens* and *ColFRI*$^{+}$ as well as *flc-3* mutant plants were transformed by floral dip (Clough & Bent, 1998) according to the common lab protocol for 'floral massage'. Primers used for cloning are listed in Table A8. The T1 generation of transgenics is available to study expression of these genes and their regulation by FLC.

List of Abbreviations

°C	degree Celsius
µ	micro
5’	5-prime
9A-V	9alanine-venus
A	adenin
Aa	*Arabis alpina*
ABA	abscisic acid
Ae	*Aethionema arabicum*
AG	*AGAMOUS*
Al	*Arabidopsis lyrata*
Am	*Arabis montbretiana*
AM	axillary meristems
AP	*APETALA*
At	*Arabidopsis thaliana*
Bhlh	basic helix-loop-helix
bp	base pair
BRC1	*BRANCHING1*
BS	binding site
bZIP	Basic Leucine Zipper
C	Cytosine
CBF1	*CRT/DRE BINDING FACTOR 1*
CAPS	cleaved amplified polymorphic sequence
CCT	CONSTANS, CONSTANS-LIKE, TOC1
cDNA	complementary DNA
CEBPα	*CCAAT/enhancer-binding protein-α*
ChIP	chromatin immunoprecipitation
Chr	chromosome
CIR1	*CIRCADIAN 1*
COL	*CO-like*
Col-0	Columbia-0
COR	cold-regulated
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats (CRISPR)- CRISPR associated
CRM	*cis*-regulatory modules
CZF2	*COLD INDUCED ZINC FINGER PROTEIN 2*
DDF1	*DWARF AND DELAYED FLOWERING 1*
DEG	differentially expressed gene/s
DMSO	Dimethyl sulfoxide
DNA	desoxyribonucleic acid
EtOH	ethanol
f	forward
FCA	*FLOWERING TIME CONTROL PROTEIN ALPHA*
FD	*FLOWERING LOCUS D*
FDR	false discovery rate
FLC	*FLOWERING LOCUS C*
FLM	*FLOWERING LOCUS M*
FOXA2	*FORKHEAD BOX A2*
FRI	*FRIGIDA*
FRI^{+}	SF2 FRI allele
FT	*FLOWERING LOCUS T*
FUL	*FRUITFUL*
FW	fresh weight
g	gram/s
G	guanine
GA	gibberellic acid
GID1	*GIBBERELLIN INSENSITIVE DWARF 1*
GNC	*GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED*
GNL	*GNC-LIKE*
GO	gene ontology

GolS3	*GALACTINOL SYNTHASE 3*
GRP2B	*GLUTAMINE-RICH PROTEIN2B*
h	hour/s
H3K27me3	histone H3 lysine 27 trimethylation
HLB	Hydrophilic-Lipophilic-Balanced
HNF	*HEPATOCYTE NUCLEAR FACTOR*
HOS1	*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1*
HvTb1	*Hordeum vulgare Theosinte Branched 1*
ID	identifyer
JA	jasmonic acid
kb	kilo basepair
KNAT1	*KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1*
l	liter/s
LD	*long day*
LFY	*LEAFY*
LOS1	*LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1*
LTI78	*LOW TEMPERATURE INDUCED 78*
M	g/mol
m	mili
m	meter/s
MADS-box	MINICHROMOSOME MAINTENANCE 1, AGAMOUS, DEFICIENS A, SERUM RESPONSE FACTOR
MCM1	*MINICHROMOSOME MAINTENANCE 1*
MCX	Mixed-mode Cation Exchange
min	minute/s
miR	*microRNA*
mRNA	messenger ribonucleic acid
Mya	milion years ago
n	number of samples
n	nano
n.s.	not significant
NA	not available
OX	oxidase
PAC	paclobutrazol
Paj	Pajares
PEP1	*PERPETUAL FLOWERING 1*
PHD	plant homeodomain
PI	*PISTILLATA*
PIF	*PHYTOCHROME INTERACTING FACTOR*
PP2A	*PROTEIN PHOSPHATASE 2A*
PRC2	POLYCOMB REPRESSIVE COMPLEX 2
P-value	probability-value
qPCR	quantitative polymerase chain reaction
QTL	quantitative trait locus
r	reverse
R^2	coefficient of determination
RF	representation factor
RGL2	*RGA-LIKE 2*
SAM	shoot apical meristem
SD	short day
SEM	Standard Error of the Mean
SEP3	*SEPALATA3*
seq	sequencing
SF2	San Feliu-2 ecotype
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
SPA2	*SPA1-LIKE 2*
SPL	*SQUAMOSA PROMOTER BINDING-LIKE*
Stdev.	Standard Deviation
STM	*SHOOT MERISTEMLESS*
SVP	*SHORT VEGETATIVE PHASE*
T	thymine

TE	transposable element
TEM	*TEMPRANILLO*
TF	transcription factor
TFL1	*TERMINAL FLOWER 1*
Th	*Tharenaya hassleriana*
TLN	total leaf number
TOE2	*TARGET OF EARLY ACTIVATION TAGGED (EAT) 2*
TSF	*TWIN SISTER OF FT*
UHPL	Ultra High Performance Liquid
V	vernalization
vern	vernalization
VIN3	*VERNALIZATION INSENSITIVE 3*
VRN1	*VERNALIZATION 1*
VRN2	*VERNALIZATION 2*
vs	versus
w	week/s
WAX	weak anion exchanger
Wt	wild type
ZAT	*ZINC TRANSPORTER OF ARABIDOPSIS THALIANA*
ZT	Zeitgeber Time

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