Evolution of the PEBP Gene Family in Plants: Functional Diversification in Seed Plant Evolution1[W][OA]

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The phosphatidyl ethanolamine-binding protein (PEBP) gene family is present in all eukaryote kingdoms, with three subfamilies identified in angiosperms (FLOWERING LOCUS T [FT], MOTHER OF FT AND TFL1 [MFT], and TERMINAL FLOWER1 [TFL1] like). In angiosperms, PEBP genes have been shown to function both as promoters and suppressors of flowering and to control plant architecture. In this study, we focus on previously uncharacterized PEBP genes from gymnosperms. Extensive database searches suggest that gymnosperms possess only two types of PEBP genes, MFT-like and a group that occupies an intermediate phylogenetic position between the FT-like and TFL1-like (FT/TFL1-like). Overexpression of Picea abies PEBP genes in Arabidopsis (Arabidopsis thaliana) suggests that the FT/TFL1-like genes (PaFTL1 and PaFTL2) code for proteins with a TFL1-like function. However, PaFTL1 and PaFTL2 also show highly divergent expression patterns. While the expression of PaFTL2 is correlated with annual growth rhythm and mainly confined to needles and vegetative and reproductive buds, the expression of PaFTL1 is largely restricted to microsporophylls of male cones. The P. abies MFT-like genes (PaMFT1 and PaMFT2) show a predominant expression during embryo development, a pattern that is also found for many MFT-like genes from angiosperms. P. abies PEBP gene expression is primarily detected in tissues undergoing physiological changes related to growth arrest and dormancy. A first duplication event resulting in two families of plant PEBP genes (MFT-like and FT/TFL1-like) seems to coincide with the evolution of seed plants, in which independent control of bud and seed dormancy was required, and the second duplication resulting in the FT-like and TFL1-like clades probably coincided with the evolution of angiosperms.

The family of phosphatidyl ethanolamine-binding proteins (PEBPs) is an evolutionarily conserved group of proteins that occur in all taxa from bacteria to animals and plants (Schoentgen and Jolles, 1995; Bradley et al., 1996; Banfield et al., 1998). Despite extensive sequence conservation, PEBP genes are involved in various biological processes, but the exact molecular function of individual genes is in most cases still unknown. In general, PEBP genes seem to act as regulators of various signaling pathways to control growth and differentiation (Yeung et al., 1999; Chautard et al., 2004).

In plants, members of the PEBP gene family have been shown to act as key regulators of the transition from the vegetative to the reproductive phase as well as being involved in determining plant architecture (Bradley et al., 1996; Kardailsky et al., 1999; Kobayashi et al., 1999). Consequently, these members have been intensively studied in Arabidopsis (Arabidopsis thaliana); in particular, FLOWERING LOCUS T (FT) and TERMINAL FLOWER1 (TFL1) are among the most thoroughly investigated PEBP proteins. Despite an amino acid identity of over 98%, these two proteins have antagonistic functions: FT promotes flowering by mediating both photoperiod and temperature signals, while TFL1 represses it. Key amino acids responsible for this functional divergence have been identified by overexpressing chimeric versions of the two proteins (Hanzawa et al., 2005; Ahn et al., 2006; Pin et al., 2010). In particular, a Tyr at the position corresponding to Tyr-85 and His-99 in FT and TFL1, respectively (Hanzawa et al., 2005), and motifs in exon 4 (segments B and C as defined by Ahn et al. [2006]) are essential for FT function. Recent analysis of a pair of FT paralogs in sugar beet (Beta vulgaris) identified three amino acids in segment B that are important for the...
an antagonistic effect of these genes on flowering (Pin et al., 2010). Although angiosperm FT- and TFL1-like genes were previously thought to be primarily involved in the control of the switch to reproductive development, recent studies on perennials and species with sympodial growth suggest a more general role in controlling the growth and termination of meristems (Lifschitz and Eshed, 2006; Lifschitz et al., 2006; Ruonala et al., 2008; Shalit et al., 2009).

Besides FT and TFL1, four more PEBP-like genes have been identified in Arabidopsis: MOTHER OF FT AND TFL1 (MFT), BROTHER OF FT AND TFL1 (BFT), ARABIDOPSIS THALLANA CENTRORADIALIS (ATC), and TWINSISTER OF FT (TSF). These genes are not as well characterized, but overexpressing ATC and BFT resulted in phenotypes similar to plants overexpressing TFL1, including late flowering, while overexpression of MFT resulted in a slight reduction of flowering time. However, loss-of-function mutations in these genes did not display any obvious phenotype (Mimida et al., 2001; Yoo et al., 2004, 2010). MFT was also reported to act during seed germination, in agreement with its expression pattern and that of its homologs in other plant species (Danilevskaya et al., 2008; Xi et al., 2010). The sixth member of the gene family, TSF, is almost identical to FT at the amino acid level. Like FT, it promotes flowering, but it makes a distinct contribution only in short-day conditions (Yamaguchi et al., 2005; D’Aloia et al., 2011).

The number of PEBP-like genes found in different plant species varies greatly, with 19 copies identified in rice (Oryza sativa) and 24 in maize (Zea mays) compared with the six members found in Arabidopsis. Phylogenetic analysis of plant PEBP genes indicates that the family is split into three main clades (Chardon and Damerval, 2005, and refs. therein): FT-like, TFL1-like, and a third clade that includes MFT-like genes. Attempts to identify PEBP genes from nonangiosperm plants have been more limited, but in mosses, liverworts, and club mosses only, MFT-like or MFT-like plus a very divergent type of PEBP-like genes have been identified (Hedman et al., 2009). In gymnosperms, two types of PEBP-like genes were identified, one type similar to MFT-like genes and another type resembling FT- and TFL1-like genes (Hedman et al., 2009). In Picea abies, these two types are each represented by two copies: PaFT₁ and PaFT₃ belong to the MFT-like group, and PaFT₂ and PaFT₄ belong to the TFL1/FT-like group (Gyllenstrand et al., 2007). PaFT₄ is the best characterized of these four genes and shows an expression pattern that correlates with both photoperiod-controlled bud set and temperature-mediated bud burst (Gyllenstrand et al., 2007). In general, however, the role of PEBP genes in nonangiosperm plants remains poorly known. Based on the conclusions from this study, we renamed these genes as follows: the two MFT-like genes (PaFT₁ and PaFT₃) are subsequently referred to as PaMFT₁ and PaMFT₂, and the two FT/TFL1-like genes (PaFT₂ and PaFT₄) are named PaTFL1 and PaTFL2, respectively.

The aim of this study is to extend the evolutionary and functional studies of plant PEBP genes to non-angiosperm plants. To elucidate the evolutionary history of plant PEBP genes, we performed extensive phylogenetic inference based on available plant PEBP-like mRNA and protein sequences. This analysis suggests a first duplication event after the divergence of club mosses and seed plants, which resulted in one MFT-like clade and one clade representing the ancestor of FT-like and TFL1-like genes. Gymnosperm FT/TFL1-like genes were placed close to the node separating FT-like and TFL1-like genes, and overexpression of PaFT₁ and PaTFL2 in Arabidopsis indicates that the function of the FT/TFL1-like proteins from gymnosperms is more similar to the function of angiosperm TFL1-like proteins. Based on these results, we suggest that a second duplication of PEBP genes probably occurred in the angiosperm lineage and that the ancestral function of the FT/TFL1-like genes was more TFL1-like. Furthermore, based on expression patterns from both angiosperms and gymnosperms, we propose that the recently described role of MFT, as a regulator of seed germination, should be expanded to a more general function in embryo development.

RESULTS

Phylogenetic Reconstruction of Plant PEBP Genes

BLAST searches using PEBP-like protein sequences from Arabidopsis against plant protein and nucleotide databases at GenBank (January 30, 2011) resulted in 265 putative full-length sequences with a complete open reading frame that were kept for phylogenetic reconstructions (a complete list of accession numbers can be found in Supplemental Table S1). The general topology of the phylogenetic tree agrees with that of Chardon and Damerval (2005) and Hedman et al. (2009), suggesting that plant PEBP genes can be divided into three main clusters: MFT-like, TFL1-like, and FT-like clades (Fig. 1).

Only two clusters of PEBP genes were retrieved in gymnosperms, one clearly associated with MFT-like genes and one positioned close to the node separating MFT-like, FT-like, and TFL1-like genes (Fig. 1). Extensive additional searches in available databases and trace archives from Picea and Pinus species, representing more than 5 million ESTs, did not reveal any additional type of PEBP-like genes in gymnosperms. The position of the second gymnosperm cluster in relation to the three major subfamilies (MFT-like, FT-like, and TFL1-like) was ambiguous. Likelihood and distance methods placed this cluster on the branch to the MFT-like cluster, while Bayesian methods placed this cluster with the FT-like genes in analyses based on amino acid sequence data or with the TFL1-like cluster in analyses based on DNA sequence. However, the support for these competing topologies was in most cases not high (Fig. 1).

Characterization of Gymnosperm PEBP Proteins

Two MFT-like genes were identified in both spruces and pines, and the phylogenetic analyses support a
duplication event predating the divergence of Picea and Pinus (Supplemental Fig. S1).

Similarly, two FT/TFL1-like genes were identified in multiple Picea species. Overall, the predicted amino acid sequences of the P. abies FT/TFL1-like genes PaFTL1 and PaFTL2 are roughly equally similar to FT and TFL1 proteins in Arabidopsis. PaFTL1 shows 85% and 88% similarity to FT and TFL1, respectively, and the corresponding figures for PaFTL2 are 86% and 85%. Comparing the sequence motifs suggested to be important for FT versus TFL1 function, PaFTL1 and PaFTL2 both contain a Tyr at the position corresponding to Tyr-85 and His-88 in FT and TFL1, respectively. In addition to Tyr-85/His-88 (Hanzawa et al., 2005), Ahn et al. (2006) identified motifs in exon 4, in particular segments B and C, that together are essential for FT function. Over segments B and C, PaFTL1 and PaFTL2 are more similar to FT than to either TFL1 or BFT, with around 65% amino acids identical to FT and around 50% identical to both TFL1 and BFT (Supplemental Fig. S2). However, specific amino acids in these segments that are highly conserved only in FT homologs, and that are potentially important for FT function, including residues corresponding to Asp-144/Gln-140 in TFL1 and FT and a triad that is essentially conserved in FT homologs (Supplemental Fig. S2; Ahn et al., 2006), were not conserved in the deduced P. abies proteins. In sugar beet, the difference between the two functionally antagonistic BvFT1 and BvFT2 proteins is linked to changes of three residues (Pin et al., 2010). The region containing these amino acids is divergent between PEBP genes with activating and repressing effects on flowering time when overexpressed in Arabidopsis (Supplemental Fig. S2).

**PaFTL1 and PaFTL2 Genes Repress Flowering When Overexpressed in Arabidopsis, But No Effect on Flowering Was Observed for PaMFT1 or PaMFT2**

As a preliminary test of protein function, constructs with the four P. abies PEBP-coding sequences inserted behind the cauliflower mosaic virus 35S promoter were introduced into Arabidopsis. For PaMFT1 and PaMFT2, which both belong to the MFT clade, no significant change in flowering time was detected (Fig. 2, A and B). Although an ANOVA indicated significant differences between lines, none of the pairwise tests between the wild type and the overexpressed line were significant (Tukey’s honestly significant difference test). For PaFTL2, however, four out of six overexpressed lines flowered significantly later than the wild type. These four lines also exhibited the highest expression levels of the transgene (Fig. 2C). Similarly, for PaFTL1, the four lines with highest expression of the transgene showed significantly delayed flowering, although not as pronounced as for PaFTL2 (Fig. 2D).

In addition, the lines that expressed PaFTL2 at the highest levels also displayed aberrant flower morphology typical for plants overexpressing TFL1 or BFT (Ratcliffe et al., 1998; Mimida et al., 2001; Yoo et al., 2010). These phenotypes included floral indeterminacy and inflorescence-in-flower-type flowers (Fig. 2, E and F). The morphology of the transgenic plant was as variable as reported for overexpressors of TFL1 (Ratcliffe et al., 1998), but the range of phenotypes observed was similar.

In conclusion, our data show that PaFTL2 can confer, at least in part, TFL1 function under overexpression conditions. Provided that this is indicative of functional conservation, PaFTL2 appears functionally more similar to TFL1 than to FT. Similarly, although the phenotypic deviations observed for overexpressors of PaFTL1 were less pronounced, the same seems to hold for PaFTL1.

**P. abies PEBP Genes Have Diverged Expression Patterns**

To approach the endogenous function of spruce PEBP genes, we analyzed their spatial and temporal
expression patterns. Expression levels of PaMFT1, PaMFT2, PaFTL1, and PaFTL2 were first measured in various tissues with quantitative reverse transcription (qRT)-PCR. RNA for these experiments was extracted from needles, vegetative and reproductive buds, seeds, pollen, and somatic embryos. Based on the results from qRT-PCR, RNA in situ hybridization was then conducted in selected tissues.

**PaFTL1 and PaFTL2**

As previous data implicated PaFTL2 in the control of annual growth rhythm (Gyllenstrand et al., 2007), expression in needles and buds was assayed in adult trees under natural conditions at three time points of the growing season. PaFTL2 exhibited relatively high expression levels in needles at all time points, while expression in vegetative as well as male and female buds was high in August but low in December and May (Fig. 3A). PaFTL1 expression was generally low in all needle and bud samples except male buds in December and May (Fig. 3B). PaFTL1 and PaFTL2 also displayed detectable but low expression levels in pollen and seeds (Fig. 4).

Using in situ hybridization, the temporal and spatial expression patterns of PaFTL1 were studied in male buds, where a high expression was observed in qRT-PCR data. Expression was first seen in microsporophyll primordia, with a seemingly even expression in entire primordia (Fig. 5A). At later stages during autumn, expression was localized inside the microsporangia, without visible expression in tapetum cells (Fig. 5, B and C). In early spring before meiosis, expression reached a very high level in microspore mother cells (Fig. 5, D and E). Additional estimates of PaFTL1 expression later in the season using qRT-PCR showed that expression in male bud remained high until bud burst and release of pollen (data not shown). Based on qRT-PCR data, PaFTL2 displayed high expression in needles as well as in vegetative and reproductive buds. In situ hybridization on needle tissue failed, but in dormant vegetative as well as reproductive buds, expression of PaFTL2 was detected primarily in the pith and procambial region. Figure 6 shows expression in male buds, and similar patterns were also observed in vegetative and female buds (data not shown).

**PaMFT1 and PaMFT2**

The MFT-like PaMFT1 and PaMFT2 both displayed low expression levels in needles and buds (Fig. 3, C and D). Both PaMFT1 and PaMFT2 were highly expressed in seeds, with very high expression levels detected both in zygotic embryo and endosperm (Fig. 4A). PaMFT1 also showed high expression levels in mature pollen (Fig. 4B). Zygotic embryos are difficult to isolate at particular stages of development, but as P. abies somatic embryos show very similar patterns of development as zygotic ones (Filova et al., 2000), we isolated RNA from somatic embryos at seven developmental stages (for a description of the stages, see Supplemental Fig. S3; Larsson et al., 2008). A clear increase in expression was first noted, in particular for PaMFT2, from stage B (4 d after the addition of abscisic acid [ABA]), concurrent with the occurrence of distinct embryonal mass in early-stage embryos, and expression remained high during embryogenesis (Fig. 4C). For PaMFT2, a decline in expression was evident in mature embryos (Fig. 4C). We also performed a detailed study of PaMFT1 and PaMFT2 expression in situ in developing somatic embryos. Although PaMFT2 seemed to exhibit generally higher expression levels than PaMFT1 in these tissues (Fig. 4C), their spatial expression patterns in embryos were similar (Fig. 7; Supplemental Fig. S4). At the first stage that could be reliably studied, expression was located to the promeristematic region (Fig. 7A). During development of the embryos, signal was subsequently detected preferentially in meristematic regions, includ-
ing the root meristem, procambium, and shoot apical meristem (Fig. 7, B–E; Supplemental Fig. S4). At later stages, expression was also detected in the developing procambium of the cotyledons (Fig. 7E). Analysis of mature zygotic embryos revealed a similar expression pattern for both genes (Supplemental Fig. S5), but prolonged exposure also revealed expression in most parts of the embryo (Supplemental Fig. S5, B and C). We also detected a very specific expression pattern in one of the \( P. \text{abies} \) MFT-like genes (\( \text{PaMFT1} \)), linked to resin ducts in male buds. In young developing male buds, distinct signals were evident at the site where resin ducts will form, between the procambium and the microsporophylls (Fig. 8A). Expression was later confined to the epithelial cells surrounding the resin ducts (Fig. 8C). No corresponding signal was detected in vegetative or female buds or in any type of bud for the three other \( P. \text{abies} \) PEBP genes.

**DISCUSSION**

**Evolutionary Expansion of the Plant PEBP Gene Family**

In line with previous data (Hedman et al., 2009), the phylogeny obtained in this study suggests that MFT-like genes are the basal clade among plant PEBP genes. MFT-like genes are present in angiosperms, gymnosperms, lycophytes, and bryophytes, whereas the FT-like and TFL1-like clades do not include any *Physcomitrella patens* or *Selaginella moellendorffii* PEBP genes. Furthermore, available EST sequence data from the fern *Pteridium aquilinum* including over 600,000 454 reads from gametophyte tissue contain only MFT-like PEBP genes (Der et al., 2011). Hence, the ancestor to FT/TFL1 genes started to diverge after the separation of the lycophytes and gymnosperms, likely in the common ancestor to seed plants.

The second group of conifer PEBP genes (including \( \text{PaFTL1} \) and \( \text{PaFTL2} \)) formed a separate cluster including one sequence from *Ginkgo biloba*. The position of the cluster in the tree is uncertain, so based on phylogenetic data, this second group of conifer PEBP genes cannot be classified as FT-like or TFL1-like. So far, no additional gymnosperm genes clustering with TFL1-like genes have been identified in gymnosperms. Although the presence of TFL1-like genes in conifers cannot be unequivocally ruled out since there is no complete conifer genome available, their presence is highly unlikely. Ambitious large-scale EST sequencing projects have...
been completed in conifers, in both spruce (for *Picea glauca*, 301,425 entries, and for *Picea sitchensis*, 175,662 entries in the National Center for Biotechnology Information [NCBI] dbEST) and pine (for *Pinus taeda*, 328,628 entries in NCBI dbEST). In addition, recent EST sequencing efforts using the 454 and Illumina sequencing platforms have added more than 5 million ESTs to the NCBI Sequence Read Archive, and none of these databases contain any conifer TFL1-like genes.

Even though the position of *PaFTL1* and *PaFTL2* in relation to the major *FT*, TFL1, and MFT clades is uncertain, functional data indicate that the proteins encoded by these genes possess a function more similar to TFL1-like genes. In particular, overexpression in Arabidopsis delayed flowering rather than promoted it, as would be expected from a FT-like gene, and some lines where the *PaFTL2* transgene was highly expressed showed flower phenotypes typical of plants overexpressing TFL1. An attractive hypothesis for the evolution of FT- and TFL1-like genes based on these observations is that the duplication and divergence between the FT-like clade and the TFL1-like clade occurred in the angiosperm lineage. The conifer FT/TFL1-like genes would then represent a lineage derived from the ancestor of both these subfamilies. According to this hypothesis, the ancestral function would be more similar to TFL1 than FT, with FT function evolving in the angiosperm lineage. In contrast, the alternative hypothesis, in which the FT and TFL1 clades diverged before the separation of angiosperms and gymnosperms, would imply both a loss of TFL1-like genes in gymnosperms and that the remaining FT-like genes acquired a TFL1-like function. Based on available data, including the fact that *PaFTL2* is expressed in both needles and vegetative buds, like several TFL1-like genes in angiosperms (Mimida et al., 2009; Mohamed et al., 2010), the first hypothesis seems more parsimonious.

**Figure 5.** In situ localization of *PaFTL1* mRNA in longitudinal sections of developing pollen cones of *P. abies*. A to E, Antisense probe. F, Sense probe. A, *PaFTL1* is expressed early in microsporophyll primordia in buds collected in August. B, In September, *PaFTL1* is expressed in initiating pollen mother cells. C to E, *PaFTL1* expression is confined to pollen mother cells throughout the winter period at least until late March, before meiosis. F, Late March sample hybridized with sense probe. Arrowheads point to areas of signal obtained with antisense probe. Bars = 100 μm.

**Figure 6.** In situ localization of *PaFTL2* mRNA in longitudinal and transverse sections of *P. abies* male cones collected after meristem termination. A and C, Antisense probe. B and D, Sense probe. A, Transverse section showing *PaFTL2* expression mainly in the pith (PH). C, Longitudinal section showing expression of *PaFTL2* in the pith and procameral region (PC). Bars = 100 μm.
Expression Patterns of *PaFTL1* and *PaFTL2* Suggest Functional Diversification

*PaFTL1* and *PaFTL2* represent a relatively recent duplication that occurred in the gymnosperm lineage. Although overexpression in *Arabidopsis* demonstrates that both *PaFTL1* and *PaFTL2* can exert similar functions in a heterologous system, their expression patterns in *P. abies* were clearly distinct. *PaFTL2* was predominantly expressed in needles and both vegetative and reproductive buds, while *PaFTL1* showed a more restricted expression, with high expression confined to male buds. The expression of *PaFTL2* is correlated with growth cessation and bud set, indicating a role in this process, as has been reported for *FT*-like genes in *Populus* (Böhlenius et al., 2006). In *Populus*, the *FT*-like *PtFT1* showed a reduction in expression correlated with the onset of growth cessation. Furthermore, trees with artificially down-regulated expression of *PtFT1* were more sensitive to shortened daylength (Böhlenius et al., 2006). In contrast, *PaFTL2* showed a strong induction of expression in response to shortened daylength that correlated with the induction of growth cessation and bud set (Gyllenstrand et al., 2007), in line with the opposite flowering response when these genes were overexpressed in Arabidopsis (Böhlenius et al., 2006; this study). It is also noteworthy that *PaFTL2* showed a similar expression pattern in both vegetative and reproductive buds, suggesting that *PaFTL2*, in contrast to angiosperm *FT*-like genes, is not primarily involved in the induction of reproductive development. On the other hand, the expression pattern of *PaFTL1* indicates a role during male cone development. Expression was first detected in microsporophyll primordia, and *PaFTL1* could potentially also be involved in specifying male reproductive development. It is notable that two *FT*-like genes in poplar also show a predominant expression in male reproductive organs (Poplar eFP browser; http://bar.utoronto.ca/efppop). The divergence of expression patterns between *PaFTL1* and *PaFTL2* illustrates the prevalence of regulatory changes in the evolution of plant PEBP genes.

*FT*-like as well as *TFL1*-like genes have been shown to possess quite variable expression patterns in different species. Some general patterns are still evident. *TFL1*-like genes seem to be mainly expressed in mer-
The MFT-Like PaMFT1 and PaMFT2 Genes Are Active during Embryo Development

Limited information is available on the function of MFT-like genes, but a general feature seems to be a predominant expression in seeds (Chardon and Damerval, 2005; Danilevskaya et al., 2008; Igasaki et al., 2008). Available functional data from Arabidopsis indicate that MFT is involved in the control of germination and that it may serve as a convergence point of ABA and GA signaling (Xi et al., 2010). Two ABA signaling components, ABI3 and ABI5, are probably directly binding to the MFT promoter and regulating MFT transcription, ABI3 as a repressor and ABI5 as an activator. Furthermore, the DELLA protein RGL2 was also reported to bind the MFT promoter to enhance expression of the gene. MFT in turn acts as a repressor of ABI5. The outcome of this complex interaction is that MFT promotes embryo growth during seed germination (Xi et al., 2010).

Our expression data in P. abies support a role for MFT-like genes not only in germination but also earlier during embry development. High expression of both P. abies genes was detected in zygotic embryos and the surrounding endosperm. Similarly, in P. abies somatic embryos, the expression of PaMFT1 and PaMFT2 was detected soon after the addition of ABA, which is crucial for the promotion of embryo maturation (Dunstan et al., 1998; Roberts et al., 1990). ABI3 has been identified as a master regulator of maturation (Giraudat et al., 1992). As ABI3 was shown to regulate MFT, it is interesting that a P. abies homolog to ABI3, PaVP1 (Footitt et al., 2003; Vestman et al., 2010), shows a similar temporal expression pattern during embryo maturation as do PaMFT1 and PaMFT2. Furthermore, the expression patterns of PaVP1 and P. abies PEBP genes coincide in embryos, dormant buds, as well as male strobili (Footitt et al., 2003). In Populus, overexpression of PtABI3 resulted in the induction of both PtFT and PtMFT during bud development induced by short days (Ruttkin et al., 2007). Thus, PaVP1 might control the expression of P. abies MFT-like genes, and possibly even other P. abies PEBP genes, as has been reported for the homologs ABI3 and MFT in Arabidopsis (Xi et al., 2010).

Expression in embryos during the maturation phase has also been reported in maize (ZCN9, -10, and -11; Danilevskaya et al., 2008) as well as in Arabidopsis (Le et al., 2010). During this stage, the development of embryos goes from a state of active cell division to cell enlargement, accompanied by an increase in ABA content. This period is also characterized by a massive accumulation of reserves and a decrease in water content. Later, during the final stage of seed development, embryos lose water and ABA and become desiccation tolerant and metabolically inactive (Gutierrez et al., 2007; Braybrook and Harada, 2008). In line with the observed ABA induction of MFT during germination (Xi et al., 2010), MFT expression seems to follow ABA levels during embryo development. However, as no obvious phenotype related to embryo development has been observed in mft mutants, a function of MFT during this phase of development remains to be determined. It is notable that mutations in genes encoding most seed-specific transcription factors do not result in a detectable phenotype (Le et al., 2010). Still, our expression data suggest a conserved function for
MFT-like genes in embryo development in gymnosperms and angiosperms.

An Ancestral Function of Plant PEBP Genes in Dormancy Regulation?

A common feature of tissues where PEBP gene expression was detected in *P. abies* is preparation for “harsh conditions,” including physiological changes such as dormancy, dehydration, and desiccation tolerance. It is well known that bud and seed dormancy share physiological characteristics (Wareing, 1956). During the maturation phase, embryos accumulate storage compounds, but they also cease cell division and water content declines. A similar adaptation to cold and dehydration occurs when the buds enter dormancy. Likewise, pollen is a dormant organ that is generally tolerant to dehydration. From the observed expression patterns of *P. abies* PEBP genes, it is tempting to speculate that the original function of plant PEBP genes is related to growth arrest and/or dormancy. In line with this idea, Shalit et al. (2009) proposed that the tomato (*Solanum lycopersicum*) orthologs of *FT* and *TFL1* (*SFT* and *SP*) regulate the balance of diverse growth processes rather than directly control the fate of cells or organs, as previously suggested.

Our data also suggest that a second subfamily of PEBP genes occurred concurrently with the evolution of seed plants, in which bud dormancy and seed dormancy required independent control. A further duplication resulting in the *FT* and *TFL1* clades, the proteins of which confer antagonistic functions, likely occurred with the evolution of flowering plants. It has been speculated that in angiosperms, with their need for rapid response to environmental signals, high levels of FT function are required and that TFL1 function evolved to alleviate the detrimental effects of FT function (Shalit et al., 2009). However, the fact that *FT/TFL1*-like genes in *P. abies* seem to code for proteins with a TFL1-like function would suggest that TFL1 function could actually be the ancestral state.

**MATERIALS AND METHODS**

**Database Searches and Phylogenetic Reconstruction**

An extensive search for plant PEBP genes was performed using Arabidopsis (*Arabidopsis thaliana*) FT, MFT, and TFL1 protein sequences as queries in BLAST searches against GenBank (on January 20, 2011). A total of 265 putative full-length plant proteins were aligned using ClustalW (Thompson et al., 1994) and back-translated to mRNA sequences for analysis at the DNA level. BLAST searches against GenBank (on January 20, 2011). A total of 265 putative full-length plant proteins were aligned using ClustalW (Thompson et al., 1994) and back-translated to mRNA sequences for analysis at the DNA level. The alignments were filtered with Gblocks using default settings to remove potentially poorly aligned positions and divergent regions (Talavera and Castresana, 2007). Phylogenies were reconstructed by Bayesian inference using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) with maximum likelihood using Phyml 3.0 (Guindon and Gascuel, 2003) and distance methods using MEGA 5.0 (www.megasoftware.net). In the amino acid analysis, Jones transition matrix was used, and both heterogeneity between sites and invariable sites were allowed. In estimation of topologies from DNA data, we used a generalized time-reversible model and estimated different rates for each codon position. For the Bayesian analysis, two Markov runs each with four chains heated according to default settings were performed. In each run, every 100th tree was saved, and posterior topologies were estimated after disregarding the first 1.5 million steps as burn in. The maximum likelihood analyses were run with the Nearest Neighbor Interchange algorithm, and support was estimated using an approximate likelihood-ratio test (Guindon et al., 2010). Distance-based phylogenetic reconstruction was based on neighbor-joining and LogDet distance for DNA data and on Jones transition matrix for amino acid data. Distance estimates were based on the first two codon positions, and bootstrap support was estimated from 500 runs. Trees were visualized with FigTree (tree.bio.ed.ac.uk/software/figtree).

**Isolation of *Picea abies* PEBP Genes**

Searches against *Pinus taeda*, *Picea glauca*, and *Picea sitchensis* EST databases revealed four unique putative PEBP genes in conifers. Partial or complete cDNAs from *P. abies* were obtained by RT-PCR using the OneStep RT-PCR kit (Qiagen). Template RNA for RT-PCR was extracted from male or vegetative buds using a slightly modified extraction method described by Azavedo et al. (2003). Partial cDNAs were extended by RACE reactions using the SMART RACE CDNA Amplification kit (Clontech) to identify the complete coding frame of the gene. In addition, the genomic sequence of each locus was determined by sequencing of PCR products.

Sequences were base called with PHRED, assembled with PHRAP, and visualized and edited in CONSED (Ewing et al., 1998; Ewing and Green, 1998; Gordon et al., 1998). The exon-intron structure for each *P. abies* PEBP homolog was determined by alignment of CDNA sequences to the corresponding genomic sequence (Supplemental Fig. S6).

**P. abies** Plant Material

Female, male, and vegetative buds, needles, and cones were collected from adult trees of *P. abies* (more than 30 years old) growing at latitude 59°53’N (Uppsala, Sweden). Buds were collected on the first date they, after dissection, could be visually determined as either female, male, or vegetative. Embryonic cell line A.95.88.22 (Egeberdottier and von Arnold, 1993) was cultured as described by Filonova et al. (2000).

**Quantitative Gene Expression Analysis**

For *P. abies*, total RNA was isolated from needles or buds following the protocol described by Azavedo et al. (2003) with minor modifications, while for Arabidopsis, the Qiagen RNeasy Mini kit was used in accordance with the manufacturer’s recommendations. cDNAs were synthesized from 0.5 μg of total RNA using SuperScript III reverse transcriptase and random hexamer primers. qRT-PCR was performed according to Cyllestrand et al. (2007) or on a MyiQ Real-Time PCR Detection system (Bio-Rad) with the thermal conditions 95°C for 7 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each reaction was performed in duplicate containing 12 μL of DyNAamo Flash SYBRGreen (DyNAamo Flash SYBRGreen qPCR kit; Finzymes), 0.5 mM of each primer, and 4.75 μL of cDNA (diluted 1:100) in a total volume of 23.75 μL. Primers used can be found in Cyllestrand et al. (2007) except for *PaFTL1*, where the primers for the MyiQ machine were as follows: forward, 5’-CCCGTACACAATTTTGACCTC-3’; reverse, 5’-CCCATCTGCTTGAAACAC-3’. Polyubiquitin was used to normalize data. Expression values were calculated as delta cycle threshold (dCT) values (CTtarget – CTcontrol).

**In Situ Hybridization**

Sense and antisense RNA probes were synthesized with in vitro transcription using the DIG RNA Labeling kit (SP6/T7; Roche Applied Science) according to the manufacturer’s recommendations and digested to 150-bp fragments according to Jackson (1991). Templates for in vitro transcription were PCR products generated from plasmids containing the coding sequences of *PaMFT1*, *PaMFT2*, *PaFTL1*, and *PaFTL2*. Primers used to generate these products are listed in Supplemental Table S2. In situ hybridization was performed according to Karlseng et al. (2009).

**Arabidopsis Transformation and Growth Conditions**

The complete cDNA of *PaMFT1*, *PaMFT2*, *PaFTL1*, and *PaFTL2* was amplified using gene-specific primers with attB sites (Supplemental Table S3). The PCR products were cloned into the pDONR 221 vector (Invitrogen). The destination vector pMED32 (Curts and Grossniklaus, 2003) was used to

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construct 35S:PaMFT1, 35S:PaMFT2, 35S:PaFTL1, and 35S:PaFTL2 plasmids. The constructs were transformed using the freeze-thaw method into Agrobacterium tumefaciens GV3101 pMP90. A. tumefaciens-mediated transformations of Arabidopsis (Columbia) plants were performed using the floral dip method (Clough and Bent, 1998), and homozygotic transgenic lines were established.

Seeds from homozygous transgenic lines and the wild type were planted on soil in a randomized manner with 24 pots in each tray. The seedlings were grown under approximately 150 μmol m⁻² s⁻¹ cool-white fluorescent light in 16 h of light/8 h of dark and 22°C (long days). Flowering time was measured by counting the final number of rosette leaves. All experiments were repeated twice.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Detailed phylogenetic trees of plant PEBP genes.

Supplemental Figure S2. Alignment of deduced amino acid sequences of segments B and C in exon 4 (Ahn et al., 2006) of selected plant PEBP genes.

Supplemental Figure S3. Stages of somatic embryogenesis used for qRT-PCR and in situ hybridization experiments.

Supplemental Figure S4. In situ localization of PaMFT1 mRNA in longitudinal sections of P. abies somatic embryos.

Supplemental Figure S5. In situ localization of PaMFT1 and PaMFT2 mRNA in longitudinal sections of zygotic embryos of P. abies.

Supplemental Figure S6. Inferred exon-intron structure of P. abies PEBP genes.

Supplemental Table S1. List of genes included in phylogenetic analyses.

Supplemental Table S2. Primers used to generate probes for in situ hybridization.

Supplemental Table S3. Primers used to clone cDNA of P. abies PEBP genes.

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LITERATURE CITED


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