Early Cone Setting in *Picea abies acrocona* Is Associated with Increased Transcriptional Activity of a MADS Box Transcription Factor[1[W][OA]]

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Conifers normally go through a long juvenile period, for Norway spruce (*Picea abies*) around 20 to 25 years, before developing male and female cones. We have grown plants from inbred crosses of a naturally occurring spruce mutant (*acrocona*). One-fourth of the segregating *acrocona* plants initiate cones already in their second growth cycle, suggesting control by a single locus. The early cone-setting properties of the *acrocona* mutant were utilized to identify candidate genes involved in vegetative-to-reproductive phase change in Norway spruce. Poly(A$^+$) RNA samples from apical and basal shoots of cone-setting and non-cone-setting plants were subjected to high-throughput sequencing (RNA-seq). We assembled and investigated 33,383 expressed putative protein-coding *acrocona* transcripts. Eight transcripts were differentially expressed between selected sample pairs. One of these (*Acr42124_1*) was significantly up-regulated in apical shoot samples from cone-setting *acrocona* plants, and the encoded protein belongs to the MADS box gene family of transcription factors. Using quantitative real-time polymerase chain reaction with independently derived plant material, we confirmed that the MADS box gene is up-regulated in both needles and buds of cone-inducing shoots when reproductive identity is determined. Our results constitute important steps for the development of a rapid cycling model system that can be used to study gene function in conifers. In addition, our data suggest the involvement of a MADS box transcription factor in the vegetative-to-reproductive phase change in Norway spruce.

The two most commonly grown and economically important conifers in Sweden, Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*), both go through a long vegetative growth phase before they begin to produce cones; the vegetative growth period is 20 to 25 years and 8 to 20 years for Norway spruce and Scots pine, respectively. Mature Scots pine trees produce cones every year, while mature Norway spruce trees produce cones only every 3rd to 5th year. The timing of cone establishment is largely synchronized within the various spruce populations and is determined by a combination of genetic and environmental factors (Lindgren et al., 1977; Högberg and Eriksson, 1994). The long generation time and the irregular cone setting between different years pose major obstacles for breeding of Norway spruce both with respect to genetic gain and retained diversity. To guarantee a sufficient seed supply, forest companies have to plant and maintain large seed orchards and in addition store large quantities of seeds to cover the demand during years of little or no seed production. Both conifer breeding programs and the production of improved seed for forest regeneration would benefit from methods to control the length of the vegetative period before the trees start to produce cones as well as the possibilities to control cone setting and cone production itself. Despite that, our knowledge of the genetic mechanisms that regulate the transition from vegetative growth to reproductive development in conifers is limited. Here, we utilize the intrinsic properties of a naturally occurring homeotic mutant of spruce, *acrocona*, which produces cones every year, to identify candidate genes of importance for reproductive phase change in spruce. In the *acrocona* mutant, female cones are regularly set on terminal positions of shoots on both top shoots and scaffold branches, at positions where wild-type spruce never produces female cones, hence the Latin name *acrocona* for "top cone." Reports of *acrocona*

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specimens from different localities in Uppland, Sweden, stem from the mid 19th century, and sporadic occurrence of the *acrocona* mutant has since then also been reported from other parts of Sweden (Fries, 1890; Joneborg, 1945). For horticultural purposes, the *acrocona* mutant has been widely propagated by grafting, and *acrocona* plants can now be found in gardens all over the temperate world. In a previous study including the *acrocona* mutant, early cone setting was mapped to spruce chromosome 6 (Acheré et al., 2004). However, the gene or genes responsible for the *acrocona* phenotype are not known.

Genetic and molecular analyses of flowering-time mutants in the angiosperm model species Arabidopsis (*Arabidopsis thaliana*) have demonstrated that the phase change from vegetative to reproductive development in flowering plants follows four distinct pathways: the autonomous pathway and those induced by photoperiod, vernalization, or gibberellin (for review, see Bäurle and Dean, 2006; Lee and Lee, 2010). In short, pathways influenced by intrinsic and environmental signals (e.g. photoperiod and temperature) regulate the onset of flowering by increasing the expression of the *FLOWERING LOCUS T* (*FT*) gene, whose encoded protein has been proposed to be the elusive mobile flower-inducing factor "florigen" (Wigge et al., 2005; Lifschitz et al., 2006). The FT protein regulates the transition of a vegetative meristem to an inflorescence meristem by activating the expression of genes belonging to the MADS box gene family of transcription factors, *APETALA1* and *SUPPRESSOR OF CONSTANS1* (*SOC1*). Hormonal induction of flowering also occurs independently of FT by the GA_3-dependent activation of *SOC1*, which, in turn, activates the floral meristem identity gene *LEAFY* (*LFY*; Moon et al., 2003). Based on advances in the understanding of the genetic programs controlling flowering in Arabidopsis, various biotechnological approaches have been undertaken to shorten the time to flowering in angiosperm trees. For instance, expression of the *FT* gene induces early flowering by shortening the juvenile phase in *Populus trichocarpa* (Böhlenius et al., 2006). Similarly, overexpression of the *Citrus unishu FT* homolog (*CiFT*) can accelerate flowering in *Poncirus trifoliata* (Endo et al., 2005). Interestingly, recent evolutionary studies suggest that the angiosperm *FT* gene is the result of a duplication event that occurred in the angiosperm lineage after the split between angiosperms and gymnosperms approximately 300 million years ago (Smith et al., 2010; Karlgren et al., 2011). This indicates that the phase change from vegetative to reproductive development in gymnosperms and angiosperms is regulated by distinct genetic mechanisms that involve both orthologous and evolutionarily unrelated genes that may or may not exert similar functions.

Studies of transcriptional regulators belonging to the MADS box gene family in Norway spruce suggest that members of this gene family have putative functions in determining the length of the juvenile period and the vegetative or reproductive identity of a shoot (Carlsbecker et al., 2003, 2004). Expression of the gene *DEFICIENS AGAMOUS-LIKE1* (*DAL1*) increases with age and conforms to a spatial pattern that marks physiological and morphological features associated with the transition to the reproductive phase. In addition, the expression of *DAL1* in transgenic Arabidopsis shortens the vegetative phase, and the plants begin to flower extremely early, sometimes forming embryonic flowers (Carlsbecker et al., 2004). Similarly, the spatial expression patterns of the Norway spruce gene *DAL10* together with phenotypic studies of Arabidopsis plants, harboring *DAL10* under the control of a constitutive promoter, suggest that this MADS box gene may be involved in specifying reproductive identity in the male and female shoots (cones) of Norway spruce (Carlsbecker et al., 2003).

Recent advances in high-throughput sequencing technologies allow for global transcriptome profiling of wild-type and mutant plants and can be used to study the differential expression of all genes involved in a particular developmental process (Wang et al., 2009). This holds true also for spruce, although analyses are hampered by the current lack of a reference genome and the fact that spruce, like other conifers, displays a high genetic diversity (Tollefsrud et al., 2008), which makes it difficult to choose a relevant wild-type comparator. Here, we have developed a bioinformatics pipeline that allows us to accurately assemble Norway spruce transcripts de novo without prior knowledge of the genomic sequence. To avoid the identification of false positives due to genetic diversity in Norway spruce, we have analyzed differentially expressed genes in a segregating sibling population of the *acrocona* mutant using massively parallel sequencing methods and also assessed selected candidate genes involved in reproductive phase change using targeted quantitative real-time (qRT)-PCR from another segregating sibling population.

RESULTS

Offspring from Controlled Crosses of the *acrocona* Mutant Show an Early Cone-Setting Phenotype

To examine the segregation pattern of the *acrocona* phenotype and to produce a population of *acrocona* siblings, two ramets of the *acrocona* clone were crossed, and the resulting progeny were grown under accelerated growth conditions in a phytotron. Nineteen out of 75 inbred *acrocona* plants (derived from crossings made in 2006) initiated cones already during the second growth cycle (Fig. 1; Supplemental Table S1). Open-pollinated *acrocona* progeny (n = 150) and control plants (n = 75) were grown together with the inbred *acrocona* plants. Only two of the open-pollinated *acrocona* progeny and none of the control plants initiated cones during the first four growth cycles. A similar segregation pattern was obtained in a subsequent growth experiment of inbred *acrocona* plants (derived from crossings made in 2009), in which three out of 16 plants initiated cones during the second growth cycle

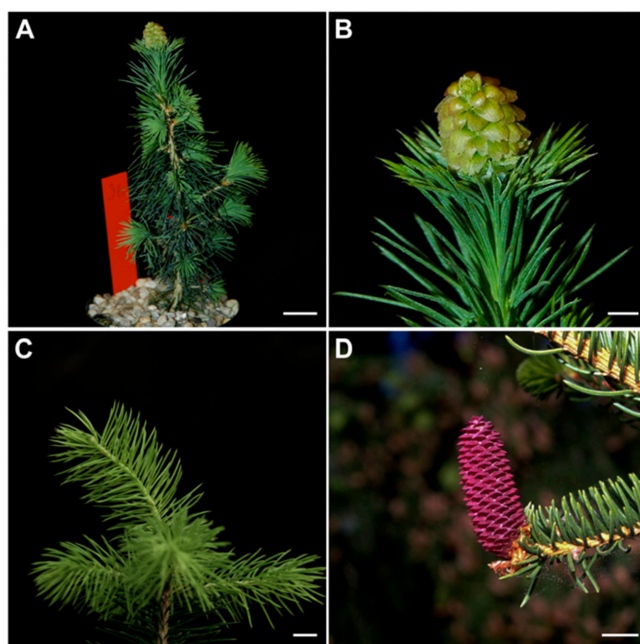


Figure 1. Cone formation in spruce. A, Apical transition cone developed on an early cone-setting inbred *acrocona* plant grown under accelerated growth conditions after three growth cycles. B, Close up of the apical transition cone in A. Note the transition from needles in the lower part to ovuliferous scales in the upper part of the apical shoot. C, Vegetative shoot phenotype on an inbred *acrocona* plant. D, Female cone on an open-pollinated wild-type tree. Bars = 2 cm (A and D) and 0.5 cm (B and C).

and a fourth plant initiated cones during the third growth cycle, while none of the control plants initiated cones. Together, the segregation of early cone setting in *acrocona* plants [19/75 and (3+1)/16] constitutes an excellent fit (χ^2 value, P = 0.003) with the Mendelian segregation expected assuming that the parents were heterozygous for the *acrocona* mutation and that only homozygous plants displayed the early cone-setting phenotype. During the fourth growth cycle, additional intermediate shoot phenotypes were initiated in inbred *acrocona* plants but not in open-pollinated *acrocona* progeny or in control plants: apical shoots with a large number of lateral short shoots and vegetative shoots with broad and bract-like needles (Supplemental Fig. S1, E–H; Supplemental Table S1).

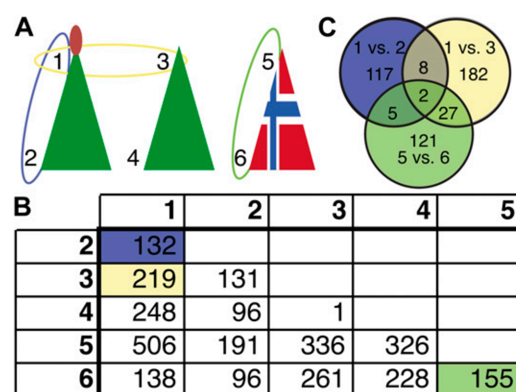
Cone-setting *acrocona* plants regularly initiated cones on the apical shoot, although individual plants produced additional cones on lateral branches (Fig. 1; Supplemental Fig. S1). Both apical and lateral shoots that initiated cones produced a transition cone phenotype, with vegetative needles in the basal part and reproductive ovuliferous scales subtended by bracts in the apical part (Fig. 1, A and B). The number of needles initiated before the transition to reproductive development differed among individual plants and among cones on plants that initiated multiple cones. Hence, in the *acrocona* plants, the transition from vegetative to reproductive identity often occurred at different stages during bud development.

Apart from the early cone-setting phenotype, the *acrocona* plants often displayed a bushy appearance, suggesting a reduced apical dominance, and reduced height as compared with open-pollinated *acrocona* progeny and control plants. The seasonal growth response to increased light and temperature was similar in both cone-setting *acrocona* plants and control plants (Supplemental Fig. S2), which indicates that the reduced height was an effect neither of daylight sensing nor of differences in the length of the growth cycle.

Transcriptome Sequencing and Analysis

In order to identify candidate genes involved in the early cone-setting *acrocona* phenotype, we performed a massively parallel sequencing (RNA-seq) of 14 poly(A^+)-enriched (i.e. mRNA) samples extracted from needles taken from inbred cone-setting *acrocona* plants, inbred non-cone-setting *acrocona* plants, and control plants. For each individual, a sample pair was extracted: one sample from the apical, potentially cone-setting shoot, and one sample from a shoot at the base of the plant, where no cone-setting occurs (Fig. 2A). The sequencing effort yielded 136 Gb of RNA sequence (between 58 and 270 Mb per sample), for an estimated average coverage on the order of 100× for exonic regions.

Since there is no Norway spruce genome sequence available, the *acrocona* transcripts from four different samples from one *acrocona* plant were reconstructed both de novo (i.e. without aligning to any reference



	1	2	3	4	5
2	132				
3	219	131			
4	248	96	1		
5	506	191	336	326	
6	138	96	261	228	155

Figure 2. Unbiased whole-transcriptome differential expression analysis. A, Schematic view of different needle samples that were tested for differential expression: apical shoot (1) and basal shoot (2) from two *acrocona* plants that initiated cones in the second growth cycle; apical shoot (3) and basal shoot (4) from three *acrocona* plants that did not initiate cones in the second growth cycle; and apical shoot (5) and basal shoot (6) from two control plants that did not initiate cones in the second growth cycle. B, Number of differentially expressed genes between all six needle samples (multiple testing corrected $P < 0.005$). C, Venn diagram with the number of shared and unique differentially expressed genes between sample 1 compared with 2, 1 compared with 3, and 5 compared with 6.

sequence) and ab initio, using a comprehensive set of 27,720 *Picea glauca* transcripts as reference (Rigault et al., 2011). With the ab initio approach, we detected 83% (22,924 transcripts) of the *P. glauca* transcripts, indicating that homologs of these transcripts were expressed in our *acrocona* RNA-seq data. With the de novo approach, we assembled 67,857 different *acrocona* transcripts (including isoforms), of which 54,775 transcripts contained at least one identified open reading frame (ORF), amounting to a total of 83,650 ORFs in total (Supplemental Fig. S3A).

Putative orthologs were detected using OrthoMCL (Li et al., 2003), which constructs groups of orthologous proteins across species. The input data sets were, in addition to our translated *acrocona* ORFs, comprehensive protein sets from *P. glauca* and Arabidopsis. The *acrocona* ORFs were represented in 19,865 orthologous groups, and we note that 14,109 (71%) of these groups also contain *P. glauca* and/or Arabidopsis proteins (Supplemental Fig. S3, B and C), indicating that at least the corresponding set of 19,439 (35%) reconstructed ORF-containing *acrocona* transcripts are real.

Conversely, 93% of the groups of orthologous proteins that contained *P. glauca* proteins also contained *acrocona* proteins. On the transcript level, 14,520 (52%) of the *P. glauca* transcripts had an ortholog in our de novo-assembled *acrocona* set. Further details about the reconstruction and analysis of the *acrocona* and wild-type spruce transcriptomes are available (J. Reimegård and O. Emanuelsson, unpublished data), and the expression levels of the identified ORFs are presented in Supplemental Data Set S1.

Unbiased Differential Gene Expression Analysis Indicates Up-Regulation of a MADS Box Gene in Early Cone-Setting *acrocona* Apical Shoots

To identify differentially expressed genes between cone-setting and non-cone-setting *acrocona* plants in an unbiased way, relative expression levels between relevant sample pairs were established from our RNA-seq data (specifically, the 83,650 identified ORFs) using Bowtie2, Cufflinks, Cuffmerge, and Cuffdiff (Trapnell et al., 2012; Fig. 2). This process yielded expression estimates for 33,383 ORFs, and we define these as the set of potential protein-coding transcripts present in our *acrocona* samples. By comparing transcript abundance in samples from apical cone-setting shoots with samples from non-cone-setting basal shoots of the same cone-setting *acrocona* plants, 132 (four up and 128 down) significantly differentially expressed transcripts were identified (Fig. 2B). Similarly, 219 (86 up and 133 down) transcripts were significantly differentially expressed in apical shoots from cone-setting *acrocona* plants compared with non-cone-setting *acrocona* plants. Expression levels and annotation of the differentially expressed genes are listed in Supplemental Data Set S2. By combining the two data sets, 10 transcripts were identified as being significantly up- or down-regulated in cone-setting shoots of *acrocona* (adjusted for multiple testing; $P < 0.005$) as compared with their prevalence in both (1) non-cone-setting regions (basal shoots) of the same plant individual and (2) potential cone-setting regions (apical shoots) with cone-setting ability but where no cones were set in other *acrocona* individuals (1 versus 2 and 1 versus 3, respectively, in Fig. 2C). Sequence comparison showed that all 10 transcripts had sequence similarity to distinct genes in the model species Arabidopsis, and we thus conclude that they likely originated from 10 different genes in *acrocona*. Two of these genes were also differentially expressed (apical versus basal) in control plants. We believe that the remaining eight genes constitute a reasonable set of first-pass candidate genes involved in the early cone-setting in *acrocona* (Table I). Seven of these genes were down-regulated in cone-setting individuals. Functional annotation and sequence similarity to known model plant proteins suggest that they have enzymatic functions of importance for cell wall composition during meristem or organ development, cell signaling, and plant stress response, and one gene encodes a protein with unknown function.

One candidate gene, *Acr42124_1*, was up-regulated in apical cone-setting shoots compared with both basal shoots of cone-setting *acrocona* plants and apical shoots of non-cone-setting *acrocona* plants. The transcript corresponding to *Acr42124_1* was 100% identical (over its entire protein-coding region as generated by our transcript reconstruction approach; BLAST E value of 0.0) to a wild-type spruce transcript identified and named *DAL19* in a previous study (P. Engström, personal communication). *DAL19* encodes a putative transcription factor belonging to the MADS box gene

Table I. *Eight differentially expressed genes in cone-setting regions compared with non-cone-setting regions*
See also the Venn diagram in Figure 2C.

Name	Differential Expression	Arabidopsis	Panther	Function
Acr34511_3	−	PRX52	PTHR31388	Peroxidase
Acr38476_2	−	AT3G49190	PTHR31650	*O*-Acyltransferase
Acr38884_3	−	AT5G36800	PTHR31999	Unknown function
Acr42124_1	+	SOC1	PTHR11945:SF19	MADS box protein
Acr55424_3	−	GH9B13	PTHR22298:SF3	Endo-1,4-β-glucanase
Acr1417_5	−	ATCAT3	PTHR11465	Catalase
Acr15193_3	−	XTH9	PTHR31062	Xyloglucan endotransglucosylase
Acr31311_1	−	AT1G72110	PTHR31650	*O*-Acyltransferase

family. Apart from the conserved MADS domain, *Acr42124_1* and *DAL19* harbor a less conserved intervening region, a K domain, and a C-terminal extension; hence, both transcripts belong to a subclass of the MADS box gene family called the MIKC type (Ma et al., 1991). Based on the sequence identity and the occurrence of all MIKC sequence elements, we concluded that the transcript *Acr42124_1* originated from the gene *DAL19*.

The MADS Box Gene *DAL19* Shows High Similarity to a Class of Angiosperm Genes That Play Important Roles in the Transition from Vegetative to Inflorescence Meristem Identity

BLAST searches indicated that *Acr42124_1*/*DAL19* (henceforth *DAL19*) is most similar to the previously cloned spruce *DAL3* gene, which, in phylogenetic analyses covering all MIKC-type genes from the model species Arabidopsis, groups close to a clade of angiosperm genes that includes, but is not limited to, the Arabidopsis genes *SOC1* and *AGAMOUS LIKE42* (*AGL42*; Tandre et al., 1995; Carlsbecker et al., 2004). In order to establish the evolutionary relationship between *DAL19*, other similar conifer genes, and angiosperm genes previously annotated to the *SOC1* clade, we performed a phylogenetic analysis using conifer and angiosperm representatives of the distantly related *AGL6* clade as an outgroup. The phylogenetic analysis showed that *DAL19* together with the spruce gene *DAL3* and other gymnosperm MADS box genes form an orthologous sister clade to the entire angiosperm *SOC1* clade (Fig. 3). This is in agreement with previously published results and suggests that both the angiosperm *SOC1* clade and the gymnosperm clade harboring *DAL19* have gone through several rounds of gene duplication after the split between gymnosperms and angiosperms.

Directed qRT-PCR Expression Analyses of Candidate Genes for the Early Cone-Setting Response Indicate MADS Box Gene Involvement in the Initiation of Cone Set and Reproductive Maturity

To provide independent *acrocona* gene expression data for *DAL19* and an additional set of eight genes previously implicated during reproductive phase change in conifers, relative transcript levels were assayed using qRT-PCR (Fig. 4). Expression levels of these nine genes were measured in needles from lateral shoots directly subtending the apical shoot. The inbred *acrocona* plants were grouped into two main categories: A, inbred *acrocona* plants initiating at least four cones; and C, inbred *acrocona* plants that did not initiate cones. Since cones were either initiated on apical shoots or, in cases of more than one cone, on lateral shoots directly subtending the apical shoot, we expected needle samples from shoots in category A (four or more cones) to be comparable to needle samples from apical shoots used in our transcriptome analysis. In samples from needles collected during the second growth cycle, the expression of *DAL19* was significantly higher in *acrocona* plants that initiated more than four cones (i.e. category A) compared with non-cone-setting *acrocona* plants of category C, where low or no expression could be detected (Student's *t* test, $P = 0.0046$). None of the other genes tested differed significantly between cone-setting plants and non-cone-setting plants. These results are also in agreement with the transcriptome analysis, which identified full-length transcripts from a majority of the genes assayed in the qRT-PCR experiments but differential expression only of transcripts corresponding to *DAL19* (Supplemental Fig. S4).

To further examine gene expression in our *acrocona* plants, samples from apical buds collected from the same shoots as the needle samples presented in Figure 4 were also assayed (Supplemental Fig. S5). In the bud sample experiments, we included a third plant category: B, inbred cone-setting *acrocona* plants initiating fewer than four cones. Expression of *DAL19* was detected in buds from *acrocona* plants that initiated more than four cones (category A), whereas no expression could be detected in buds from *acrocona* plants that initiated fewer than four cones (category B) or no cones (category C), indicating that *DAL19* was specifically up-regulated in putative cone-setting shoots. Interestingly, Norway spruce genes paralogous to *DAL19*, such as the gene corresponding to transcript *Acr4141_1* and *DAL3*, did not show any differential expression between cone-setting and non-cone-setting *acrocona* plants in any of our qRT-PCR experiments (Fig. 4; Supplemental Fig. S5).

The MADS box genes *DAL10* and *DAL1*, which both have suggested functions in different aspects of reproductive phase change, were analyzed in our qRT-PCR experiments (Fig. 4; Supplemental Fig. S5). *DAL10* was expressed in needles from both cone-setting and non-cone-setting *acrocona* plants during the second growth cycle. No expression could be detected in buds from the same growth cycle. However, during the third growth cycle, significantly higher *DAL10* levels were detected in plants that already had initiated at least one cone (i.e. in shoots belonging to categories A and B) compared with *acrocona* plants lacking cones (category C), which expressed *DAL10* only at very low levels (Student's *t* test, $P = 0.0426$; Fig. 4; Supplemental Fig. S5). Interestingly, expression of *DAL1* was consistently high during the second growth cycle in both needles and buds of one individual plant that initiated as many as 10 cones (individual A1 in Fig. 4; Supplemental Fig. S5).

Other genes that have been implicated in reproductive development and phase change are the angiosperm *LFY* orthologs *NEEDLY* (*NLY*) and *PaLFY*. Both *NLY* and *PaLFY* were expressed at varying levels in all examined samples, and no distinct changes between cone-setting and non-cone-setting plants could be detected for *NLY* (Fig. 4; Supplemental Fig. S5). Expression levels of *PaLFY,* however, were significantly higher in buds from the third growth cycle of cone-setting *acrocona* plants as compared with non-cone-setting plants (Student's *t* test, $P = 0.007$; Fig. 4;

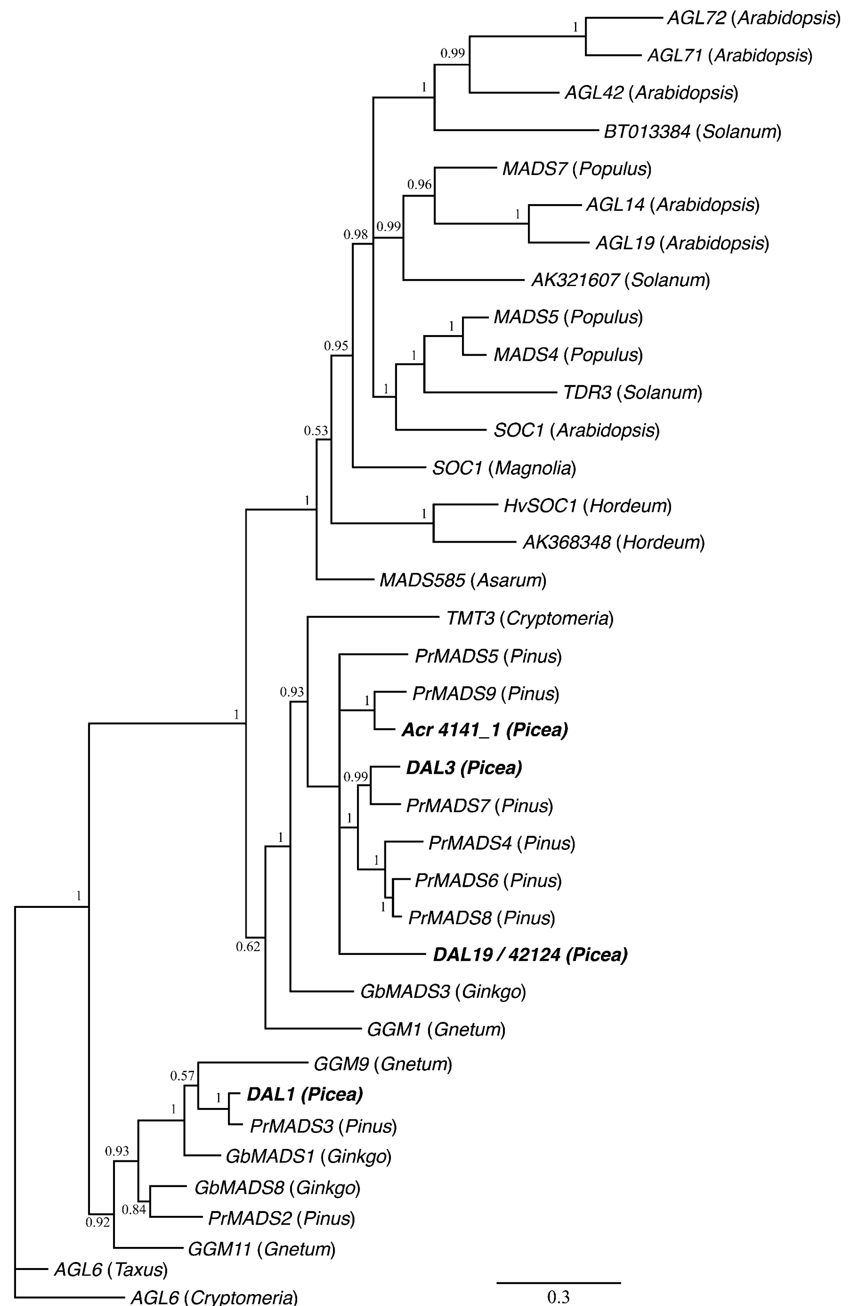


Figure 3. Phylogenetic relationship of MADS box genes from angiosperms and gymnosperms that show orthology to the *DAL19* gene. Shown is a 50% majority rule tree derived using Bayesian phylogenetics. Numbers beside each node indicate posterior probabilities. The taxon of origin is shown in parentheses after each gene name, and spruce gene names are highlighted in boldface. Names and accession numbers are found in Supplemental Table S2.

Supplemental Fig. S5). The putative reproductive organ identity gene *DAL2* or the *FT/TFL1*-like gene *PaFTL2* did not show expression patterns that correlated with the cone-setting phenotype (Supplemental Fig. S4). The expression of candidate genes was also assayed using qRT-PCR in bud and needle samples of control plants (Supplemental Fig. S6). In these experiments, we could not detect any significant differences in expression levels between tissue samples, although individual plants expressed different genes at different levels.

Altogether, the qRT-PCR experiments corroborate the differential expression of *DAL19* in early cone-setting *acrocona* plants before or at the onset of cone initiation and indicate that *DAL10* is activated in apical buds of *acrocona* plants that have made the phase change from vegetative to reproductive development.

DISCUSSION

Theodor Fries and colleagues first recognized the *acrocona* mutant in the mid-19th century from the aberrant shape and position of cones and vegetative shoots. Fries (1890) described the aberrant structures as monstrous cones. Here, we interpret these phenotypes as more or less covering the transition between vegetative and reproductive shoot identity. Hence, the

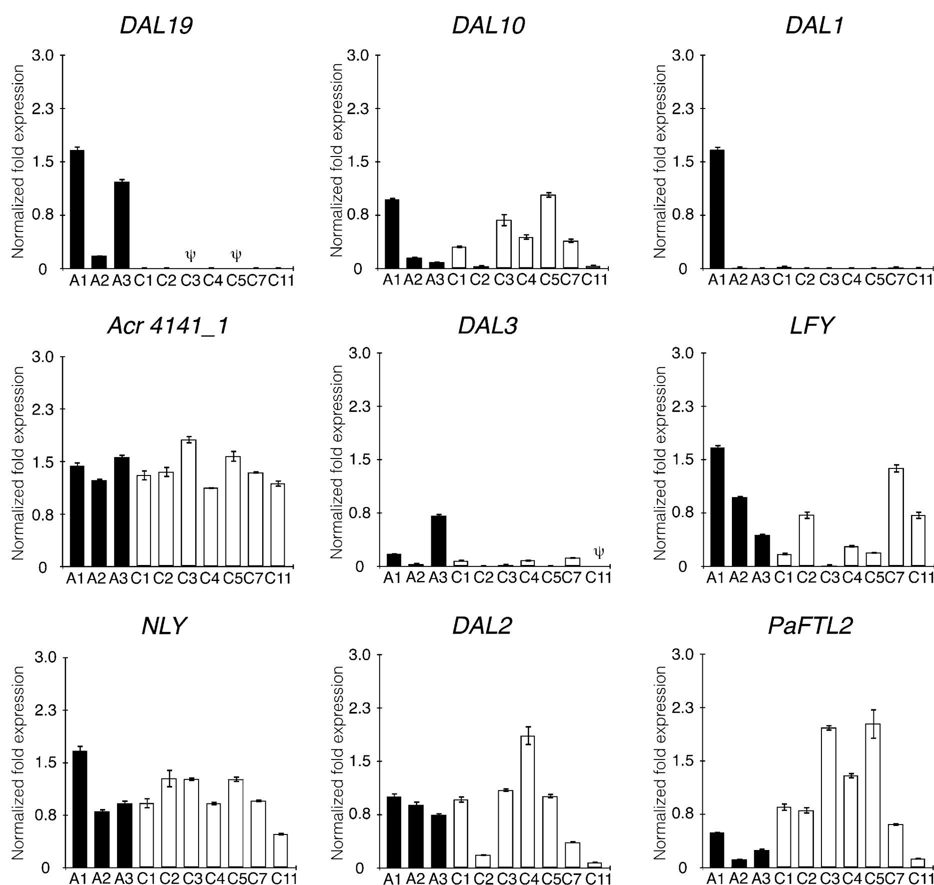


Figure 4. qRT-PCR analyses of nine genes in needles from the second growth cycle. The samples were collected from inbred cone-setting *acrocona* plants initiating four or more cones (A) and inbred vegetative, non-cone-setting *acrocona* plants (C). Numbers beside the categories A and C indicate individual *acrocona* plants. The expression of *DAL19*, *DAL10*, *DAL1*, *Acr4141_1*, *DAL3*, *LFY*, *NLY*, *DAL2*, and *PaFTL2* was assayed. Needles were sampled from one lateral shoot subtending the apical shoot and collected during the vegetative growth of the shoot before winter dormancy. Expression levels are presented as unscaled expression values based on mean values of three technical replicates. ψ represents no detectable expression. Error bars denote SE of technical replicates. Expression values of each gene are normalized against the expression of three reference genes, *POLYUBIQUITIN*, *ACTIN*, and *HISTONE2A*.

female cones formed on the parental ramets can be categorized either as complete cones or as partial transition cones, which is in agreement with the structures reported both by Fries (1890) and Joneborg (1945). Both types of cones have the possibility to produce seeds. This is also true for inbred *acrocona* plants, which produced seeds after they had been planted outdoors and received normal growth cycles. One additional interesting aspect of the adult plants used to produce the segregating sibling population of inbred *acrocona* plants is that the adult plants initiate cones frequently (i.e. every growth season), even though cone setting in spruce, by and large, is coordinated and occurs only once every 3 to 5 years. One molecular interpretation of the combined phenotypes observed in adult *acrocona* plants is that one or more of the signals that act as reproductive integrators is ectopically active. By making inbred crosses of two *acrocona* ramets, we hoped to enhance the cone-inducing properties of the *acrocona* mutant and produce a population of segregating *acrocona* plants that could be used for further molecular studies. In addition to the frequent cone setting observed in the adult *acrocona* ramets, one-fourth of the plants in our inbred crosses made the phase change from vegetative growth to reproductive growth extremely early and initiated cones on apical shoots already during their second growth cycle. Occurrences of additional *acrocona* phenotypes, such as the intermediate shoot phenotypes that develop in the segregating plants during the fifth growth cycle, suggest that the *acrocona* trait is semidominant. In contrast, linkage analysis between genetic markers and the early cone-setting phenotype has previously suggested that early cone setting is a dominant monogenic trait (Acheré et al., 2004), while early cone setting in inbred *acrocona* mutants has been reported to occur with a much lower frequency (3%) than reported here (Flachowsky et al., 2009). This indicates that the accelerated growth conditions are of importance for the penetrance of the early cone-setting phenotype or that different variants of the *acrocona* mutant have been collected over the past 150 years.

The finding of an early cone-setting mutant in the gymnosperm Norway spruce is interesting in two respects. First, it may be developed into a technical tool for studying gene function in a conifer. Second, the mutant phenotype itself provides an opportunity to study vegetative-to-reproductive phase change in a conifer system. Functional studies of individual genes in conifers largely rely on cell-based systems such as somatic cell lines or comparative studies using the heterologous angiosperm model system (Sundström and Engström, 2002; Sundström et al., 2009). Adult characteristics are difficult and time consuming to study, since most conifers enter the reproductive phase after 15 to 25 years of juvenile growth. The short generation time of inbred *acrocona* plants makes this an

interesting system that, in combination with embryogenic cell lines (to allow for mass propagation of *acrocona* clones) and efficient transformation protocols, can be developed into a rapid-cycling conifer model system. One key aspect of this is to characterize the genetic mechanism underlying the early cone-setting phenotype in the *acrocona* mutant. To accomplish this, we used large-scale sequencing methods and bioinformatics analyses to compile and present the transcriptome of cone-setting and non-cone-setting *acrocona* plants as well as of control plants of Norway spruce.

We performed massively parallel sequencing (RNA-seq) on needle samples from inbred *acrocona* plants and control plants and obtained an estimated coverage of the exonic regions of, on average, 100× for different samples. We used state-of-the-art transcript reconstruction methods, trying both ab initio and de novo approaches. With our final transcript reconstruction process, we found support through orthology in *P. glauca* and/or Arabidopsis for 35% of our *acrocona* transcripts. This is rather low; including more species in the orthology analysis would probably increase this percentage, as would relaxing the requirements for orthology. Also, many detected *acrocona* ORFs are probably fragments, as the length distribution of the ORFs with no orthologs has a large peak in the lower end of the length range (Supplemental Fig. S3C, "No orthologs").

Almost all groups of orthologous proteins containing *P. glauca* proteins also contained *acrocona* proteins (de novo reconstructed set), which on the transcript level translates to a detection rate of 52% of the to-date most comprehensive published *P. glauca* transcriptome (Rigault et al., 2011). The ab initio approach to transcript reconstruction provided, at 83%, a greater coverage of the *P. glauca* transcripts, further supporting our suggestion that the ortholog detection process is rather conservative. Given that we only have a single tissue type (needles from apical and basal shoots) and a limited number of conditions or developmental stages, this is a reasonable fraction, indicating that we have reached a sufficient sequencing depth and that our sequence data analysis is reasonable, albeit not perfect. Also, of the 7,501 orthologous groups shared between Arabidopsis and *P. glauca*, 95% are present in *acrocona*, indicating that most of the well-conserved proteins are present in the *P. glauca* transcriptome. Turning the argument around, out of the 8,296 orthologous groups shared between *acrocona* and Arabidopsis, 86% are also found in *P. glauca*, indicating that our de novo-assembled transcriptome is at least as representative of the total spruce transcriptome as the published *P. glauca* transcriptome. Altogether, since we are interested in the mRNAs expressed in needles just before *acrocona* sets cones, and since the quality of the de novo-assembled transcriptome is on par with the *P. glauca* transcriptome, we decided to use our de novo-assembled transcriptome as a reference for the differential expression analysis, thus minimizing the risk of completely missing any tissue- or development-specific mRNAs.

We were interested in identifying transcripts that were significantly differentially expressed in apical cone-setting shoots compared with non-cone-setting shoots in *acrocona* plants and that at the same time were not differentially expressed (apical versus basal shoots) in control plants. As a result, we could drastically reduce the number of candidate genes associated with early cone setting from 33,383 down to eight, and among these, the MADS box gene *DAL19* stood out as the most interesting candidate. Our unbiased approach (RNA-seq coupled with de novo transcript assembly) enabled us to suggest *DAL19* as a candidate gene, which, since it was not previously published or deposited in any database, would have been practically impossible using a directed search (e.g. microarrays or any PCR-based method) or our ab initio-assembled transcripts. The process of extracting candidate genes through differential expression was rather conservative, and by easing the requirements, we would have ended up with a larger number of candidates. One possible candidate that could have been annotated as being up-regulated in cone-setting shoots is the MADS box gene *DAL1*, which shows a similar expression pattern to that of *DAL19* in our transcriptome analysis, albeit with a higher variance between sample pairs, precluding it from being detected as statistically significant. This notion is supported by the qRT-PCR experiments that revealed high expression of *DAL1* in buds and needle samples of one individual plant that initiated high numbers (more than 10) of cones during the second growth cycle.

In our phylogenetic analysis, *DAL19* together with three other Norway spruce paralogs form a sister clade to the angiosperm *SOC1/AGL42* clade. Several of the angiosperm genes in this clade have experimentally been shown to act as floral integrators during the transition from vegetative to reproductive phase (Dorca-Fornell et al., 2011), although other genes (e.g. *AGL14* and *AGL19*) also have functions during root development (Gan et al., 2005). This is in agreement with the prevailing notion that the MADS box gene family has evolved through a series of gene duplications followed by subfunctionalization or neofunctionalization and that, although phylogenetic position may be indicative of a common ancestral function, orthologs can have acquired distinct and different activities (Irish and Litt, 2005). Here, we present evidence to suggest that the gymnosperm lineage orthologous to the *SOC1/AGL42* clade has gone through several independent gene duplications. Furthermore, based on the expression profiles in our unbiased gene expression analysis (RNA-seq) and qRT-PCR experiments, we show that *DAL19* is the only spruce paralog in the clade that shows a clear up-regulation in cone-setting shoots as compared with non-cone-setting shoots.

Recent experimental evidence suggests that SOC1 is a floral integrator that regulates the activity of both floral meristem identity genes as well as floral organ identity genes (Immink et al., 2012). In our qRT-PCR experiments, we assayed the expression of previously

described Norway spruce orthologs to the floral meristem identity gene *LFY*: *PaLFY* and *NLY*. Both *PaLFY* and *NLY* are expressed in all tissues examined; however, the level of *PaLFY* expression increased slightly but significantly in buds of cone-setting *acrocona* plants during the third growth cycle. Known Norway spruce orthologs to the angiosperm floral organ identity genes did not show any differential expression in our experiments. Nor did we, in our unbiased approach, identify any differential expression of putative Norway spruce orthologs to microRNA156-targeted SQUAMOSA PROMOTOR BINDING-LIKE transcription factors, which recently have been shown to regulate *SOC1* activity and vegetative-to-reproductive phase change in angiosperms (Huijser and Schmid, 2011). As previously noted, gymnosperms also lack an apparent ortholog to the angiosperm *FT* gene (Karlgren et al., 2011), and further studies of gymnosperm-specific genes are crucial for the understanding of reproductive phase change in conifer trees. Interestingly, we observed differential expression of the *DAL10* gene, which belongs to a gymnosperm-specific clade of the MADS box gene family. *DAL10* has been suggested to be a marker for reproductive shoot identity (Carlsbecker et al., 2003) and is expressed in buds after, but not before, the phase change from vegetative to reproductive growth. Hence, the data presented here suggest that *DAL10* is activated as a result of reproductive competence, whereas upregulation of *DAL19* coincides with or precedes reproductive phase change. It is tempting to speculate that the ancestral function of the SOC1/AGL42 clade was to determine the length of the juvenile growth period and to regulate the phase change from vegetative to reproductive growth. Here, we present evidence to suggest that *DAL19* may be involved in a similar process in gymnosperms, which would imply that at least part of this gene function was present already in the last common ancestor of angiosperms and gymnosperms.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Two parental ramets, derived from shoots collected from a homeotic mutant of Norway spruce (*Picea abies* var *acrocona*) that have been grafted onto rootstocks, were used in this study. Grafted plants were planted in the Genetic Garden in Uppsala (latitude 59°51′0′′ N) and in the park of Alnarp (latitude 55°42′0′′ N) around 1975. The genetic uniformity between one plant in the Genetic Garden and one plant in the Alnarp park was confirmed by microsatellite analysis, which revealed no discrepancy between studied loci (n = 7; data not shown). To establish a segregating sibling population of inbred *acrocona* plants, controlled crossings between the two selected ramets of the *acrocona* mutant were performed. Pollen collected from the ramet growing in the Alnarp park was used as pollen donor. Controlled crosses were made by isolation of female cones from surrounding pollen as they started to elongate and mature during early spring. The *acrocona* pollen was subsequently sprayed onto the female cones during the pollination phase. Open-pollinated progeny were derived from open-pollinated seeds from the *acrocona* plant in the Genetic Garden and in the Alnarp park. Seeds were collected from mature cones late in autumn. Seeds collected from open-pollinated trees in the seed orchard FP-65 Rörby (latitude 59°54′ N), which harbor 27 unrelated clones with an average latitudinal origin of 59°24′ N, were used for regenerating control plants.

Seeds were germinated for 1 month and then potted in stone wool (Grodiana) in the phytotron. The potted plants were initially grown under continuous light (240 μmol m^{-2} s^{-1}) at 20°C and 75% relative humidity for 8 weeks. This was followed by a 16-h night treatment and with gradually lowered temperature essentially as described (Ununger et al., 1988; i.e. 1 week at 10°C, 1 week at 5°C, 1 week at 2°C, followed by 1 week at 10°C). During the subsequent growth cycles, the plants followed the following growth regime: 4 weeks with 8/16-h day/night conditions at 20°C/15°C; 7 weeks with progressive increase of the night period by 1 h per week at 20°C/15°C; 2 weeks with 8/16-h day/night conditions at 20°C/15°C; followed by a 16-h night treatment and gradually lowered temperature as described above. Under these accelerated growth conditions, the plants were cultured during four to five growth cycles.

All plant material for molecular analyses was collected when the shoots had reached approximately 90% of their final length, which corresponds to the time point when bud identity is determined in Norway spruce (Lindgren et al., 1977; Högberg and Eriksson, 1994). At the end of the growth cycle, it is only possible to determine the reproductive or vegetative identity of a shoot by detrimental microdissection of the developing bud. Therefore, the collected plant material was snap frozen in liquid nitrogen and stored at −70°C until the cone-setting status of the plant/shoot could be determined in the following growth cycle.

Phenological data (e.g. height, bud set, and branching pattern) were recorded each week under the growing periods during the first three growth cycles from 75 inbred *acrocona* plants (derived from crossings made in 2006), 150 open-pollinated *acrocona* progeny, and 75 control plants. During each growth cycle, one lateral shoot, directly subtending the apical shoot, was sampled from each plant. Isolated needles and buds from these shoots were used in subsequent qRT-PCR analyses.

Four inbred *acrocona* plants (two cone-setting plants and two non-cone-setting plants, derived from crossings made in 2009) together with two control plants were used in the transcriptome analysis. During the second and third growth cycles, needles (five to seven) directly subtending the forming bud on the top apical shoots (i.e. potentially cone-setting shoots) and from vegetative lateral shoots were sampled. Ten *acrocona* samples were sequenced: two samples from apical cone-setting shoots, three samples from apical non-cone-setting *acrocona* shoots, and five samples from basal *acrocona* shoots. Four samples were sequenced from control plants: two samples from apical non-cone-setting shoots and two samples from basal shots. Thus, in total, 14 samples were assayed in the transcriptome sequencing.

RNA Isolation

Total RNA for qRT-PCR analyses was isolated using the Spectrum Plant Total RNA kit (Sigma-Aldrich). Total RNA was subjected to on-column DNase digestion according to the manufacturer's instructions (Sigma-Aldrich). The integrity of total RNA was assessed by Bioanalyzer (Agilent). Complementary DNA was prepared from 0.5 to 1 μg of total RNA using the Maxima First Strand Synthesis Kit for RT-qPCR (Thermo Scientific).

Total RNA for massively parallel sequencing was isolated using a slightly modified protocol described by Azevedo et al. (2003). RNA was subjected to DNase digestion according to the manufacturer's instructions (DNA-free; Life Technologies).

Library Generation and Massively Parallel Sequencing

RNA libraries for sequencing were prepared using TruSeq RNA kits (Illumina) according to the manufacturer's instructions with the following changes: the protocols were automated using an MBS 1200 pipetting station (Nordiag) as described previously (Stranneheim et al., 2011), and all purification steps and gel cuts were replaced by magnetic bead cleanup methods (Borgström et al., 2011).

The clustering was performed on a cBot cluster generation system using an Illumina HiSeq paired-end read cluster generation kit according to the manufacturer's instructions (Illumina). The 14 samples were sequenced on an Illumina HiSEquation 2000 as paired-end reads to 100 bp, using all eight lanes of a flow cell. Each sample was divided into four equal aliquots and sequenced in four different lanes, thus with seven samples in each lane using multiplexing tags to separate samples logically. All lanes were spiked with a 1% to 2% phiX control library. One lane failed (low cluster density) and was resequenced 31 d later. The sequencing runs were performed according to the manufacturer's instructions. Base conversion was done using Illumina's OLB version 1.9.

De Novo Transcript Assembly and ORF Identification

The transcript reconstruction process has been described in detail (J. Reimegård and O. Emanuelsson, unpublished data). Briefly, sequence reads

from Illumina HiSeq2000 were filtered with cutAdapt (Marcel, 2011) and an in-house script to remove whole reads or parts of reads with low quality. Only pairs where both reads passed the quality test were further analyzed. Trinity (Grabherr et al., 2011; build 11.05.27) was used to build the de novo-assembled transcripts. Potential ORFs were extracted from the transcripts with getOrf from the EMBOSS package (Rice et al., 2000). An ORF was accepted if it contained a start codon, an ORF longer than 150 nucleotides, and a stop codon. Multiple ORFs per transcript were allowed provided that they did not overlap on the transcript. ORFs on one transcript were selected in a hierarchal order based on their length, where the longest was selected first. If multiple ORFs from different transcripts had identical sequences, only one was kept. Each ORF was translated into peptide sequence using Transeq from the EMBOSS package.

Generating Groups of Orthologous Proteins

Groups of orthologous proteins from *acrocona*, *Picea glauca*, and Arabidopsis (*Arabidopsis thaliana*) were generated by OrthoMCL version 1.4 (Li et al., 2003). The Arabidopsis proteins (The Arabidopsis Information Resource 10) were downloaded from phytozome.net (Lamesch et al., 2012). *P. glauca* proteins were generated by subjecting its transcripts (Rigault et al., 2011) to the same ORF detection protocol as described for *acrocona* (above) and then translated into protein sequences.

Functional Annotation of *acrocona* Peptide Sequences

The identified *acrocona* peptide sequences were analyzed with Panther 7.2 (Mi et al., 2010). Genes that were identified as belonging to a Panther class were annotated with Gene Ontology terms according to Panther 7.2 Gene Ontology annotation. Genes with no Panther class were annotated based on orthologs in Arabidopsis and *P. glauca* (see above). If an *acrocona* protein belonged to an ortholog group that contained multiple Arabidopsis proteins, then the *acrocona* protein was assigned the functional annotation of the Arabidopsis protein with the highest BLAST (version 2.2.24) score.

Differential Gene Expression Analysis

The quality-filtered reads were mapped onto the ORFs generated from the transcriptome (see above) with Bowtie2 (Langmead and Salzberg, 2012), allowing only for concordant mapped pairs (–no-mixed–no-discordant). Cufflinks together with Cuffmerge (Trapnell et al., 2012) were used to identify the full-length transcripts, and Cuffdiff was used to identify differently expressed genes. Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html) was used to create the Venn diagrams, while expression patterns for genes were generated using the cummeRbund package for R (Trapnell et al., 2012).

Phylogenetic Analysis

Annotated MADS box genes with high similarity to *DAL19* from selected angiosperms and gymnosperms were retrieved from GenBank using BLAST searches (Altschul et al., 1997; for accession numbers, see Supplemental Table S2). Selected sequences belonging to the closely related gymnosperm *AGL6* clade were also included in the analysis to serve as outgroup sequences. The obtained sequences were translationally aligned using the MAFFT module within Geneious (Geneious Pro version 5.6 created by Biomatters; http://www.geneious.com). The data set was then reduced such that partial sequences and putative close orthologs and paralogs were omitted. The resulting data set comprised 37 taxa and included 666 characters, excluding the highly variable C-termini-encoding sequences.

Phylogenetic analysis of the resulting nucleotide alignment was carried out using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The selected model of evolution was GTR + I + G, which assumes a general time reversibility (GTR), a certain proportion of invariable sites (I), and a gamma approximation (G) of the rate variation among sites. Four chains of the Markov Chain Monte Carlo were run in parallel, sampling one tree every 500 generations for 2.5 million generations starting with a random tree. The search reached stationarity after approximately 250,000 generations. The first 250,000 generations (the burn in) were omitted in generating the consensus phylogeny.

qRT-PCR

qRT-PCR was performed using the iQ5 Real-Time Detection System on iCycler iQ 96-well PCR plates with adhesive seals (Bio-Rad Laboratories). Primers used to quantify expression levels are presented in Supplemental Table S3. The expression data of each gene were normalized against the expression of three reference genes, *ACTIN*, *POLYUBIQUITIN*, and *HISTONE2A* (Supplemental Table S3). Amplifications were carried out using the DyNAmo Flash Sybr Green qPCR kit (Thermo Scientific). PCR cycling conditions were as advised by the manufacturer, with annealing and extension at 60°C for 30 s. The reactions were run for 40 cycles, and at the end of each run, melt curves were generated to ensure product uniformity. Samples were added to the plates in triplicate. In all studies, interrun connector samples were included to correct for the use of multiple plates. All calculations and normalizations were done using the iQ5 software based on the "Pfaffl methods" (Bio-Rad).

Sequence data from this article can be found in the Sequence Read Archive data libraries under accession number SRA064221.

Supplemental Data

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

Supplemental Figure S1. Phenotypes of inbred *acrocona* plants grown under accelerated growth conditions.

Supplemental Figure S2. Seasonal growth responses of *acrocona* plants and control plants.

Supplemental Figure S3. Transcriptome sequence analysis.

Supplemental Figure S4. Expression pattern of protein-coding transcripts.

Supplemental Figure S5. qRT-PCR expression data of candidate genes from bud samples in cone-setting and non-cone-setting inbred *acrocona* plants.

Supplemental Figure S6. qRT-PCR expression data of candidate genes from needle and bud samples in non-cone-setting wild-type plants.

Supplemental Table S1. Phenotype frequencies of inbred *acrocona* plants.

Supplemental Table S2. Gene names and accession numbers used in the phylogeny.

Supplemental Table S3. Primer sequences used for qRT-PCR analyses.

Supplemental Data Set S1. Expression levels of all identified ORFs.

Supplemental Data Set S2. Expression levels and annotation of the differentially expressed genes.

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