Short Day–Mediated Cessation of Growth Requires the Downregulation of AINTEGUMENTALIKE1 Transcription Factor in Hybrid Aspen

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Abstract

Day length is a key environmental cue regulating the timing of major developmental transitions in plants. For example, in perennial plants such as the long-lived trees of the boreal forest, exposure to short days (SD) leads to the termination of meristem activity and bud set (referred to as growth cessation). The mechanism underlying SD–mediated induction of growth cessation is poorly understood. Here we show that the AIL1-AIL4 (AINTEGUMENTALIKE) transcription factors of the AP2 family are the downstream targets of the SD signal in the regulation of growth cessation response in hybrid aspen trees. AIL1 expression is induced in the shoot apical meristem and leaf primordia, and exposure to SD signal downregulates AIL1 expression. Downregulation of AIL1 gene expression by SDs is altered in transgenic hybrid aspen plants that are defective in SD perception and/or response, e.g. PHYA or FT overexpressors. Importantly, SD–mediated regulation of growth cessation response is also affected by overexpression or downregulation of AIL1 gene expression. AIL1 protein can interact with the promoter of the key cell cycle genes, e.g. CYCD3.2, and downregulation of the expression of D-type cyclins after SD treatment is prevented by AIL1 overexpression. These data reveal that execution of SD–mediated growth cessation response requires the downregulation of AIL1 gene expression. Thus, while early acting components like PHYA and the CO/FT regulon are conserved in day-length regulation of flowering time and growth cessation between annual and perennial plants, signaling pathways downstream of SD perception diverge, with AIL1 transcription factors being novel targets of the CO/FT regulon connecting the perception of SD signal to the regulation of meristem activity.

Introduction

The ability to adapt to changes in the environment is crucial to the survival of both animals and plants. Plants, unlike animals, are sessile organisms and have therefore evolved highly sophisticated mechanisms to anticipate seasonal changes and modulate their patterns of growth and development. Day length is one of the key environmental cues utilised by plants to anticipate seasonal changes and regulates several key developmental transitions associated with plant adaptation and reproduction. One of the most fascinating examples of this is provided by perennial plants, e.g. the long-lived trees of the boreal forest, in which the day length signal regulates the developmental transition from active growth to a more resilient dormant state prior to the onset of winter [1]. These perennial plants anticipate the approach of winter by detecting the reduction in day length (i.e. the short day signal, or SD signal) in the autumn and when the day length falls below the critical day length required for the promotion of growth, cell division in the meristems ceases [2]. The most visible indicator of short day–induced growth cessation is the formation of a bud that encloses the apical meristem and leaf primordia [3]. The importance of day length sensing for the survival of perennial plants is illustrated by the increased mortality due to delayed growth cessation in transgenic hybrid aspen plants that are unable to sense reductions in day length [4].

Intriguingly, there are numerous similarities at the regulatory level between day length mediated control of growth cessation in perennial plants and one of the most well studied developmental transitions in plants - the transition from vegetative growth to floral development. For example, key flowering time regulators such as the CONSTANS (CO), FLOWERING LOCUS T (FT) and the group of photoreceptors known as PHYTOCHROMES (PHYs) that are involved in day length mediated regulation of flowering time regulation in Arabidopsis [5,6,7,8], are all also involved in SD–induced growth cessation in trees [4,9,10,11]. In poplar species two closely related orthologs of FT (FT1 and FT2) have been found and recent analysis in hybrid aspen clone 353 indicates that FT2 could be primarily involved in SD–mediated growth cessation whereas FT1 is primarily involved in flowering [11]. In hybrid aspen (clone T89, used in this study), it has been shown that short day mediated downregulation of FT gene expression (FT1 and FT2) triggers the induction of growth cessation whereas overexpression of FT1 eliminates the plants’ ability to respond to the SD signal and thus prevents timely growth cessation [9]. Both CO and PHYTOCHROME A (PHYA) act upstream of the FT genes in SD-mediated induction of growth.
Author Summary

Day length is a critical and robust environmental cue utilised by plants to modulate their patterns of growth and development. In perennial plants such as long-lived trees of the boreal forest, reduction in day length (short day signal/SD) induces the cessation of growth prior to the advent of winter. This developmental switch is of major adaptive significance as inability to undergo growth cessation leads to mortality in these plants. Our knowledge of how SD signal induces growth cessation is rudimentary. Here we show that AIL1 (AINTEGUMENTALIKE1), a plant-specific transcription factor, is a downstream target of the SD signal and can regulate the expression of key cell proliferation related genes. Intriguingly, the early acting components in day length regulated processes such as flowering and growth cessation are conserved between annual and perennial plants. However, our results show that the pathways downstream of short day perception diverge between these day length–controlled developmental transitions. These results have important implications for the evolution of the perennial life cycle and demonstrate how the same signal, namely day length, can regulate diverse developmental switches in annual and perennial plants.

Downregulation of the expression of the AINTEGUMENTALIKE (AIL) gene family coincides with the cessation of growth and bud set following SD treatment in Populus

The Populus genome contains 13 genes belonging to the ANT-subgroup of the AP2 transcription factor family [20]. Four of these genes are here designated as AIL1-AIL4 (AINTEGUMENTALIKE 1-4) as they belong to the same clade as the Arabidopsis ANT transcription factor (Figure S1). We investigated the expression of AIL1 as well as the expression of the related genes AIL2-AIL4 in the apex of hybrid aspen plants after SD treatment (Figure 1A and Figure S2). RT-PCR data indicates that AIL1 (Figure 1A) as well as AIL2-AIL4 expression (Figure S2) are downregulated along with that of cell cycle markers CYCD3:2 and CYCD6:1 after SD treatment (Figure 1B, panels B and C) and this downregulation coincides with the cessation of growth and bud set in the apex of hybrid aspen T89 trees [21].

Downregulation of AIL gene expression is perturbed in hybrid aspen plants with altered SD response

We then investigated the effect of perturbed SD perception or response on the regulation of AIL gene expression after SD treatment. For this we used transgenic hybrid aspen that are unable to respond to the SD signal due to overexpression of PHIA or FTT1 cDNA as well as plants that are hypersensitive to the SD signal and undergo premature growth cessation due to the downregulation of FT expression [FTRNAi] [4,9]. Since all 4 AIL genes are highly similar and displayed similar expression pattern after SD treatment, we chose to perform detailed analysis of AIL1 regulation. RT-PCR analysis indicated that the downregulation of AIL1 expression after SD treatment is severely attenuated in the apex of PHIA and FTT1 overexpressors in contrast with the wild type (Figure 2A). In contrast the FTRNAi plants that respond more rapidly to SD treatment than wild type [9] display a stronger and earlier reduction in the expression of AIL1 (Figure 2B). These results strongly suggest that AIL1 expression is a potential downstream target of the SD signal transduced via the CO/FT module in cessation of growth and bud set in the apex of hybrid aspen.
AIL1 expression in hybrid aspen apex is confined to zones of actively dividing cells

We further investigated expression of AIL1 in different tissues and found that AIL1 is primarily expressed in the apical region of hybrid aspen (Figure 3A). Since the downregulation of AIL1 gene expression was closely associated with cessation of growth and bud set, we analysed the domain of AIL1 gene expression in the apex. For this analysis we generated transgenic hybrid aspen expressing a transcriptional fusion between 2.5 Kb upstream sequence of AIL1 gene from Populus trichocarpa and the uidA (β-glucuronidase/GUS) reporter gene. In the transgenic hybrid aspen, the reporter gene expression was mostly confined to the zone of dividing cells in the apex, the vascular tissues and the leaf primordia (Figure 3B). The expression pattern of pAIL1:UidA reporter construct correlates well with the previously described expression pattern of CYCA1 which serves as a marker of dividing cells in the apex of hybrid poplar plants [22] indicating that AIL1 expression is associated primarily with cell proliferation.

Perturbing AIL gene expression affects SD-mediated regulation of growth cessation response

Analysis of AIL1 gene expression suggested that its downregulation could be important for the SD mediated cessation of growth and bud set. We tested this hypothesis by generating transgenic hybrid aspen that would maintain high levels of AIL1 expression even after SD treatment in contrast with the wild type by expressing AIL1 cDNA under the control of the 35S promoter (these transgenic lines are henceforth referred to as AIL1oe). Several independent lines were obtained and tested for high expression of AIL1 (Figure S3A shows data for two chosen lines); two lines were chosen for detailed analysis of their response to SD treatment. Unlike the wild type plants that undergo growth cessation and form an apical bud after 6 week of SD treatment, the apices of AIL1oe fail to undergo proper growth cessation and bud set after 6 weeks of SD treatment (Figure 4A–4C). We also generated transgenic hybrid aspen overexpressing AIL3 (Figure S3) to investigate whether other members of the gene family share the same function (these transgenic lines are henceforth referred to as AIL3oe lines). AIL3oe display a similar phenotype to AIL1oe during SD treatment (Figure 4D–4F).

We also investigated the effect of downregulation of AIL gene expression on SD mediated bud set. The functional redundancy between AIL genes suggested by similar regulation and effect on bud set in AIL1oe and AIL3oe plants lead us to generate transgenic hybrid aspen plants in which the expression of all 4 AIL genes was targeted for downregulation using artificial microRNA (amiRNA). Two amiRNA constructs (255 and 256) were expressed in hybrid aspen and two lines (255-6 and 256-23) with reduced expression of AIL1 gene expression (Figure S4) were selected for further analysis of growth cessation response. The transition from active growth to bud set after SD treatment in the wild type and lines 255-6 and 256-23 was investigated using a method separating different stages of bud development [23]. Compared to the wild type, the lines 255-6 and 256-23 displayed a more rapid transition from active growth to bud set and a majority of the plants in the two transgenic lines made the transition to intermediate and late stage of bud set at least a week (or more) earlier than the wild type plants after SD treatment (Figure S5). This data along with the perturbed growth cessation response in AIL1oe and AIL3oe plants indicate that downregulation of AIL gene expression is necessary for SD mediated growth cessation response.

AIL1 is the downstream target of SD signal in the activation of growth cessation responses

The altered growth cessation responses in AIL1oe plants could either be due to the failure of these plants to perceive the short day signal or to a failure in properly responding to it. To distinguish between these two possibilities we compared the response of FT2 expression to SD treatment in the leaves of wild type and AIL1oe. Downregulation of FT2 expression in the leaves is the earliest known marker for the detection of the SD signal and recent results have implicated its downregulation in SD mediated growth cessation [9,11,12]. Our data show that both the wild type (Figure 5A) and AIL1oe lines (Figure 5B) exhibit similar decreases in their levels of FT2 transcripts following SD treatment. This result indicates that unlike FT genes, AIL1 does not act early in SD response but is rather a downstream target of the SD signal.

AIL1 is a potential regulator of D-type cyclin gene expression

The expression of several cell proliferation related genes, e. g. D-type cyclins, that are key cell cycle regulators [24,25,26,27] is downregulated in a similar manner to AIL genes during SD mediated cessation of growth in hybrid aspen [15], Figure 1B, 1C. We therefore investigated whether the AIL1 transcription factor could be involved in the regulation of the D-type cyclin genes and if so whether their expression is perturbed in AIL1oe lines after SD treatment. Therefore we analysed the expression of two D-type cyclins, CYCD3:2 and CYCD6:1, in the apex of wild type hybrid aspen analyzed by real-time PCR after 0 (OSD), 14 (14SD), 28 (28SD) and 42 (42SD) days of short day treatment. AIL1oe lines (Figure 1B) exhibit similar decreases in their levels of CYCD3:2 and CYCD6:1 transcripts following SD treatment. This implies that AIL1oe plants display a more rapid transition from active growth to bud set as compared to the wild type and lines 255-6 and 256-23 but this transition is still delayed when compared to wild type plants. It is likely that several other genes in addition to the D-type cyclins are downregulated in the AIL1oe plants due to their failure to perceive the SD signal or to a failure in properly responding to it. The goal of this paper was to examine the role of AIL1 in the SD mediated growth cessation response in hybrid aspen and the fact that it might be downregulated in AIL1oe plants indicates that both these possibilities need to be investigated further.
cyclins, CYCD3:2 and CYCD6:1 after SD treatment in AIL1oe plants after 6 weeks of SD treatment. Our RT-PCR data (Figure 6) showed that while the expression of CYCD3:2 and CYCD6:1 is downregulated in the wild type after 6 weeks of SD treatment, this was not the case in the AIL1oe plants. This result indicates that AIL1 could be involved in the regulation of D-type cyclins and the failure to downregulate AIL1 expression after SD treatment leads to a corresponding failure to downregulate the expression of these key cell cycle regulators.

**AIL1 transcription factor can interact with D-type cyclin promoters**

The observation that CYCD3:2 and CYCD6:1 expression after SD treatment is perturbed in AIL1oe plants prompted us to investigate whether the AIL1 transcription factor can interact with CYCD promoters from hybrid aspen using electrophoretic mobility shift assays (EMSA). We expressed HA-tagged AIL1 protein in Arabidopsis protoplasts and used the extracts in gel shift assays using 3 different different fragments from a hybrid aspen CYCD3:2 promoter (results from two fragments are shown here). Our data show that extracts containing AIL1 protein specifically display a gel shift with the promoter fragment consisting of 200 bp of sequence situated upstream of the start codon of CYCD3:2 (Figure 7). Together with the CYCD3:2 and CYCD6:1 gene expression data, the gel-shift analysis strongly suggests these cyclin genes might be potential downstream targets of the AIL1 transcription factor in hybrid aspen.
Discussion

Short day mediated cessation of growth and budset prior to the onset of winter is a key developmental transition that is critical to the survival of perennial plants in boreal forest [1]. In this work, we identify \textit{AIL} genes belonging to the AP2 transcription factor family as downstream targets of the SD signal transduced via the CO/FT module and that downregulation of their expression is necessary for cessation of growth and bud set in hybrid aspen.

In poplar, there are 4 closely related \textit{AIL} genes and our data indicates that all four \textit{AIL} genes could have similar function at least in SD mediated growth cessation response as suggested by the similar phenotypes of \textit{AIL1} and \textit{AIL3} overexpressing plants as well as in plants in which these genes are targeted for downregulation. However we cannot exclude the possibility that there could be functional differences between the different \textit{AIL} genes with respect to other biological processes as we have not been able to specifically downregulate individual genes of the \textit{AIL} family and study the effect on growth and development so far. It is particularly important to note in this respect that even closely related genes can diverge both in expression profiles and at a functional level as suggested by the careful analysis of FT1 and FT2 in poplar species which indicate that FT1 could be primarily involved in reproductive growth whereas FT2 controls growth cessation [11].

Several observations suggest that the downregulation of \textit{AIL} gene expression following SD treatment is necessary for the activation of growth cessation responses. \textit{AIL1} (and most likely other genes of this family as well) is primarily expressed in dividing and meristematic cells in hybrid aspen (Figure 3) and the downregulation of their expression coincides temporally with the SD-mediated induction of growth cessation responses in hybrid aspen (Figure 1 and Figure 4), including the termination of elongation growth, bud set and the downregulation of core cell cycle genes such as the D-type cyclins (Figure 1). Furthermore, \textit{AIL1} downregulation after SD treatment is attenuated in \textit{FT} or \textit{PHYA} overexpressors (Figure 2) that fail to respond properly to SD treatment [4,9]. Importantly, growth cessation response is

![Figure 4. Bud formation in wild type, \textit{AIL1 (AIL1oe)}, and \textit{AIL3 (AIL3oe)} overexpressing transgenic hybrid aspen plants after 6 weeks in short days. Six weeks of SD-treatment (8 h light) leads to bud formation in wild type T89 (A), whereas no bud set is observed in \textit{AIL1oe} line 2B or 3B (B and C). Similarly in SD treatment consisting of 14 hours day length, bud formation is observed after 6 weeks SD-treatment in apices of wild type T89 (D) whereas no bud set is observed in \textit{AIL3oe} line 9 (E) or \textit{AIL3oe} line 10 (F). Position of the apex is indicated by white arrows. doi:10.1371/journal.pgen.1002361.g004](image-url)
perturbed when AIL1 or AIL3 expression is maintained at high levels even after SD treatment and earlier bud set is observed in transgenic hybrid aspen with reduced expression of AIL1 (Figure 4 and Figure S5). All of these results are consistent with the AIL genes being downstream targets of the SD signal in the control of the growth cessation response.

Three hypotheses can be proposed to explain the role of AIL genes in SD mediated control of growth cessation response and why growth cessation response is perturbed when the AIL1 oe and AIL3 oe expression is maintained at high level even after SD treatment as in AIL1 oe and AIL3 oe lines. Firstly, the AIL genes could act upstream of FT2, in which case the increased expression of AIL1 as in AIL1 oe could counteract the downregulation of FT2 by the SD signal. However, this hypothesis is incompatible with the observation that the downregulation of FT2 subsequent to SD treatment proceeds as normal in AIL1 oe lines (Figure 5). Alternatively, the AIL genes could act independently of FT2 and their increased expression in AIL1 oe and AIL3 oe could prevent the downregulation of the targets of FT2 following SD treatment. Alternatively, the AIL genes are the targets of SD signal downstream of CO/FT regulon leading to their downregulation after SD treatment. Our results support the latter hypothesis because SD treatment results in the downregulation of all AIL genes and this downregulation of AIL gene expression is severely attenuated in plants that overexpress FT1. Further evidence for the connection between the CO/FT regulon and AIL1 expression was obtained by analysis of FTRNAi lines that respond more rapidly than the wild type to SDs and in these lines, AIL1 expression is significantly reduced compared to wild type after 2 weeks of SD treatment (Figure 2B). Finally the downregulation of the AIL1 expression leads to earlier transition from active growth to bud set strongly suggesting that the AIL genes are the downstream targets of the SD signal. Thus our results suggest a mechanism in which AIL genes act downstream of the CO/FT regulon and that downregulation of AIL gene expression culminates in growth cessation and bud set after SD treatment.

FT has been shown to act as a transcriptional co-regulator in Arabidopsis [28]. In poplar species, two FT genes are present of which FT2 is rapidly downregulated after SD treatment; thus FT2 could either directly regulate AIL at the transcriptional level in hybrid aspen or alternatively downstream targets of FT2 could regulate AIL gene expression. Our data supports the latter suggestion because the kinetics of downregulation of FT2 and AIL gene expression subsequent to SD treatment is not consistent with direct regulation of AIL gene expression by FT2. While FT2 is typically downregulated within 3–7 days in the leaves after the commencement of SD treatment ([9,12], Figure 5), it takes 2–3 weeks until downregulation of the AIL genes becomes apparent in the apex (Figure 1). Moreover, induction of FT2 in the leaves has little effect on expression of most of AIL genes [11], which might again suggest an indirect regulation of AIL expression by FT2. Thus these results suggest that there may be one or more genes that are direct targets of FT2 and act upstream of the AIL genes regulating their expression in the apex. Determining the identity of these targets of FT2 and regulators of AIL expression in the apex is an important objective for future research in this area. The downstream targets of FT in daylength mediated regulation of flowering time such as SOC1 and the floral meristem identity genes FUL and AP2 [13] are well known. However these are unlikely to be the targets of the CO/FT regulon in the regulation of the AIL genes in the apex unless the tree homologs of these genes have acquired novel functions and have been recruited to regulate meristem activity by controlling AIL gene expression.

AIL1 is expressed in dividing cells (Figure 2), can potentially interact with the promoters of D-type cyclins (Figure 7) and maintaining high level of AIL expression prevents the downregulation of D-type cyclin expression after SD treatment (Figure 6) suggesting that AIL1 has a role in regulation of key cell cycle regulators. Indeed, data from Arabidopsis also shows that the putative AIL1 ortholog ANT can positively regulate cell division as
We therefore propose that the downregulation of AIL genes, overexpression leads to increased duration of cell division [17].

Figure 7. Interaction of HA-tagged AIL1 with CYCD 3:2 promoter fragment by electrophoretic mobility shift assay (EMSA). (A) AIL1 + 5 fmol biotin-labeled CYCD 3:2 promoter fragment F1, (B) AIL1 + biotin-labeled CYCD 3:2 promoter fragment F1 + 500 fmol unlabeled F1, (C) AIL1 + 5 fmol biotin-labeled CYCD 3:2 promoter fragment F2, (D) AIL1 + biotin-labeled CYCD 3:2 promoter fragment F2 + 500 fmol unlabeled F2, (E) biotin-labeled F1, (F) biotin-labeled F2 and (G) F1 and F2 in relation to the start codon of CYCD 3:2. Lanes A-D have additional non-specific competitor added to the reaction mix. Cell extracts from protoplast expressing HA-tagged AIL1 were used for gel-shift analysis. Additional lanes between D and F on the gel are omitted from the picture. doi:10.1371/journal.pgen.1002361.g007

its overexpression leads to increased duration of cell division [17]. We therefore propose that the downregulation of AIL gene expression after SD treatment leads to the downregulation of a subset of D-type cyclins such as CYCD3:2 and CYCD6:1. The downregulation of the expression of core cell cycle regulators such as the abovementioned cyclins would then culminate in cessation of growth and bud set. However it is unlikely that the D-type cyclins are the only targets of AIL1 because the expression of several other cell cycle genes is also downregulated after SD treatment [15]. Additionally transcriptional network analysis indicates that several other cell cycle genes might be regulated by the AIL1 transcription factor [29]. Moreover, preliminary investigations suggest that altering CYCD3:2 expression alone is not sufficient to activate the growth cessation response.

Substantial progress has recently been made in understanding how SD signal is perceived, and downregulation of FT2 expression after SD treatment has been identified as a key early event in the induction of growth cessation response [9,11]. However, the components targeted by SD signal downstream of the CO/FT regulon in the induction of growth cessation response have remained elusive, especially factors that would link the downregulation of FT2 expression to cessation of growth. Indeed analyses of hybrid poplar clones that differ in timing of bud set have suggested an important role for such factors in differential growth cessation [12]. Our finding that the AIL genes are the targets of the SD signal that is transduced via the CO/FT module in growth cessation response and bud set therefore represents an important step in elucidating the mechanism underlying this key developmental transition in perennial plants as this links the CO/FT module to the regulation of cell cycle through the AIL genes in SD mediated cessation of growth and bud set.

The CO/FT module is an important component of the molecular machinery that allows plants to respond to changes in day length, and its role in day length mediated control of flowering time is well established [30]. Therefore it was not surprising that the same CO/FT module is also involved in controlling the timing of SD-mediated growth cessation in perennial trees, as this is another key developmental transition that is regulated by the day length signal. However, given that flowering and growth cessation processes are distinct morphologically, it appears unlikely that the downstream targets of this module in the regulation of flowering would be the same as those involved in the growth cessation response. Our findings suggest that the AIL transcription factors, which have the potential to regulate the expression of cell cycle genes, were co-opted at some point in evolutionary history to serve as mediators of the day length signal. This co-option would have allowed the versatile CO/FT module to regulate a novel developmental transition. These results demonstrate that an evolutionary “mix and match” strategy involving combining different regulatory modules can allow a small number of regulatory modules to control a wide range of diverse biological processes. In conclusion, our data demonstrates the divergence of the regulatory pathway downstream of the conserved CO/FT module between day length controlled floral transition and growth cessation response and identifies AIL1 as a potential regulator of cell cycle related genes and a novel target of the short day signal downstream of the CO/FT module in regulation of growth cessation in perennial trees.

Materials and Methods

Plant material and growth conditions

Cuttings of hybrid aspen (Populus tremula x tremuloides) clone T89 (wild type) and the transgenic lines were grown in half-strength Murashige/Skoog medium (½ MS) under sterile conditions for approximately 4 weeks and then transferred to soil. After four weeks in greenhouse the plants were moved to growth chambers (18 hour light/6 hour night, 20°C). After one week the chamber settings were shifted to short day conditions (8 hour light/16 hour night, 20°C or 14 hour light/10 hour night, 20°C). Growth cessation was determined by measurement of elongation growth and/or bud set. Pictures of apices to assess bud formation were taken using Canon EOS digital camera. For tissue specific expression analysis of AIL genes, samples were taken from tissue culture grown plants 4 weeks after cuttings were transferred to new media.

Identification of AIL-genes and phylogenetic analysis

AIL-genes were identified by blasting the Arabidopsis AINTEGUMENTA gene (AT4G37750) against the Populus genome. Gene models [http://genome.jgi-psf.org/Popt1_1/Popt1_1.home.html] were manually chosen based on intron-exon structure (JGI protein ID for each model can be found in Figure S1). Sequences were aligned and a bootstrapped phylogenetic tree generated using ClustalX [31]. The phylogenetic tree was visualised using TreeView [http://darwin.zoology.gla.ac.uk/~page/treview/].
Generation of All1oe and All3oe lines

The full length cDNA for All1 transcription factor was cloned into the donor vector pDONR201 (Invitrogen.com) before transfer into the destination vector pK2GW7 [32]. The resulting vectors were introduced into agrobacterium GV3101pmp90RK [33] followed by the transformation of hybrid aspen clone T89 [34]. The same strategy was used to generate All3oe lines with the exception of entry clone construction that in this case was performed using the pENTR/D-TOPO cloning kit (Invitrogen.com).

GUS promoter analysis

The All1 promoter was amplified using the primers: FW: CACCCGGGGAATGATAGGCTGACAA and RP:CCCAAAATCTTGTGCTACTTCG and cloned into the pENTR/D-TOPO vector (Invitrogen.com). The fragment was transferred into the pK2GWFS7 binary vector [32]. The construct was transformed into hybrid aspen using Agrobacterium mediated transformation as described before [34]. Apices from transgenic lines expressing the reporter gene were collected from greenhouse grown trees approx. 5 weeks after potting. The apices were incubated approx. 3 h at 37°C in GUS-solution (1 mm X-gluc, 1 mm K3Fe(CN)6, 1 mm K4Fe(CN)6 50 mm sodium phosphate buffer (pH 7.0), and 0.1% (v/v) Triton X-100). The samples were then rinsed with water, dehydrated to 50% (v/v) ethanol, fixed for 10 min in FAA (5% (v/v) formaldehyde, 5% (v/v) acetic acid, and 50% (v/v) ethanol), and cleared in 100% (v/v) ethanol. Once cleared, the samples were embedded in LR-White/10% PEG 400 resin in polypropylene capsules (TAAB) The apices were then sectioned on a Microm HM550 microtome (Microm International GmbH, Germany) at approx 20 μm, floated on water, heat-fixed to glass slides, mounted in Entellan neu (Merck, Germany) Sections were visualized with Zeiss Axiospan light microscope and captured with a digital camera, AxioCam together with the Axiovision 4.5 software (Zeiss, Germany).

RNA isolation and real-time PCR analysis

Total RNA from poplar apices was extracted using the Aurum Total RNA kit (Bio-Rad). Care was taken to collect tissue samples for RNA isolation at the same time (usually between 13–16 PM) for each experiment. 100–500 ng of RNA was DNase treated with RNase free DNasel (Fermentas) and used for cDNA synthesis using iScript cDNA synthesis kit (BioRad) or qScript cDNA synthesis kit (Quanta BioSciences). Reference genes were validated using GeNorm Software [35]. The reference gene chosen was UBIQ in all experiments except for the analysis of the overexpression of All1 and All3 in the All1oe and All3oe lines, where 18S rRNA was used as the reference gene. Analysis of expression in FTRANAs used two reference genes, UBQ and TIP41 like. SYBR green (Bio-Rad or Quanta BioSciences) was used as non-specific probe in all reactions and relative expression values were calculated using the Δct-method [9]. A complete list of primers used in RT-PCR analysis can be found in Table S1.

Generation of transgenic hybrid aspen plants with reduced expression of All genes

To downregulate the expression of All genes, artificial microRNAs were designed using the online tools at http://wmd.weigelworld.org/cgi-bin/mirnatools. Briefly primers (Table S3) were used to generate artificial microRNAs directed against all the 4 All genes and cloned into the plant transformation vector pK2GW7 according to the cloning protocol at http://wmd3.weigelworld.org/. Two different miRNA constructs (named 255 and 256) were made and transformed into hybrid aspen clone T89 as described earlier. Following transformation several hybrid aspen lines with reduced expression of All1 were obtained and one line each for the two constructs 255 and 256 were selected for the analysis of bud set after SD treatment (lines 255-6 and 256-23).

Analysis of bud set in hybrid aspen plants with reduced expression of All genes

Bud set was scored using the method described by [23]. We used a score of 3 to indicate active growth (complete lack of bud set) and 0 to indicate a completely closed bud and score of 2 or 1 to indicate intermediate stages. For this analysis, bud set was scored every 7 days in a minimum of 5 or more plants for a period of 7 weeks.

Generation of HA-tagged All1 and overexpression in Arabidopsis protoplasts

All1 full length cDNA was amplified using the following primers: pTAI1(EcoR) FW: CATGGATCCATGAAATCTACGGGT-GATAA and pTAI1(SalI) RP:CATGGTGCACCTTCATGCTTTCATGTC. The resulting fragments were cloned into pRT104-3xHA [36]. Transfection into Arabidopsis protoplasts were performed as described [36,37] using 8 μg of purified plasmid. Cells were lysed in a lysis buffer containing 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 10% glycerol 1 mM DTT, 0.1% Igepal and 1X PIC (Protease Inhibitor Cocktail). After centrifugation the supernatant was collected and immediately frozen in liquid nitrogen. The expression of the HA-tagged All1 protein was confirmed with western blot and resulting cell extracts were used for subsequent analysis.

Generation of labeled CycD3:2 promoter fragments

CycD3:2 promoter sequences were identified using the JGI populus genome database (http://genome.jgi-psf.org/Poptr1_1/ Poptr1_1.home.html). Approx. 200 base pair fragments were amplified using primers specified in Table S2. The fragments were gel-purified using E.Z.N.A. Gel Purification Kit (Omega Bio-Tek) followed by phenol-chloroform extraction and ethanol precipitation prior to use in gel-shift assays. Five pmol of purified fragments were biotin labeled using the Biotin 3’ End DNA Labeling Kit (Pierce). Labeling and labeling efficiency determination was performed according to the manufacturers recommendation.

Electrophoretic mobility shift assay

The biotin-labelleed promoter fragments were mixed with protoplast cell extracts containing All1-1A or control extracts from non-transfected protoplasts. For the binding reaction the following conditions were used: 10 μl protoplast cell extract, 0.5 μl biotin-labelleed DNA (10 fmol/μl), 0.4 μl non-specific competitor (poly (dl-dc), 1 mg/ml), 0.5 μl BSA (20 mg/ml) and lysis buffer to a total of 20 μl. For specific competition, 500 fmol non-labelled fragment was added to the reaction. Binding was performed on ice for 10 min followed by 30 min in room temperature. The samples were run on a non-denaturing polyacrylamide gel (5%-0.5xTBE) and transferred to a Hybond N+ membrane (GE Healthcare, Sweden). Crosslinking and detection was performed using the LightShift Chemiluminescent EMSA kit (Pierce.com).

Supporting Information

Figure S1 Phylogenetic analysis of the ANT family of AP2 transcription factors in Arabidopsis and Populus. Arabidopsis ANTEGUMENTA (ANT) groups with four Populus genes, which were named ANTEGUMENTALIKE 1–4 (AIL1-AIL4). AIL1 and AIL3 analysed in detail are marked in bold letters.

(TIF)
Figure S2  Expression of AIL genes is downregulated after short day treatment during growth cessation. Expression of AINTEGUI-MENTALIKE genes (AIL1-AIL4) was analysed in the apex of wild type hybrid aspen after 6 weeks of SD treatment (8th day). Y-axis indicates the expression levels after 6 weeks of short day treatment normalized to the level prior to the start of short day treatment (SD 0). In each case average from three independent experiments is shown and error bars represent standard deviation. (TIF)

Figure S3  Expression of AIL1 and AIL3 in transgenic hybrid aspen. Expression in the apex of AIL1 in the wild type hybrid aspen (T89) and two transgenic lines (AIL1oe line 2B and 3B) expressing AIL1 cDNA under the control of 35S promoter. Y-axis shows the transcript levels of AIL1 relative to that of the reference gene (18S rRNA). Data from 2 independent experiments is shown. (B) Expression of AIL3 in wild type hybrid aspen (T89) and two transgenic lines (AIL3oe line 9 and line 10) expressing the AIL3 cDNA under the control of 35S promoter. Y-axis shows the ratio of AIL3 expression relative to that of the reference gene (18S rRNA). The expression values are average of 3 biological replicates and error bars represent the standard deviation for the three biological replicates. (TIF)

Figure S4  Expression of AIL1 in amiRNA expressing lines. A) Expression of AIL1 in apices of tissue culture grown wild type (T89) and amiRNA expressing hybrid aspen (253-6). A). Expression of AIL1 in apices of tissue culture grown wild type (T89) and amiRNA expressing hybrid aspen (256-23). Expression values are median of three technical replicates. (TIF)

Figure S5  Analysis of bud set in amiRNA expressing lines. Analysis of bud set phenotype in wild type hybrid aspen (T89), and amiRNA lines 253-6 and 256-23. The transition from active growth to completion of bud set was divided into 4 stages where 0 is a completely developed bud and 3 correspond to an actively growing apex. Number of plants used for each genotype are: T89 n = 5, 253-6 n = 7 and 256-23 n = 6. X-axis denotes weeks in short days and Y-axis denotes number of plants at a particular stage of bud set transition. Colors specifying the stage of bud set are denoted. (Day length = 14h) (TIF)

Table S1  Real-time PCR primer sequences. *Please note that cross-reactivity could occur between primer pairs used for the detection of AIL1 and AIL2 gene expression. (DOCX)

Table S2  Primers for CYCD3:2 promoter fragments. (DOCX)

Table S3  Primers for amiRNA construction. (DOCX)

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Author Contributions

Conceived and designed the experiments: AK LB RPB. Performed the experiments: AK LB. Analyzed the data: AK LB RPB. Contributed reagents/materials/analysis tools: AK LB RPB. Wrote the paper: AK RPB.

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