Differential regulation of *Knotted1*-like genes during establishment of the shoot apical meristem in Norway spruce (*Picea abies*)

Emma Larsson · Folke Sitbon · Sara von Arnold

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Abstract Establishment of the shoot apical meristem (SAM) in *Arabidopsis* embryos requires the KNOXI transcription factor SHOOT MERISTEMLESS. In Norway spruce (*Picea abies*), four KNOXI family members (HBK1, HBK2, HBK3 and HBK4) have been identified, but a corresponding role in SAM development has not been demonstrated. As a first step to differentiate between the functions of the four Norway spruce *HBK* genes, we have here analyzed their expression profiles during the process of somatic embryo development. This was made both under normal embryo development and under conditions of reduced SAM formation by treatment with the polar auxin transport inhibitor NPA. Concomitantly with the formation of an embryonic SAM, the *HBK2* and *HBK4* genes displayed a significant up-regulation that was delayed by NPA treatment. In contrast, *HBK1* and *HBK3* were up-regulated prior to SAM formation, and their temporal expression was not affected by NPA. Ectopic expression of the four *HBK* genes in transgenic *Arabidopsis* plants further supported similar functions of *HBK2* and *HBK4*, distinct from those of *HBK1* and *HBK3*. Together, the results suggest that *HBK2* and *HBK4* exert similar functions related to the SAM differentiation and somatic embryo development in Norway spruce, while *HBK1* and *HBK3* have more general functions during embryo development.

Keywords Embryo patterning · *KNOTTED1*-like homeobox (*KNOX*) · Norway spruce · 1-N-naphthylphthalamic acid (NPA) · Polar auxin transport (PAT) · Shoot apical meristem (SAM)

Introduction

The shoot apical meristem (SAM) is established early during plant embryogenesis. It includes a group of self-renewing cells, which eventually give rise to all above-ground parts of a plant. The regulation of establishment and maintenance of the SAM has been thoroughly studied in the angiosperm *Arabidopsis thaliana*. However, angiosperms and gymnosperms separated approximately 300 million years ago (Smith et al. 2010), and our knowledge about the corresponding processes in gymnosperms is limited.

The establishment of the embryonal SAM in *Arabidopsis* is dependent on the expression of the homeodomain containing transcription factor *SHOOT MERISTEMLESS (STM)*; Barton and Poethig 1993; Long et al. 1996). *STM* is one of four class I *KNOTTED1*-like homeobox (*KNOXI*) genes in *Arabidopsis*, the other members being *KNOTTED-like from Arabidopsis thaliana 1/BREVIPEDICELLUS (KNAT1/BP), KNAT2 and KNAT6*. *KNOXI* genes are expressed in the SAM and down-regulated before leaf initiation. In addition, they are expressed in the margins of compound leaves, where they presumably prevent cell differentiation (Hay and Tsiantis 2010). *STM* is the best studied gene within the *KNOXI* gene family. Since the other members act redundantly with *STM* in the...
development of both vegetative and reproductive meristematic tissues, it has been difficult to assign specific functions to each gene (Hay and Tsiantis 2010).

STM is the first KNOXI gene to be expressed during embryogenesis. It marks the entire SAM, and its expression is essential for establishment of the embryonal SAM (Barton and Poethig 1993; Long et al. 1996). KNAT6 is also expressed in the embryonal SAM, but not until bilateral symmetry has been established (Belle-Boix et al. 2006). It marks the SAM boundaries and acts redundantly with STM to maintain meristem identity and organ separation (Belle-Boix et al. 2006). BP is expressed in the shoot apex at the seedling stage (Dockx et al. 1995). There is no disruption of the SAM in bp mutants (Venglat et al. 2002), although BP can restore the SAM function when ectopically expressed in stm mutants (Scofield et al. 2008). Mutants of knat2 have no obvious phenotype, probably because of redundancy with KNAT6 (Byrne et al. 2002). However, ectopically expressed KNAT2 induces a conversion of ovules to carpels, suggesting a function related to carpel development (Pautot et al. 2001), and also STM has been shown to be important for carpel development (Scofield et al. 2007). The delineation of embryonal shoot and root meristems in Arabidopsis is correlated with auxin maxima, established by polar auxin transport (PAT; Möller and Weijers 2009). We have previously shown that PAT also is of major importance for the correct patterning of the embryonal shoot and root in Norway spruce (Larsson et al. 2008). To further characterize the molecular regulation of SAM establishment in conifers, it is important to know more about the expression of KNOXI genes during SAM formation.

Four KNOXI genes, HBK1, HBK2, HBK3 and PaKN4 (from now on denoted HBK4 for simplicity), have been identified in Norway spruce (Guillet-Claude et al. 2004; Hjortswang et al. 2002; Sundás-Larsson et al. 1998). HBK1, HBK2 and HBK3 are expressed in embryogenic cultures, stems, roots, and female and male cone buds, but not in needles (Hjortswang et al. 2002). In addition, HBK1 shows a tissue-specific expression in vegetative meristems (Sundás-Larsson et al. 1998). Overexpression of HBK3 leads to enlarged SAMs in somatic embryos and an accelerated differentiation of early embryos from proembryonic masses (PEMs), while down-regulation of HBK3 precludes embryo differentiation (Belmonte et al. 2007). Furthermore, it has been shown that HBK2 is expressed only in embryogenic cell lines that are competent to form fully mature cotyledonary embryos, while HBK1 and HBK3 are expressed also in developmentally arrested lines (Hjortswang et al. 2002). However, there are so far no reports on the expression pattern of HBK4.

Phylogenetic analyses have shown that the four KNOXI genes in spruce form a monophyletic group and likely have diversified after the split between angiosperms and gymnosperms (Guillet-Claude et al. 2004). The authors suggested that gene losses have occurred in conifers after new paralogs were gained and further proposed that HBK1 and HBK3 would exert similar and redundant functions, while HBK2 and HBK4 should be more diverse.

Here, we have analyzed the expression of all four HBK genes during Norway spruce embryo development and correlated their expression to PAT inhibition and the establishment of a functional SAM. The expression profiles, together with expression studies in transgenic Arabidopsis plants, indicate that HBK1 and HBK3 act in similar pathways, while HBK2 and HBK4 have several features in common, but are distinct from HBK1 and HBK3. Furthermore, the temporal expression of HBK2 and HBK4 during early embryo development suggests functions connected to SAM establishment and somatic embryo formation.

Materials and methods

Plant materials

The embryogenic cell line 28:05 of Norway spruce (Picea abies L. Karst) was used throughout this study. It was stored in liquid nitrogen and thawed approximately 6 months before the start of the experiments. After thawing, the cell cultures were treated as described previously (von Arnold and Clapham 2008). Briefly, proembryogenic masses (PEMs) were maintained in liquid proliferation medium containing the plant growth regulators (PGRs), 2,4-dichlorophenoxyacetic acid (2,4-D) and N\textsuperscript{6}-benzyladenine (BA) at 10.0 and 4.4 μM, respectively. The cultures were transferred to fresh medium weekly. To stimulate differentiation of early somatic embryos from PEMs, the cultures were transferred to pre-maturation medium lacking PGRs for 1 week. For development of late somatic embryos and maturation, the cultures were plated on solidified maturation medium containing 30 μM of abscisic acid (ABA).

To study the effect of PAT on the expression of the HBK genes, embryogenic cultures were treated with 20 μM 1-N-naphthylphthalamic acid (NPA; Sigma-Aldrich, Schnellendorf, Germany) during both the pre-maturation and maturation phases, as described by Larsson et al. (2008). Samples for gene expression studies were collected from eight consecutive stages from both control and NPA-treated embryos (Fig. 1). The developmental stages of NPA-treated embryos were largely defined according to the sizes of phenotypically normal control embryos. Whole mount of tissue was sampled from proliferating PEMs (stage 1) and early embryos (stage 2). From stage 3 and onwards, the
Embryos were sampled individually and sorted according to their developmental stage to increase the specificity in subsequent expression analyses. Samples were stored at \(-80^\circ\text{C}\) until use.

Cell line 88:1, in which proliferation of PEMs continue on pre-maturation medium (Hjortswang et al. 2002), was used to analyze the expression of the \textit{HBK} genes in an arrested embryogenic cell line. Samples were taken after 1 week of proliferation in the presence of PGRs, after 1 week in pre-maturation medium, and after 1 week on maturation medium. Samples from cell line 28:05 were used as control.
RNA isolation and cDNA synthesis

For quantitative real-time PCR (qRT-PCR), total RNA was extracted according to a modified protocol from Azevedo et al. (2003). Briefly, samples snap frozen in liquid nitrogen were disrupted in TissueLyZer II (Retsch, Haan, Germany). Extraction buffer was added to each sample, which was again frozen in liquid nitrogen, thawed at 42°C, disrupted and finally incubated at 42°C for 90 min, followed by subsequent RNA isolation. For real-time PCR (RT-PCR), total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer.

An aliquot (10 µg) of each sample was treated with DNase using the DNA-free protocol by Ambion (Ambion inc, Austin, TX, USA), and 1 µg of the DNA-free RNA was used to synthesize cDNA using the qScript™ cDNA Synthesis Kit (Quanta BioSciences, Inc. Gaithersburg, MD, USA) according to the protocol provided by the manufacturer.

Quantitative real-time PCR

qRT-PCR was performed using the DyNAmo™ Flash SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) in a BIO-RAD iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Primers used to quantify expression levels are presented in Table 1a. Expression data were normalized against the expression of the reference genes CELL DIVISION CONTROL2 (CDC2), ELONGATION FACTOR-1 ALFA (EF1-α) and PHOSPHOGLUCOMUTASE, previously selected based on their stability (Vestman et al. 2011) as determined using the geNorm software (Vandesompele et al. 2002). PCR cycling conditions were as advised by the manufacturer in the DyNAmo™ Flash SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) with annealing and extension at 60°C for 30 s. The reactions were run for 40 cycles followed by the generation of a melting curve to ensure product uniformity. All samples were added in triplicate to each plate, and each gene was measured in three independent biological replicates. The analyses were performed using the iQ5 software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analyses of the results from the qRT-PCR were performed using the SAS (2008) software general linear model (GLM) procedure. The effects of treatment and sample on each gene expression level were analyzed using GLM with treatment and sample as factors. The different genes were analyzed separately.

Vector construction for overexpression in Arabidopsis

Full-length cDNAs of HBK2, HBK3 and HBK4 were amplified from early somatic embryos of Norway spruce, and full-length cDNA of HBK1 was amplified from a pBluescript clone kindly provided by Dr. Annika Sundás-Larsson (Uppsala University, Uppsala, Sweden) using primers designed from published sequences (Accession numbers AF063248, AF483277, AF483278, AF483279, AY680389 and AY680400). Primers are presented in Table 1b. Amplified products were subcloned into the pJET1.2/blunt cloning vector using the CloneJET™ PCR Cloning Kit (Fermentas Helsingborg, Sweden). All cDNA clones were subsequently re-amplified using primers with attB-adapters and inserted into the 35S promoter containing Gateway vector pGWB2 (Nakagawa et al. 2007) using the Gateway® technology according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Primers are presented in Online Resource Suppl. Tab. 1c. The resulting vectors were introduced by freeze–thawing into Agrobacterium tumefaciens strain C58:C1. Arabidopsis plants, ecotype Columbia (Col), were transformed using the floral-dip method and transgenic seedlings were selected on kanamycin-containing plates. Resistant seedlings were planted in soil and phenotypically analyzed.

Results and discussion

We have previously shown that formation of a functional SAM during embryo development in Norway spruce is dependent on PAT (Larsson et al. 2008). Briefly, treatment with the well-established PAT inhibitor NPA delayed the development of somatic embryos, and mature somatic embryos showed abnormal morphology with fused or aborted cotyledons and no histologically visible SAM. These embryos could neither germinate nor develop an epicotyl, indicating lack of a functional root meristem and SAM.

A model system for studying genes correlated with SAM formation in conifer embryos

To analyze the expression of the four HBK genes during embryo development in Norway spruce, and whether the expression was different in embryos lacking a functional SAM, both control somatic embryos and somatic embryos treated with NPA were separated into eight consecutive developmental stages (Fig. 1). This classification increases the resolution during late embryogeny and maturation compared to our previous work (Larsson et al. 2008).

Proliferating PEMs were considered as stage 1 (Fig. 1a). Early embryos that had started to differentiate from the larger PEM structures represented stage 2 (Fig. 1b). At stage 3 (Fig. 1c), the embryos had developed further and consisted of an embryonal mass with a smooth surface and a long suspensor made up of highly vacuolated cells. The
embryonal mass and the suspensor were separated by tube cells, which are specific for gymnosperms. Stage 4 (Fig. 1d) was represented by late embryos with a dense embryonal mass, which was continuously growing while most of the suspensor cells had been degraded by programmed cell death, a feature that is essential for the formation of viable somatic embryos (Bozhkov et al. 2005; Smertenko et al. 2003). Stage 5 (Fig. 1h) was characterized by maturing embryos, which had clearly visible cotyledon primordia surrounding a convex SAM primordium. At this stage, all suspensor cells had been degraded. During stages 6–8 (Fig. 1i–k), the embryos continued to increase in size, the cotyledons, which could be seen as small protuberances at stage 6 (Fig. 1i), expanded further at stage 7 (Fig. 1j) and finally fully spread out at stage 8 (Fig. 1k).

The development of NPA-treated embryos was divided into eight consecutive stages mainly defined according to the size of control embryos. There were no distinct differences between control embryos and NPA-treated embryos until the cotyledon primordia were clearly visible (cf. Fig. 1b–g). However, compared to the cylindrical control embryos (Fig. 1b, c), NPA-treated embryos often carried more suspensor cells, resulting in cone-shaped early embryos (Fig. 1e, f). In general, the NPA-treated embryos developed more slowly and the suspensor did not degrade at the same rate as for control embryos. As the embryos matured, it became evident that the cotyledons were fused, giving the apex a doughnut appearance when observed from above (Fig. 1n). At stage 8, when the cotyledons of control embryos burst, the doughnut thinned out at the edges and there was a deep cavity at the center (Fig. 1o). We have previously shown by histological analysis that the SAM is missing in such embryos (Larsson et al. 2008).

Expression of HBK2 and HBK4 coincides with the SAM formation

The relative expression level of HBK1, HBK2, HBK3 and HBK4 was analyzed by quantitative real-time PCR in both control embryos and in embryos that had been treated with NPA throughout their development. Since we have previously shown that NPA treatment of PEMs does not affect SAM formation (Larsson et al. 2008), stage 1 cultures remained untreated. Thus, the expression of each gene at each stage and treatment could be related to the expression at stage 1.

HBK1 was expressed in proliferating PEMs and the expression was significantly up-regulated from stage 3 and then down-regulated in mature embryos (Fig. 2a). The relative expression of HBK3 (Fig. 2c) was similar to that of HBK1, although HBK3 became significantly up-regulated first at stage 4 and then remained at a high expression level. Treatment with NPA did not significantly alter the expression of either gene. These results show that the accumulation of both HBK1 and HBK3 mRNA precedes the differentiation of the SAM. However, since NPA-treated embryos lack a visible SAM (Larsson et al. 2008), the absence of an NPA-response on HBK1 and HBK3 expression suggests that the genes are not specifically related to SAM establishment, and thus have a more general role in embryo development.

In contrast, HBK2 was expressed in proliferating PEMs, but as early embryos started to differentiate at stage 2, the expression decreased and reached its lowest level at stage 3 and 4 (Fig. 2b). At stage 5, when the cotyledons started to develop and the SAM primordium was bulging out from the center of the cotyledon crown, the HBK2 expression increased drastically and the mRNA level was 20 times higher compared to that at stage 3 and 4. From stage 6, the relative expression of HBK2 was two to three times that compared to stage 1, and almost 40 times higher as compared to that at stage 3 and 4. Compared to control embryos, treatment with NPA led to a significantly higher HBK2 expression at stage 2, but a significantly lower expression at stage 5 and 6. Taken together, the expression of HBK2 started to increase between stages 4 and 5 in control embryos, coinciding with the appearance of the SAM primordium, while it was not until stage 6 when the fused cotyledon ring became visible that HBK2 expression increased in NPA-treated embryos. Interestingly, NPA treatment did not alter the expression of HBK2 when all stages were analyzed as a group, indicating that NPA treatment delayed the up-regulation of HBK2. The delay in HBK2 expression could either be a direct effect of the blocked PAT, or it could mirror an indirect effect, where up-stream regulators of HBK2 are directly affected by the blocked PAT. It has been shown in tomato (Solanum lycopersicum) that there is a narrow window during leaf development when the leaf is responsive to KNOXI activity (Shani et al. 2009). Assuming a similar spatial and temporal window during embryo development in Norway spruce, our results suggest that HBK2 must be up-regulated at stage 5 for an appropriate differentiation of SAM.

The relative expression level of HBK4 (Fig. 2d) was low in proliferating PEMs, but increased as embryo morphogenesis began. At stage 5, when the SAM was visible at the center of the small cotyledon protuberances, HBK4 was significantly up-regulated compared to stage 1, and the expression level then remained high throughout the maturation process. There was a statistically significant difference between the HBK4 mRNA accumulation in NPA-treated embryos and in control embryos. The expression of HBK4 was initially down-regulated between stages 1 and 2 in NPA-treated embryos. Furthermore, the up-regulation of HBK4 observed in control embryos at stages 5 and 6, when SAM and cotyledon differentiation began, was partly
inhibited in NPA-treated embryos. It was not until the embryos were mature that the relative expression level became comparable in NPA-treated and control embryos. These results suggest that also HBK4 should be up-regulated at a certain time point for the SAM to be established.

The qRT-PCR results show that the highly similar genes, HBK1 and HBK3, are expressed in a similar way during embryo development, and that their temporal expression levels are not affected in embryos lacking a functional SAM. This suggests that HBK1 and HBK3 have more general roles during embryo development, rather than specific functions during embryonal SAM establishment. In contrast, both HBK2 and HBK4 become up-regulated during the establishment of the embryonal SAM. This temporal up-regulation is comparable to that of STM in Arabidopsis (Long et al. 1996), indicating that HBK2 and HBK4 have roles in SAM establishment. In addition, the increase in the relative expression of HBK2 and HBK4 is delayed in NPA-treated embryos lacking a SAM, which support that appropriate timing of the expression of HBK2 and HBK4 is important for SAM establishment.

HBK2 and HBK4 are specifically expressed in cell lines competent to form fully mature cotyledonary embryos.

It has been shown that HBK1 and HBK3 are expressed both in embryogenic cell lines that form fully mature cotyledonary embryos, and in blocked cell lines that never pass the PEM-to-embryo transition stage (Hjortswang et al. 2002). This is in contrast to HBK2, which is only expressed in embryogenic cell lines that form fully mature embryos. To further investigate the similarities and differences between the four HBK genes, the temporal expression of HBK4 was compared to that of HBK1, HBK2 and HBK3 in a control line (28:05) and in a blocked cell line (88:1). HBK1 and HBK3 were expressed in both cell lines, while HBK2 and HBK4 were expressed only in cell line 28:05 (Fig. 3). These results support a similar regulation of HBK1 and HBK3, and of
HBK1 expressing only showed mild serration. In addition, the plants leaves, while most of the HBK2 and HBK4 expression of HBK2 and HBK4 genes have some features in common with angiosperm KNOXI genes when constitutively expressed in Arabidopsis. However, since the spruce genes did not induce SAM differentiation, we assume that HBK genes are too diverse compared to angiosperm KNOXI genes to be able to stimulate such induction. In addition, the delayed abscission of the HBK2 and HBK4 expressing flowers is a characteristic, not previously presented for KNOXI overexpressors. This suggests that HBK2 and HBK4 can function in pathways other than those previously described for KNOXI proteins. Taken together, our data show that HBK1 and HBK3 have similar expression profiles during somatic embryo differentiation and development in Norway spruce. Although HBK1 and HBK3 can function in pathways similar to angiosperm KNOXI genes when expressed in Arabidopsis, their temporal expression patterns during somatic embryogenesis in Norway spruce suggest that HBK1 and HBK3 have a more general function during embryo development than to specify the SAM. In contrast, HBK2 and HBK4 are specifically expressed in embryogenic cell lines competent to develop into fully mature cotyledonary embryos, and their temporal expression profiles are correlated with the formation of the embryonal SAM. This suggests that HBK2 and HBK4 are essential for somatic embryogenesis and the formation of a functional SAM in Norway spruce.

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Table 1 Phenotypic characteristics of transgenic Arabidopsis plants expressing the Norway spruce HBK1 to HBK4 cDNA from the CaMV 35S promoter

| Gene   | Frequency of plants with | Number of plants |
|--------|--------------------------|------------------|
|        | Weakly serrated rosette |                  |
|        | Severely serrated rosette |                  |
|        | Serrated cauline |                  |
|        | Degenerated floral organs |                  |
| Control | 4.2 | 0.0 | 5.6 | 0.0 | 71 |
| HBK1   | 22.2 | 59.3 | 55.6 | 44.4 | 54 |
| HBK2   | 54.1 | 32.4 | 56.5 | 0.0 | 37 |
| HBK3   | 31.3 | 59.4 | 70.7 | 60.4 | 64 |
| HBK4   | 48.1 | 24.1 | 67.3 | 0.0 | 54 |

*Plants expressing a 20-bp tag from the CaMV 35S promoter.*
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