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Molecular Regulation of Embryo Development in Norway Spruce

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The present work describes the isolation of putative transcription factors expressed during somatic embryo development in the gymnosperm Norway spruce (*Picea abies*).
Two Norway spruce homeobox (*PaHB*) genes belonging to the homeodomain-glabra2 (HD-GL2) family, were isolated. Both genes display a highly conserved intron pattern characteristic of their phylogenetically related angiosperm HD-GL2 genes. The two predicted gymnosperm proteins are also highly similar to the angiosperm HD-GL2 proteins. In proembryogenic masses, both genes are expressed in all embryogenic cells. In early maturing somatic embryos, *PaHB1* becomes restricted to the protoderm layer and *PaHB2* is not expressed. At a later stage, *PaHB1* expression remains on the protoderm whereas *PaHB2* transcripts are mainly detected in the underlying cortical layers. A stepwise peripheral to central radial patterning takes place during embryo development in Norway spruce. Ectopic expression of *PaHB1* led to an early block in somatic embryo development suggesting that the inner layers of the embryos must be devoid of *PaHB1* to proceed through embryogenesis.
The conservation of protoderm-specific expression in HD-GL2 and lipid transfer protein (*LTP*) genes from divergent plants suggests putative common cis-regulatory elements in these genes. Sequence comparisons between the isolated *PaHB1*, *Pa18* (encoding a predicted LTP) promoters and the angiosperm counterparts allowed us to identify candidate motifs for protoderm expression. The *AtML1* promoter and *PaHB1* promoter, both fused to the reporter gene *GUS*, were transferred into Norway spruce and Arabidopsis respectively, enaling reporter gene analysis.
The Norway spruce *viviparous1* (*Pavp1*) single-copy gene shows similar gene structure and protein domain organization as the angiosperm counterparts. The expression profile of *Pavp1* further suggests a similar role of *vp1* genes in maturation and desiccation processes in seed plants.

Keywords: homeobox, pattern formation, VP1, Norway spruce, embryogenesis, gymnosperm.

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« …So you think you have the solution
But it's just another illusion…»

Bob Marley and The Wailers
(Rastaprofessor and his jahmen)

Abstract

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Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Ingouff M, Farbos I, Lagercrantz U and von Arnold S. 2001. *PaHB1* is an evolutionary conserved HD-GL2 homeobox gene expressed in the protoderm during Norway spruce embryo development. *Genesis* **30**: 220-230.

II. Ingouff M, Farbos I, Wiweger M and von Arnold S. The tissue-specific expression of two HD-GL2 family homeobox genes reveals a stepwise peripheral to central radial patterning during embryo development in Norway spruce. (Manuscript).

III. Ingouff M, Farbos I, Wiweger M and von Arnold S. Study on the conservation of cis-regulatory regions directing tissue-specific expression of the HD-GL2 homeobox genes in seed plants. (Manuscript).

IV. Footitt S, Ingouff M, Clapham D and von Arnold S. The Norway spruce (*Picea abies* [L.] Karst) *viviparous 1* gene (*Pavp1*); its expression during somatic embryogenesis and in nonembryogenic tissues. (Manuscript).

Contents

Introduction

Animals and higher plants have a fundamental difference in the early developmental processes. In mammals, a major feature of embryo development is the migration of cells to generate three-dimensional shape. In higher plants, on the other hand, cells are entirely nonmotile (except during fertilization) implying that plants have evolved different molecular networks to form an embryo.
Despite these differences, evolution of shape in animals and plants both result from evolution of developmental processes. Since development is largely under genetic control, changes in developmental control genes may be a major aspect of evolutionary changes in morphology (Gilbert et al., 1996; Doebley and Lukens, 1998).
In recent years, gene families; that encode transcription factors controlling various developmental processes; have been found in animals and plants. In plants, the homeobox and MADS-box families are two important families of developmental regulators (Chan et al., 1998; Theißen et al., 2000).
The homeobox, shorthand for "homeotic box", is a sequence of 183 nucleotides encoding 61 amino acids (Scott et al., 1989). The homeobox encodes the homeodomain responsible for binding DNA and thereby influencing DNA transcription. The homedomain folds into three α-helices. The first two helices are separated by a loop and the last two are separated by a turn.
Animal homeobox genes play a variety of developmental roles, typically involving the regulation of cell pattern and the activation of other genes instrumental in the formation of the basic body plant (for an example see, Mastick et al. 1995). In animals, most of the homeobox genes are arranged in clusters. The increasing complexity in the homeobox cluster number and architecture has been hypothesized to be related to the increasing complexity in body plans among phyla of animals (Ruddle et al, 1994).
Homeobox genes have been isolated in unicellular and multicellular plant organisms. In contrast to animals, plant homeobox genes are not gathered in clusters. Therefore, the relationships between the diversification of this gene family and the evolution of the plant body plan are unknown.

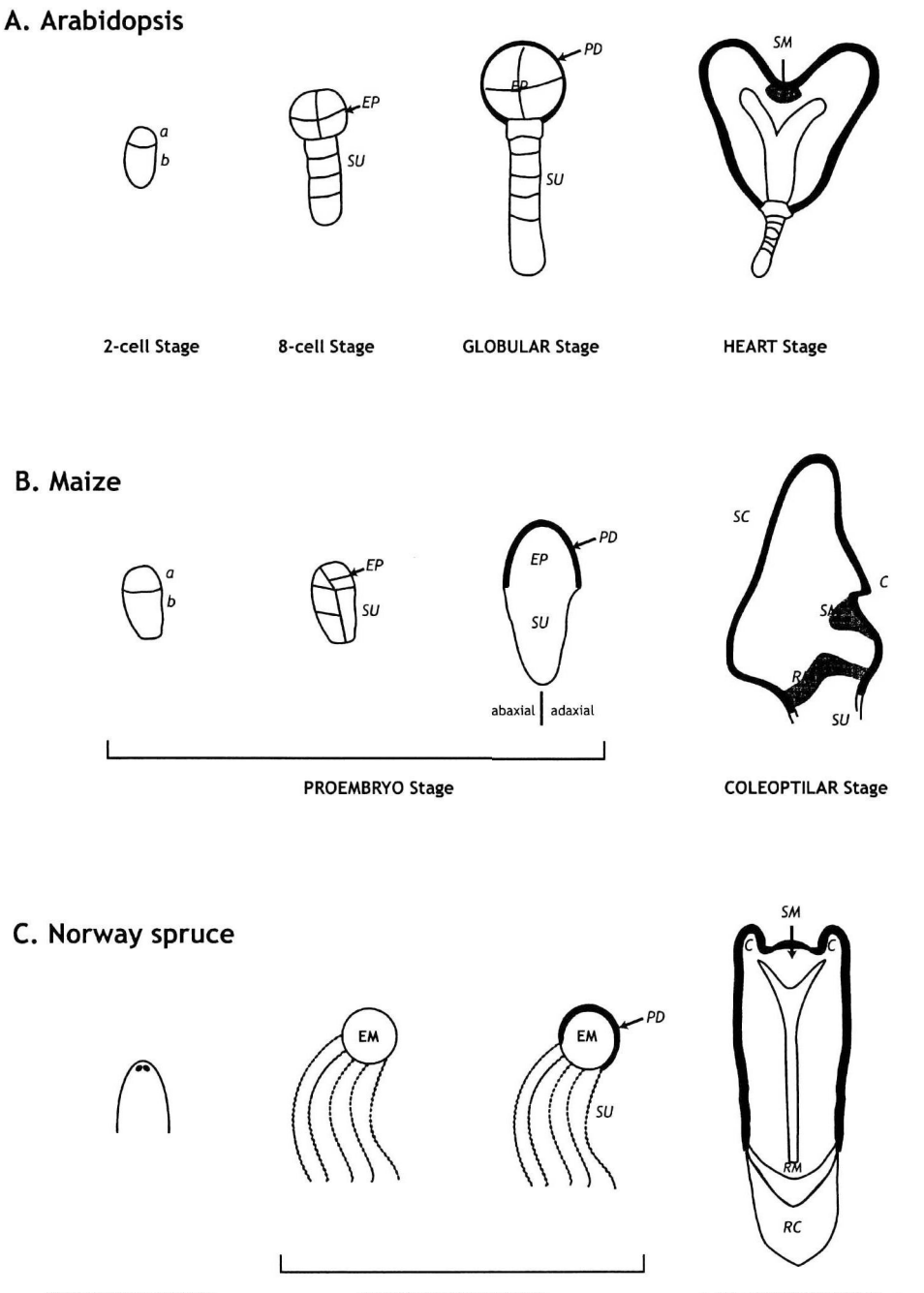
Embryo development in seed plants

During embryogenesis, the primary body plan, established early during embryo development, can be conceptually divided into the formation of shoot and root meristems and the definition of the three primary tissues: the outer protoderm, that later differentiates into the epidermis, the inner mass of ground tissue, which produces the cortical and endodermal tissues, and the centrally located procambium which generates the vascular tissue (Jürgens, 1994).

A fundamental question in embryogenesis is the process of pattern formation (Jürgens et al., 1994), which establishes the spatial relationships of the different organs and tissues of the embryo.

The embryo development process in angiosperms and gymnosperms has been thoroughly described by following the pattern of cell division and the use of cytological methods (reviewed in, Romberger et al., 1993, Steeves and Sussex, 1989; Mordhorst et al., 1997). To provide a reference for comparing embryo development in angiosperms and gymnosperms, I shall first describe major events of the development of the dicot *Arabidopsis* and the monocot maize embryo and then the Norway spruce gymnosperm embryo development will be treated separately. Selected stages of embryo development in angiosperms (*Arabidopsis* and maize) and gymnosperms (Norway spruce) are schematically represented in figure 1.

Figure 1: Schematic overview of embryo development in angiosperms (*Arabidopsis* and maize) and gymnosperms (Norway spruce). Illustrations adapted from Laux and Jürgens (1997); Randolph, (1936) and Singh, (1978). a, apical cell; b, basal cell; C, cotyledon; EM, embryonal mass; EP, embryo proper; PD, protoderm; RC, root cap; RM, root meristem; SC, scutellum; SM, shoot meristem; SU, suspensor.



A schematic representation of embryo development in *Arabidopsis* (Laux and Jürgens, 1997) and maize (Randolph, 1936; van Lammeren, 1986) are presented in figure 1A and figure 1B respectively.
In *Arabidopsis* and maize embryogenesis, the zygote undergoes an asymmetric division, forming a smaller apical and a larger basal cell. In *Arabidopsis*, the apical cell undergoes three rounds of divisions to form the eight-celled embryo proper. In maize the pattern of cell division during early embryogenesis is less strict, leading to a cone-shaped structure in which the suspensor and the embryo proper are not clearly delineated (proembryo stage). Therefore it is difficult to trace the origin of the organs back to a definite cell or a group of cells.
Protoderm formation is the first recognizable stage of histogenesis in plant embryo development (West and Harada, 1993). In *Arabidopsis*, the protoderm is formed by the early globular stage. During the proembryo stage of maize embryogenesis, the protoderm is delineated on the apical end of the embryo as a surface layer of cells. Once established, this surface layer is characterized by a predominant anticlinal (perpendicular to the surface) cell division pattern in both species. Consequently newly formed daughter cells in the protoderm remain in this layer, setting up an independent cell lineage (Steeves and Sussex, 1989). In addition, the protoderm geometrically sets apart the inner cells from which the peripheral ground tissue and procambial core are derived.
At the globular stage embryo in *Arabidopsis*, the cells of the inner mass, underlying the protodermal cells, are further dividing to form the precursors of vascular cells (procambium) in the center and the surrounding ground tissue. By the heart stage, the radial pattern of tissue layers is completed and the root primordium is generated. At this stage the two cotyledons start to grow and the embryo passes from a radial to a bilateral symmetry. The shoot meristem precursor cells reside between the cotyledon primordia. However, a morphological distinction of the shoot meristem is only visible by the torpedo stage. The shoot and root meristems are aligned to the embryo proper-suspensor axis
At the end of the proembryo stage the maize embryo looks like a club-shaped mass showing little differentiation. Histologically a group of meristematic cells becomes visible at the adaxial side of the transition stage embryo (Randolph, 1936; van Lammeren, 1986). Slightly later this mass of cells differentiates into two distinct cell masses: the upper part located in the adaxial outgrowth generates the shoot apical meristem (SAM) and the lower part, just above the suspensor, forms the root apical meristem. Consequently, the polar meristems of the maize embryo are positioned obliquely in comparison to the embryo proper-suspensor axis. The major portion of the embryo, which does not contribute to the main axis of the embryo proper, enlarges and becomes the scutellum (Randolph, 1936). At the coleoptilar stage, a notch on top of the SAM is the first sign of the coleoptile, which forms a ring of tissues around the meristem.

Embryo development in gymnosperms

Singh (1978) divided the gymnosperm embryo development process into three phases: proembryogeny (stages before elongation of the suspensor), early embryogeny (stages after elongation of the suspensor and before the establishment of the root meristem) and late embryogeny (establishment of the root and shoot meristems and further development of the embryo until maturity). A schematic representation of selected stages of embryo development in Norway spruce is shown in figure 1C. In gymnosperms, proembryos are generally characterized by a free-nuclear stage (Singh 1978), whereas in most of the angiosperms wall formation immediately occurs after the first cell division (Steeves and Sussex, 1989). After several divisions, the proembryo becomes cellularized. During early embryogeny, the embryo forms a distinct embryonal mass (analogous to the embryo proper in angiosperms) on the end of the suspensor system. Later, the embryonal mass is surrounded with a surface layer of cells that functions as protoderm layer; although cell divisions may not be exclusively anticlinal as in angiosperms (Rombeger et al., 1993). The definition of the protoderm typically is the first evidence of differentiation (Rombeger et al., 1993). Late embryogeny in gymnosperms corresponds to the "post-globular" embryo development in angiosperms (Singh, 1978; Rombeger et al., 1993). Early during this period, the root and shoot meristems are delineated and the plant axis is established. A root organization center is first formed which gives rise to the root meristem. The cotyledon primordia arise in a ring around the distal end of the embryo. Following the differentiation of the inner primary tissues, the embryonic shoot apex is formed at the top of the embryo (Rombeger et al., 1993).

Embryo maturation in seed plants

The maturation program comprises the synthesis of seed storage products that will be utilized during germination, the acquisition of desiccation tolerance, the prevention of precocious germination and the induction of dormancy (Harada, 1997). An essential hormonal regulator in the maturation phase is abscisic acid (ABA). The synthesis and deposition of storage and late embryogenesis abundant (LEA) proteins are usually regulated through ABA- and water stress-induced gene expression (Doderman et al., 1997). Seed storage proteins have been isolated in gymnosperms and angiosperms and show considerable sequence conservation (Misra, 1994).

In conclusion: Embryo development is different within angiosperms and between angiosperms and gymnosperms. For example, maize and Norway spruce embryos do not display regular cell division patterns in contrast to *Arabidopsis*. The

number of cells at maturity is much larger in maize and Norway spruce than in *Arabidopsis*.
However, a comparison of embryo development between angiosperms (*Arabidopsis*, maize) and gymnosperms (Norway spruce) suggests that in all cases the protoderm layer is the first tissue delineated during radial pattern formation.

Mutational dissection of embryo pattern formation

A variety of experimental systems have been employed to understand the making of the plant embryo (for review, see Mordhorst et al., 1997). Chemical and insertional mutagenesis strategies have provided unprecedented leaps in the understanding of zygotic embryogenesis in angiosperms. Numerous mutants with altered developmental programs have been generated in genetic model species such as *Arabidopsis*, maize and rice (Goldberg et al., 1994; Hong et al., 1995; Meinke, 1995; Neuffer et al., 1997; Laux and Jürgens, 1997). Mutations affecting either the radial pattern or the apical-basal pattern and the maturation process have been described mainly for the *Arabidopsis* embryo.

Mutants affecting radial patterning

Few mutants with specific radial-pattern defects have been described in *Arabidopsis*. Interestingly, these mutants were originally isolated for their seedling-root phenotype but subsequently found to display the same radial pattern defect at the embryonic stage.
Radial patterning is initiated with the formation of a surface layer of epidermal precursor cells overlying non-epidermal cells. Up to now, no mutants lacking or specifically affected in the protoderm layer have been described.
The *anthocyaninless2* (*anl2*) mutant has an aberrant radial root patterning (Kubo et al., 1999). The *anl2* roots produce extra cells called intervening cells, located between the cortical and epidermal layers. The nature of the extra cell layer is unknown at present. The *pinocchio* (*pic*) mutant has no cortex in the primary root (Benfey et al., 1993). The radial tissue organization of the primary roots can be traced back to the embryonic stage (Scheres et al., 1995a). These layer-patterning defects in the primary root probably have an embryonic origin. The embryo of *fass/ton* mutants (Torres-Ruiz and Jürgens, 1994; Traas et al., 1995) has additional cortical cells and an enlarged vascular cylinder. The *hydra1* mutant is similar to *fass*, as it also exhibits an abnormal radial pattern with disrupted vascular strands (Topping et al., 1997)
In *Arabidopsis*, the single-layered ground tissue is established by the globular stage. At a later stage of embryogenesis, the ground tissue cells undergo asymmetric divisions, producing an outer cortical cell layer and an inner endodermal layer (Scheres et al., 1995b). Mutations in two genes *SCARECROW* (*SCR*) and *SHORT ROOT* (*SHR*), affect different aspect of ground tissue

formation. The *shr* mutant embryo fails to establish the endodermis (Benfey et al., 1993; Scheres et al., 1995b). In the *scr* mutant embryo, the ground-tissue cells fail to divide to give the cortex and endoderm, and the single layer of ground tissue expresses features of both endodermis and cortex (Benfey et al., 1993; Scheres et al., 1995b). These two genes encode putative transcription factors belonging to the GRAS (*GIBBERELLIN-INSENSITIVE*, *REPRESSOR of gal-3*, *SCARECROW*) family (Di Laurenzio et al., 1996; Pysh et al., 1999; Helariutta et al., 2000). *SCR* is expressed in the cortex/endodermal initial cells and in the endodermal cell lineage but surprisingly *SHR* transcripts are detected in the procambium (Di Laurenzio et al., 1996; Heliariutta et al., 2000). The specific expression of *SHR* in the procambium is required for the maintenance of *SCR* endodermal expression (Helariutta et al., 2000). Thus the centrally located vascular primordium appears to participate in subepidermal radial patterning. Putative orthologues of *SCR* have been identified in maize (Lim et al., 2000) and in pea (Sassa et al., 2001). Although the relationships between initial cells and each tissue have not been established in the root of pea and maize, the similar amino acid sequence and expression pattern of *SCR* genes in two dicots (*Arabidopsis*, pea) and one monocot (maize) suggest functional conservation of *SCR* in the differentiation of the endodermis among angiosperms.

The *wooden leg* (*wol*) mutant is characterized by a decrease of vascular precursor cells that are all specified as xylem at the expense of phloem (Scheres et al., 1995b). The *WOL* gene encodes a putative two-component histidine kinase (Mähönen et al., 2000).

All these mutations affecting the embryo radial pattern support the idea that radial patterning progresses from the periphery to the center.

Mutants affecting apical-basal patterning

The apical-basal pattern is defined by the positioning of the shoot meristem and cotyledons, the hypocotyl and the root including the root meristem. A screen for gene mutations deleting domains of the embryonic apical-basal pattern has been performed in *Arabidopsis* (Mayer et al., 1991; Mayer et al., 1993). Four mutants, namely *gnom* (*gn*), *monopteros* (*mp*), *gurke* (*gk*) and *fackel* (*fk*) have been described. The *gk* mutant does not form a shoot meristem and cotyledons whereas mutant alleles of the *GN* gene delete the root and the cotyledons. The *GN* gene encodes a brefeldin A (BFA)-sensitive guanine-nucleotide exchange factor (Shevell et al., 1994; Steimann et al., 1999). Coordinated polar localization of the auxin efflux carrier PIN is impaired in *gn* embryos (Steimann et al., 1999). Since BFA also affects the polar localization of PIN1, one aspect of GN action may involve polar transport of auxin. The *gn* phenotype can be mimicked by altering auxin tranport in early embryo of the closely related *Brassica juncea* (Hadfi et al., 1998).

Despite a disturbed apical-basal pattern of embryo polarity, *AtLTP1* (lipid transfer protein) expression is unaffected and becomes confined to the protoderm layer of

the *gn* embryos (Vroemen et al., 1996) suggesting that establishment of the apical-basal and radial patterns can be achieved independently.
The *mp* mutant gives a seedling lacking primary roots but able to form roots postembryonically (Berleth and Jürgens, 1993). The *MP* gene encoding an auxin-response transcription factor acts within the context of embryogenesis (Hardtke and Berleth, 1998). The genetic and molecular data from the *mp* and *gn* mutants indicate that auxin plays a variety of roles early during embryo development.
In the *fk* mutant, the cotyledons appear directly attached to the root. The abnormal phenotype of the *fk* mutant starts with a lack of asymmetric cell division at the globular-embryo stage. The isolation of the *FK* gene, encoding a sterol reductase, revealed that the *fk* phenotype resulted from an early block in sterol biosynthesis (Jang et al., 2000; Schrick et al., 2000). It points to a critical role for sterols in embryogenesis.
The mutations in *CLAVATA1* (*CLV1*) (Clark et al., 1993) and the homeobox *WUSCHEL* (*WUS*) (Laux et al., 1996) genes affect the development of the shoot meristem during embryogenesis and postembryonic development. The *CLV1* and *WUS* genes encode a membrane receptor serine/threonine kinase and a homeobox gene respectively. The *shoot meristemless* (*stm*) homeobox gene, leading to a similar shoot-meristem defective phenotype when mutated, is specifically expressed in the cells at the top of the late-globular embryo that likely represent the incipient shoot meristem (Barton and Poethig, 1993; Long et al., 1996). In all cases, the development of the cotyledons is not affected, suggesting that the cotyledons and the shoot meristem are independently specified.
Another mutant called *hobbit* (*hbt*), is affected in the basal region and is therefore incapable of forming a root meristem (Willemsen et al., 1996)

Mutations affecting maturation process

Genetic studies have revealed that in *Arabidopsis*, the *ABA-INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*) and *LEAFY COTYLEDON1* (*LEC1*) loci play major roles in regulating maturation (reviewed in Wobus and Weber, 1999). All three promote embryo-specific processes and simultaneously repress germination. They also interact to regulate several processes during seed maturation, including accumulation of chlorophyll, desiccation tolerance, sensitivity to ABA and expression of storage proteins (Parcy et al., 1997; Wehmeyer and Vierling, 2000).
The pleitropic effects of *abi3 Arabidopsis* mutants on seed maturation (Koorneef et al., 1984) have also been described for the *viviparous1* (*vp1*) locus in maize (Robertson, 1955). The *vp1* mutants are blocked in the maturation progran. As a result, the mutant embryo proceeds precociously into seedling development (Robertson, 1995). The ABI3 and VP1 proteins as well as their presumed orthologs share a similar domain organization (Giraudat et al., 1992; Mc Carty et al., 1991; Hill et al., 1996; Luerßen et al., 1998).

Molecular markers of the protoderm layer

Molecular markers with a protoderm-specific expression have been isolated in angiosperms (mainly *Arabidopsis* and maize).
The *Arabidopsis AtML1* homeobox gene is expressed in all the embryonic cells at the octant stage to become restricted to the protoderm at the globular stage (Lu et al., 1996). The maize *ZmOCL1* gene, highly similar to *AtML1*, is also specifically expressed in the protoderm (Ingram et al., 1999).
A similar switch in expression, from a uniform to a protoderm-specific expression, has been described for members of the lipid transfer protein (LTP) family in *Arabidopsis* (*AtLTP1*, Thoma et al., 1994; Vroemen et al., 1996), carrot (*EP2*, Sterk et al., 1991) and maize (*LTP2*, Sossountzov et al., 1991). Sabala et al. (2000) isolated the *Pa18* gene, encoding a putative LTP protein, and showed a typical switch of expression pattern towards the protoderm layer during Norway spruce embryo development.

In conclusion: Although the apical-basal and radial tissue patterning processes overlap in time, they can be independently established. Thus radially arranged tissues are present in mutants disturbed in the apical-basal pattern (Mayer et al., 1991). The analysis of *Arabidopsis* mutants defective in radial tissue patterning suggest a centripetal tissue patterning process.
The understanding of embryo pattern formation in other seed plants is not as advanced as in the model plant *Arabidopsis*. To what extent the mechanisms of *Arabidopsis* embryo pattern formation can be extrapolated to other more divergent angiosperms and to seed plants in general is currently unknown.
The characterization of homologues of *Arabidopsis* genes resulting in a specific pattern defect, when mutated, is one possibility to facilitate the comparison of embryo formation across seed plants. In species where mutants are not available the function of genes can be studied using somatic embryogenesis and transformation technology.

Plant homeodomain families

The plant homeobox genes can be divided in two large groups encoding homeodomains and homeodomain-leucine zipper (HD-Zip) proteins, respectively (Morelli et al., 1998).

The homeodomain-leucine zipper (HD-Zip) family

The homeodomain-leucine zipper (HD-Zip) family consists of proteins featuring a dimerisation domain, the leucine zipper, immediately adjacent to the homeodomain. Interestingly the association of these two motifs (a HD and a leucine zipper) in transcription factors has not been identified in other organisms than plants. It might suggest that they participate in plant-specific processes.
In *Arabidopsis*, the HD-Zip family has been divided into four distinct subfamilies, named HD-Zip I, II, III and IV, based on HD-Zip domain comparisons and intron positions, (Sessa et al., 1994). This classification was further illustrated with the characterisation of additional HD-Zip genes from other angiosperms but also from non-angiosperm plants. Dozens of HD-Zip I and II genes have been reported in dicots and monocots (reviewed in Chang et al., 1998). HD-Zip III genes have mainly been described in *Arabidopsis* (Sessa et al., 1998; Zhong and Ye, 1999; Otsuga et al., 2001) though rice EST clones showing high amino acid identity to class III proteins are now available. Recently, HD-Zip I, II, III genes have been isolated from lower plants (a moss, Sakakibara et al., 2001; a fern, Aso et al., 1999). No gymnosperm HD-Zip I, II, III genes have been reported so far. However the presence of these three gene classes in angiosperms and mosses indicates that these subfamilies originated before the divergence of the vascular plant and moss lineages. Therefore, it is likely that HD-Zip genes belonging to these three classes are present in gymnosperms.
The HD-Zip IV subclass has also been named the Homeodomain-Glabra2 family (HD-GL2) by Lu et al. (1996). The HD-Zip IV genes have been reported in several species such as *Arabidopsis* (Lu et al., 1996; Tavares et al., 2000), maize (Ingram et al., 1999; Ingram et al., 2000), the orchid *Phalaenopsis* (Nadeau et al., 1996) and sunflower (Valle et al., 1997).
Attempts to isolate HD-Zip IV genes, using PCR and degenerate primers corresponding to conserved regions of the homeobox, in two non-angiosperm plants (a fern, Aso et al., 1999; a moss, Sakakibara et al., 2001) were unsuccessful so far.

Domains in the HD-Zip proteins

Class I and II

Proteins that belong to the HD-Zip I and II class are 300 amino acids long and share a very similar HD-Zip domain (Chang et al., 1998). An acidic domain,

likely to act as a transcriptional activation domain (Ptashne, 1988), is usually present upstream and/or downstream the HD-Zip motif (Chang et al., 1998). Dual repressor/activator ability was also described for rice HD-Zip II protein (Meijer et al., 1997). The class I proteins are usually not conserved outside the HD-Zip motif (Chang et al., 1998). In class II proteins, additional common sequences can be found downstream from the HD-Zip domain; these have the amino acid CPSCE motif, and a C-terminal end segment (Chang et al., 1998).
The DNA-binding mechanisms have been studied *in vitro* in HD-Zip proteins. In *Arabidopsis*, the HD-Zip I and II proteins recognize pseudopalindromic DNA sequences specific for each class (Sessa et al., 1993; Sessa et al., 1994; Sessa et al., 1997; Johannesson et al., 2001). HD-Zip II proteins from the resurrection plant *Craterostigma plantagineum* (Frank et al., 1998) and rice (Meijer et al., 1997), and HD-Zip I proteins from sunflower (Palena et al., 1999) and soybean (Tang et al., 2001) bind DNA with specificities similar to their respective *Arabidopsis* HD-Zip class proteins. It is likely that most of the class I and II HD-Zip proteins would show similar binding characteristic at least *in vitro*.
The DNA-binding activities analyzed *in vivo* for one HD-Zip I protein (Aoyoma et al., 1995) and two HD-Zip II proteins (Steindler et al., 1999; Meijer et al., 1997) confirmed that the DNA-binding sites described *in vitro* for these two subclasses were also recognized *in vivo*.
The DNA-binding ability of these proteins was greatly enhanced in the form of dimers in *Arabidopsis* (Sessa et al., 1993; Sessa et al., 1994; Sessa et al., 1997), sunflower (Gonzalez et al., 1997; Palena et al., 1999) and rice (Meijer et al., 1997; Meijer et al., 2000). The HD-Zip I and II proteins seem to form mainly homodimers in *Arabidopsis* (Sessa et al., 1993; Sessa et al., 1997) and in rice (Meijer et al., 2000). Heterodimerisation has been demonstrated between members from the same class, in HD-Zip I proteins in *Arabidopsis* (Johannesson et al., 2001) and in HD-Zip II proteins in different species such as rice (Meijer et al., 1997; Meijer et al., 2000) and *C. plantagineum* (Frank et al., 1998).
A negative autoregulatory mechanism has been recently described for one *Arabidopsis* HD-Zip II protein, that might contribute to rapid switching off of this gene when its induction signal stops (Ohgishi et al., 2001).

Class III and IV

The class III and IV HD-Zip proteins are about 600-700 amino acids long with an N-terminal HD-Zip motif. In contrast to the class I and II proteins, the spatial organization of HD-Zip motif in class III is characterized by insertions of four amino acids, one between the helix 2 and helix 3 of the HD and another between the HD and the leucine zipper domain (Baima et al., 1995; Sessa et al., 1998).
Unlike the other three HD-Zip classes, the HD-Zip domain in class IV features a HD linked to a truncated leucine zipper-like domain. This dimerisation domain is formed by two subdomains interrupted by a loop consisting of hydrophobic amino acids with a conserved CX2CG peptide (where X means any amino acids) (Chang et al., 1998).

Downstream from the HD-Zip motif, an additional domain has been detected in these two classes. This 200-bp long motif is called the START domain for StAR-related lipid transfer. The identification of this domain was derived from a functional prediction study showing significant similarities between these HD-Zip proteins and the mouse StAR (steroidogenic acute regulatory) protein and its closest orthologue MLN64 from human (Ponting and Aravind, 1999; Tsujishita and Hurley, 2000). A recent sequence profile search and structure comparison and prediction study has suggested that the START domain probably functions as a ligand-binding domain (Iyer et al., 2001). Proteins containing a START domain have been shown to bind different ligands such as sterols and phosphatidylcholine (Kallen et al., 1998; Akeroyd et al., 1981). The proposed ability of the START domain to bind lipids has not been studied for the HD-Zip class III and IV proteins. However, mutations in the region coding for the START domain of a HD-Zip III gene *PHABULOSA* (McCornell et al., 2001) suggest that the START domain is required for the activity of the protein.

It is noteworthy that the START domain is present in the Zip III and IV proteins, and shares a very high level of identity within each class but a very low level across classes. It suggests that if the predicted function of the START domain is to bind lipids, the nature of the ligand is likely to be specific for each class.

A consistent level of similarities remains downstream from the START domain of the class III and IV proteins, suggesting the presence of additional domains with unknown functions.

The DNA-binding target was determined *in vitro* for one HD-Zip III protein (Sessa et al., 1998). Moreover, this study also showed that this protein binds as a dimer.

Recognition sites for the HD-Zip IV proteins have not been reported. However, the Zip domain of the GL2 protein can functionally replace the Zip domain of a HD-Zip II protein and induce the formation of dimers and DNA-binding (Di Cristina et al., 1996). Similarly, dimerisation and DNA-binding abilities were conferred by the same region of the sunflower protein HAHR1 (*Helianthus annuus* homeobox from roots) (Palena et al., 1997) indicating that family IV proteins also bind DNA as dimers (Di Cristina et al., 1996).

Functions of the HD-Zip proteins

Class I and II

In *Arabidopsis* the class I and class II HD-Zip genes constitute a large family of at least 26 members designated *Arabidopsis thaliana homeobox* (*ATHB*) (Johannesson, 2001). The characterization of null mutations in selected genes is a powerful tool for elucidating gene function by assessing the resulting phenotype. However, mutations in HD-Zip I and II genes leading to distinguishable phenotypic effects in *Arabidopsis* have not been reported to date. A compensation

of the abolished gene function by other closely related HD-Zip genes might be an explanation.
In the absence of mutant phenotypes, transgenic plants either over- or underexpressing HD-Zip I and II genes can provide clues to their functions. Expression of *ATHB-2* is regulated by changes in the red to far red light ratio and overexpression of *ATHB-2* enhances cell expansion in the hypocotyl (Carabelli et al., 1996; Steindler et al., 1999). ATHB-2 would be active in the phytochrome-regulated growth responses such as shade avoidance and neighbour detection that lead to adaptative changes in the development of a plant (Steindler et al., 1999). Overexpression of *ATHB-13* affects cotyledon shape by inhibiting lateral expansion of epidermal cells in sugar-treated seedlings, showing that ATHB-13 can affect plant development in response to sucrose (Hanson et al., 2001). Rice plants overexpressing *Oshox1* showed alterations in leaf morphology, but also retarded growth (Meijer et al., 1997)
Exogenous hormones and abiotic stresses transcriptionally regulate some HD-Zip genes. The genes *ATHB-7* (Söderman et al., 1996) and *ATHB-12* (Lee and Chun, 1998) are induced by ABA, and *ATHB-1* by ethylene (Morelli et al., 1998). The *ATHB-6* gene is induced by ABA, water deficit and osmotic stress (Söderman et al., 1999). Two HD-Zip genes in *C. plantagineum* are drought-inducible (Frank et al., 1998).

Class III

In *Arabidopsis*, the HD-Zip III class consists of at least five highly similar genes *ATHB-8*, *ATHB-9/PHAVOLUTA* (*PHV*), *ATHB-14/PHABULOSA* (*PHB*) and *ATHB-15* (Sessa et al., 1994; Sessa et al., 1998; McConnell et al., 2001; Baima et al., 2000) and *REVOLUTA* (*REV*)/*INTERFASCICULARFIBERLESS* (*IFL1*) (Zhong and Ye, 1999; Talbert et al., 1995; Ratcliffe et al., 2000). It is noteworthy that at least four of them, *IFL1/REV* (Zhong and Ye, 1999; Otsuga et al., 2001), *ATHB-8* (Baima et al., 1995), *ATHB-9/PHV* (Baima et al., 2001) and *ATHB-14/PHB* (McConnell et al., 2001) are expressed in the vascular system. At the torpedo stage of embryo development, a specific expression is detected in the procambium for *PHB* (McConnell et al., 2001), *REV* (Otsuga et al., 2001) and *ATHB-8* (Baima et al., 1995). Later, their transcripts mark vascular precursor cells within developing organs (Baima et al., 1995; Zhong and Ye, 1999; Otsuga et al., 2001; McConnell et al., 2001).
Ectopic expression of *ATHB-8* in transgenic *Arabidopsis* and tobacco plants results in a higher production of primary and secondary xylem suggesting that overexpression of *ATHB-8* promotes vascular cell differentiation (Baima et al., 2000). Knockout *Arabidopsis* plants were obtained for *ATHB-8*, but no phenotype was noticed (Baima et al., 2001). The *ifl1/rev* mutant plants show a block of interfascicular fiber differentiation, alteration of secondary xylem differentiation but also pleiotropic effects such as reduced numbers of cauline branches and reduced numbers of secondary rosette inflorescence (Talbert et al., 1995; Zhong and Ye, 1999). The *phb-1d* mutation affects vascular tissue formation and

differentiation but also alters leaf polarity such that adaxial characters develop in place of adaxial leaf characters (McConnell and Barton, 1998; McConnell et al., 2001). The different phenotypes of aerial organs in these mutants may be a consequence of altered vascular cell differentiation. Taken together, these data suggest that the HD-Zip III family may participate in the cell fate determination of the vascular tissue.
Auxin is considered as a major factor for the vascular tissue differentiation in plants (Aloni, 1987). The *ifl1* mutants are also dramatically affected in auxin polar transport in the inflorescence stems, and auxin polar transport inhibitors alter the normal differentiation of interfascicular fibers in the inflorescence stems of wild-type *Arabidopsis* (Zhong and Ye, 2001). Transgenic tobacco plants for the *ATHB-8* gene mimicked phenotypic effects when plants treated with inhibitors of auxin polar transport (Baima et al., 2000).
Taken together, the HD-Zip III genes would be key components of the auxin-signalling pathway leading to the formation of the procambium in the embryo and vascular cell differentiation in the plant body.

Class IV

The members of the HD-Zip IV class all feature a HD-Zip motif, however their dimerisation domain differs from the canonical leucine zipper as found in the other three HD-Zip classes. Lu et al. (1996) considered it as a separate family called the HD-GLABRA2 (HD-GL2) (Lu et al., 1996). This family was originally composed of O39 from the orchid *Phalaenopsis* (Nadeau et al., 1996), and GL2 (Rerie et al., 1994) and ATML1 (for Meristem L1 layer) (Lu et al., 1996) from *Arabidopsis*. In the following part, I shall use the term HD-Zip IV to define this group. In the *Arabidopsis* genome, I identified six additional putative HD-Zip IV genes in addition to the ten previously described members (*AtML1*, Lu et al., 1996; *ATHB-10/GL2*, Di Cristina et al., 1996; Rerie et al., 1994; *FWA*, Soppe et al., 2000; *GL2-1*, *GL2-2*, *GL2-3*, *GL2-4*, *GL2-5*, Tavares et al., 2000; *ANL2*, Kubo et al., 1999; *PROTODERMAL FACTOR2*, Accession number AB056455).
The *Arabidopsis* homeobox gene *AtML1* transcripts are first detected in the apical cell of the embryo proper after the first asymmetric division of the zygote. In the early globular stage, the expression of *AtML1* becomes specifically restricted to the protoderm. At the seedling stage, *AtML1* is expressed in the L1 layer of the shoot apical meristem (Lu et al. 1996).
Three HD-GL2 genes *ZmOCL1*, *ZmOCL4* and *ZmOCL5* (OCL for outer cell layer) were isolated from maize and shown to have a very similar protodermal/epidermal specific expression as the one described for *AtML1* (Ingram et al., 1999; Ingram et al., 2000). However, *ZmOCL4* and *ZmOCL5* transcripts preferentially localize in the protoderm of the adaxial and abaxial face of the embryo, respectively. The expression of *ZmOCL3* is suspensor-specific and *ZmOCL2* transcripts accumulate in the subepidermal layer of the shoot meristems (Ingram et al., 2000).

The *O39* gene from the orchid *Phalaenopsis*, is expressed during early steps of differentiation of ovule primordia, mainly in the placenta epidermis, the protoderm of ovule primordia and in the outer cell layer surrounding the archesporial cell (Nadeau et al., 1996).
Several mutants for HD-Zip IV genes are available in *Arabidopsis* providing indications about their functions. The *anl2* mutant has an aberrant radial root patterning (Kubo *et al.*, 1999). The *anl2* primary root produces extra cells called intervening cells, located between the cortical and epidermal layers. In the *anl2* mutant, the anthocyanin accumulation is significantly decreased in subepidermal layers of rosette leaves (Kubo et al., 1999). A late flowering phenotype was described for *lab1-1D* mutant constitutively expressing *ANL2* (Weigel et al., 2000). A similar phenotype was observed in *fwa* mutant (Soppe et al., 2000). However, the *anl2* loss-of-function phenotype described by Kubo et al. (1999) is unrelated to flowering, suggesting that the cause of late flowering in the *lab1-1D* mutant might not reflect the primary gene function. The *Arabidopsis ATHB-10/GL2* mutant alleles are affected in trichome development, seed coat mucilage production (Rerie et al., 1994) and root hair formation (Di Cristina et al., 1996). Studies with promoter *GL2::GUS* reporter gene fusion further discovered *GL2* promoter activity gene in specific cells of the outer integument of the seed coat and the stomata complex, (Windsor et al., 2000; Hung et al., 1998). During embryogenesis, the expression of *GL2* was detected within protodermal cells at the base of the heart-stage embryo (Lin and Schiefelbein, 2001). These data suggest that the patterning of epidermal cell types begins at an early stage of embryogenesis (Lin and Schiefelbein, 2001).

In conclusion: The HD-Zip family is divided into four classes whose corresponding members seem to be involved in distinct developmental processes in angiosperms. The HD-Zip I and II genes are proposed to be regulators of plant growth in response to changes in the environment during postembryonic phases of the life cycle in plants (Chan et al., 1998; Morelli et al., 1998). The HD-Zip III and IV families would participate in the regulation of specific patterning processes during embryogenesis and later during plant development. The HD-Zip III class would be involved in the development and differentiation of vascular tissues (Baima et al., 2000) and the HD-Zip IV class in the regulation of epidermal and subepidermal cell fate, and differentiation.

Additional families

The TALE family: KNOX and BELL subfamilies

The TALE (three amino acid loop extension) proteins are characterized by three extra residues between helix 1 and helix 2 of the HD (Bürglin, 1997). In plants they are encoded by the class I and class II *KNOTTED1*-like (*KNOX*) genes and the *BELL* genes.
The *KNOX* genes were originally identified through the cloning of the *Knotted1* gene in maize, whose mutant phenotype is the formation of ectopic knots on the surface of maize leaves (Volbrecht et al., 1991). *KNOX* genes have been identified in many highly divergent plants including Norway spruce (Sundås-Larsson et al., 1998), a moss (Champagne and Ashton, 2001) and a unicellular green algae (Serikawa and Mandoli, 1999). Based on sequence homology and expression pattern, two classes have been distinguished: class I genes specialized in the establishment and maintenance of meristematic identity and in the initiation of leaf primordia and class II genes having a more global expression pattern and no clear functional role in plant development (reviewed in Reiser et al., 2000). KNOX proteins and *KNOX* mRNAs have been shown to be able to move from cell to cell in maize (Lucas et al., 1995) and in tomato (Kim et al., 2001) respectively. Negative regulators of *KNOX* genes have been described and encode *myb* domain proteins (reviewed in Barton, 2001).
The *BELL* genes are poorly studied. The *Arabidopsis BELL1* is required for integument specification of the ovule (Western and Haughn, 1999) and the *ATH1* gene (Quaedvlieg et al., 1995) induces delayed flowering upon overexpression (Smeekens et al., 1998).
Interactions through homo/heterodimerisation between KNOX class I and class II proteins as well as between KNOX class I and BELL proteins have been described recently in barley (Müller et al., 2001).

The PHD-finger family

The plant homeodomain finger (PHD finger) proteins are defined by an N-terminal cysteine scaffold linked to the HD, combined with an upstream leucine zipper (Korfhage et al., 1994; Halbach et al., 2000). This family consists of four maize genes *ZmHOX* and genes of other species, such as *Arabidopsis* (Schindler et al., 1993) and parsley (Korfhage et al., 1994). The *ZmHOX* genes share identical expression pattern, being highly activated in maize shoot and root meristems from embryogenesis to the reproductive phase (Klinge and Werr, 1995) suggesting a function in plant development and growth. Interestingly, the *ZmHOX2* genes encode a polypeptide with two homeodomains.

The WUSCHEL family

The WUSCHEL (WUS) family is represented by the *Arabidopsis WUS* gene (Mayer et al., 1998). Based on mutant and molecular analyses, WUS appears to be required for the establishment of a functional embryonic shoot apical meristem by specifying stem cell identity and the stem cell maintenance in the shoot and floral meristems (Laux et al., 1996; Mayer et al., 1998).

Additional families

Windhövel et al. (2001) described a new class of homeodomain family, the Zinc finger homeodomain (ZF-HD) family, characterized by 4 extra amino acids inserted in the loop between helix 1 and helix 2 of the homeodomain. Accordingly, this gene family could also be named the FALE (four amino acids loop extension) class. In addition to this typical homeodomain, ZF-HD proteins feature two highly conserved amino acid motifs predicted to fold into two one-zinc finger domains involved in homo/heterodimer formation. These genes would be involved in the establishment of the characteristic expression of the C4 phospho*enol*pyruvate-carboxylase gene in the photosynthetic organs.
The PALE (penta amino acids loop extension) homeodomain family has been designated according to an extra loop of five amino acid inserted between the first helices of the homeodomain (Hertzberg and Olsson, 1998). They would participate in xylem and phloem formation in poplar.
The Nodulin homeobox genes in soybean and lotus encode deduced proteins with atypical homeodomains (Jorgensen et al., 1999). In particular, the highly conserved phenylalanine in position 49 is substituted by a leucine and an amino acid insertion between the first two helices.

In conclusion: The homeobox family, originally described in animal systems (Gehring, 1994), is well represented in the plant kingdom. The plant homeobox genes show expression patterns suggesting an analogous role to the animal counterparts, in the regulation of key developmental processes.
A large number of genetic and molecular data emerged from the *Arabidopsis* plant system. Studies dealing with other species especially non-angiosperm species, are rare. However, the available data already show that families of homeobox genes (KNOX and HD-Zip I, II and III) involved in distinct developmental processes were already present in primitive land plants such as mosses. Additional comparative studies will probably provide clues on the function of plant homeobox genes during development in divergent plants and maybe unravel common and/or specific developmental processes in the different groups of the plant kingdom.

Results and discussion

In the homeobox section, I described the homeodomain-glabra2 (HD-GL2) family.
The tissue-specific expression of angiosperm HD-GL2 genes and mutant analyses suggest that the HD-GL2 would be involved in the regulation of epidermal and subepidermal cell fate and differentiation during embryonic and postembryonic development.
In our laboratory, we are using somatic embryogenesis of the gymnosperm Norway spruce to study embryo development. Filonova et al. (2000) proposed a developmental pathway for somatic embryogenesis and showed that somatic and zygotic embryo development in Norway spruce are highly similar except at the earliest stages of development corresponding to the proembryogeny stage in zygotic embryogenesis.
The isolation of HD-GL2 genes and the characterization of their expression pattern might provide tissue-specific molecular markers useful for studying embryo pattern formation in Norway spruce and tools to compare embryo development in seed plants.
Another interesting step of seed development is the maturation process where the *viviparous* angiosperm genes have been shown to play a pivotal role (reviewed in Wobus and Weber, 1999). The isolation of Norway spruce *viviparous1* (*Pavp1*) homologue can provide insights into this process in conifers.

PaHB1 and *PaHB2* are conifer HD-GL2 genes (I,II)

To isolate HD-GL2 genes expressed during somatic embryogenesis in Norway spruce (*Picea abies*), two sets of degenerate oligonucleotides derived from a nucleotidic sequence alignment between *AtML1*, *GL2* and *O39* (Lu et al., 1996; Rerie et al., 1994; Nadeau et al., 1996) and RT-PCR were used. Two clones were identified in Norway spruce proliferating embryogenic cell cDNA. These clones were named *PaHB1* and *PaHB2* (*Picea abies* homeobox).
A sequence similarity search using BLAST (Altschul et al. 1990) revealed that the *PaHB* genes encode putative HD-GL2 proteins highly similar to the angiosperm counterparts. The predicted PAHB proteins present a typical HD-GL2 protein domain organisation: an N-terminal homeodomain linked to a non-canonical leucine zipper domain, and a putative StAR-related lipid transfer (START) domain (Lu et al., 1996; Ponting et al., 1999; Tsujishita et al., 2000). Upstream the homeodomain, PAHB1 has an acidic region rich in glutamate and aspartate, which is similar to the corresponding region of O39, GL2-2 and ZMOCL5 (figure 1, paper I). Similarly, a short stretch rich in aspartate and glycine precedes the homeodomain at the N-terminal end of PAHB2, which is reminiscent of the N-terminal regions of HD-GL2 proteins. Regions surrounding the homeodomain featuring acidic amino acid/alanine/proline stretches have been shown to

correspond to activation and/or repression domains in some HD-Zip I and II proteins (Aoyama et al., 1995; Meijer et al., 1997; Meijer et al., 2000).
The putative PAHB proteins share striking sequence similarities with the HD-GL2 angiosperm proteins, inside and outside the homeodomain. Protein sequence comparisons with the PAHB proteins and angiosperm counterparts are presented in figure 1. The N-terminal part of the PAHB proteins is less conserved and was excluded in the analyses. The overall PAHB1 protein shares more than 70% identity with the *Arabidopsis* proteins ATML1, PDF2 and GL2-2 and the monocot proteins O39 and ZMOCL5. The PAHB2 protein is more similar to other angiosperm HD-GL2 proteins. The PAHB2 protein is 68% identical to ANL2 from *Arabidopsis* and shares 63% identity with GL2-1 from *Arabidopsis* and OCL1 from maize. However, the PAHB proteins are also highly similar to each other, (62 % identity).

	Arabidopsis			*Phalaenopsis*	Maize
	ATML1	PDF2	GL2-2	O39	OCL5
PAHB1	73	74	71	75	70

	Arabidopsis		Maize		
	ANL2	GL2-1	OCL1	OCL2	OCL3
PAHB2	68	63	63	54	59

Figure 2: Percentage of identity between Norway spruce PAHB proteins and angiosperm homeodomain-glabra2 (HD-GL2) proteins. In the analyses, the region upstream from the homeodomain, which is usually highly variable within the HD-GL2 family was omitted.

A sequence similarity search using the BLAST function of UTR (untranslated region) database (Pesole et al., 2000) was performed on the 5'- and 3'-UTR of *PaHB1* and *PaHB2*. No significant sequence similarity was detected in their 5'-UTR. The sequence analyses of 3'-UTR revealed a highly conserved 17-bp long motif in *PaHB1* and *PaHB2* mRNA (figure 1, manuscript II). This motif was only found in certain angiosperm genes of the HD-GL2 family. Homeobox genes expressed during embryogenesis in metazoans sometimes feature regulatory elements located in their 3'-UTR that influence RNA localization (Bashirullah et al., 1998), RNA translation (Dubnau et al., 1996; Rivera-Pomar et al., 1996) and RNA stability (Fontes et al., 1999). The conservation of this short sequence among HD-GL2 genes across divergent seed plant families underlines the importance of its role.
To visualize the evolutionary relationships between the conifer PAHB proteins and the angiosperm HD-GL2 counterparts, we generated an unrooted phylogenetic tree by the neighbor joining (NJ) distance method using the conserved domains of the protein sequences (figure 2a, manuscript II). The phylogenetic analysis revealed that the HD-GL2 family consists of at least three distinct subgroups, each subgroup gathering proteins from monocot and dicot (*Arabidopsis*) plants. The PAHB1 protein groups with one subgroup consisting of

ATML1, GL2-2 and PDF2 from *Arabidopsis* and OCL5 and O39 from maize and *Phalaenopsis*, respectively. The PAHB1 protein together with these angiosperm proteins further forms a monophyletic subclass with GL2-3 from *Arabidopsis*. In contrast, PAHB2 is more closely related to ANL2 and GL2-1 from *Arabidopsis* and OCL3 from maize. The PAHB2 protein together with these angiosperm proteins form a monophyletic subclass with OCL1 and OCL2 from maize.

A third major monophyletic subgroup consists of two well-supported subdivisions. The first subdivision is represented by the *Arabidopsis* proteins GL2-4 and GL2-5 and one maize protein OCL4 and the second by GL2 from *Arabidopsis*, HAHR1 from sunflower and GHGL2 from cotton. Phylogenetic analyses performed with different methods could not resolve the position of FWA within the HD-GL2 family. In the NJ analysis, FWA is positioned with low support with the subgroup represented by PAHB2 whereas in the maximum parsimony (MP) analysis, FWA clusters with the third subgroup. It is then unclear if this highly divergent protein constitutes a distinct subgroup of the HD-GL2 family.

In the maximum parsimony analysis, the general tree topology was unchanged but the defined subgroups were supported with lower bootstrap values (data not shown).

To provide additional information about phylogenetic relationships between HD-GL2 genes, we determined the intron positions in the coding sequence of *PaHB1* and *PaHB2* by partial sequencing of a genomic DNA fragment amplified by PCR. The intron pattern of spruce *PaHB* genes was compared to that of angiosperm HD-GL2 genes currently available: a maize gene, *ZmOCL1* (Ingram et al., 1999), and ten *Arabidopsis* genes (Rerie et al., 1994; Di Cristina et al., 1996; Lu et al., 1996; Kubo et al., 1999; Soppe et al., 2000; Tavares et al., 2000). The *Arabidopsis* HD-GL2 genes present a very similar overall intron-exon organization with nine positionally conserved introns numbered from 1 to 9 (Tavares et al., 2000). The intron positions 1, 2, 4, 5, 7 and 9 are found in all the members whereas intron positions 3, 6 and 8 can be present/absent in different combinations. The position of these introns in relation to the deduced amino sequence of the genes is presented in figure 2b (manuscript II).

The *PaHB1* intron pattern features all the strictly conserved intron positions (1, 2, 4, 5, 7 and 9) as well as the less conserved ones (intron positions 3, 6 and 8). This intron pattern is only present in four *Arabidopsis* genes (*AtML1*, *PDF2*, *GL2-2* and *GL2-3*) which all belong to the same subgroup as *PaHB1*. It is noteworthy that the intron position 3 seems to have been lost in *AtML1*.

The *PaHB2* exon/intron boundaries are located at the positions 1, 2, 3, 4, 5, 7 and 9 (figure 2b, manuscript II). This intron pattern is highly similar to those of *ANL2*, *GL2-1*, *ZMOCL1* and *FWA*, although *PaHB2* features an additional intron position (position 3). Thus, the gene structure of *PaHB1* and *PaHB2* further supports the fact that *PaHB1* and *PaHB2* are phylogenetically associated with *AtML1*-like genes and *ANL2*-like genes respectively.

The fact that PAHB proteins are phylogenetically more closely related to angiosperm counterparts than to the other spruce protein suggests that at least two

ancestor proteins belonging to these two distinct subgroups were already present before angiosperms and gymnosperms separated, about 300 million years ago (Stewart and Rothwell, 1993).

Tissue-specific expression of *PaHB* genes (I,II)

One pressing question was if the *PaHB* genes have a similar spatial expression pattern as defined or suggested for some of their phylogenetically related angiosperm counterparts.

PaHB1 and *PaHB2* expression were detected in all analysed parts of the seedling (needles, epicotyl, hypocotyl and root), in reproductive organs (male and female strobili) and during somatic embryogenesis in proliferating cells and in maturing embryos (figure 2, paper I; figure 3, manuscript II).

The spatial expression pattern of *PaHB1* and *PaHB2* was further investigated by *in situ* hybridization during somatic embryo development in Norway spruce (paper I, figure 4; manuscript II, figure 4). In proembryogenic masses (PEM) of proliferating embryogenic cultures *PaHB1* and *PaHB2* transcripts are detected in all embryonic cells (figure 4a, paper I for *PaHB1*; data not shown for *PaHB2*). In early somatic embryos, *PaHB2* is mainly expressed in the embryonal mass and in the embryonal tube cells (figure 4a, manuscript II). However, since the vacuolated cells are damaged during the sample fixation, we cannot exclude that *PaHB2* is expressed in suspensor cells.

Owing to difficulties in obtaining proper sections of early somatic embryos, *PaHB1* expression data are unclear at that stage. However *PaHB1* transcripts were detected in proliferating cell cultures consisting of PEM and early somatic embryos (Filonova et al., 2000) suggesting that *PaHB1* is also expressed in the embryonal mass of somatic embryos.

In early maturing somatic embryos when the root-organization center is formed, *PaHB1* expression becomes restricted to the protoderm layer (figure 4b, paper I). When the tissue-specific expression of *PaHB1* was established in maturing somatic embryos, *PaHB2* transcripts were not detected (figure 4b manuscript II). Later, in cotyledonary-stage mature somatic embryos when all the primary tissues are formed and the root/shoot axis is delineated, *PaHB1* expression is maintained in the protoderm layer (figure 4c, paper I) and *PaHB2* expression is mainly restricted to the cortical layers of the hypocotyl and the root (figure 4c, 4d, manuscript II). A stronger *PaHB2* expression was detected on the outermost layer of the cortex. No *PaHB2* expression was detected in the cotyledons (figure 4c, manuscript II) or the shoot apical meristem (data not shown).

When the *PaHB2* sense and antisense probe were used, a hybridization signal was obtained in the distal part of the column and in the pericolumn of the root cap. However, the staining appeared stronger when the sections were hybridized with the antisense probe, indicating that the *PaHB2* gene is probably expressed in specific cells in the root cap (figure 4c, manuscript II).

The expression pattern of *PaHB2* was also investigated in the primary root of germinated seeds (figure 4f, manuscript II). The cortex-specific accumulation of *PaHB2* transcripts observed in the mature embryo becomes more evident in the protruding primary root. Moreover *PaHB2* is expressed in all the cortical layers of the root. No *PaHB2* mRNA was detected in the vascular tissue. *PaHB2* expression was also clearly excluded from the root meristem zone.
The genes *PaHB1* and *PaHB2* might participate in the establishment/maintenance of the radial pattern by specifying cell identity.

Conservation of tissue-specific expression of HD-GL2 genes in seed plants (I,II)

Despite the long evolutionary distance from angiosperms and conifers, the *AtML1* and *ANL2* genes from *Arabidopsis* share a highly similar gene structure and protein domain organization with the spruce genes *PaHB1* and *PaHB2*, respectively. The fact that each PAHB protein is phylogenetically more closely related to angiosperm counterparts than to the other spruce protein suggests that at least two distinct HD-GL2 proteins were already present before angiosperms and conifers separated, about 300 million years ago (Stewart and Rothwell, 1993).
Similarly to *PaHB1*, *AtML1* expression pattern switches from a uniform expression in the embryo proper at the early stages of embryogenesis to a protoderm-specific expression at the early globular stage (Lu et al., 1996). This typical tissue-specific expression was also described for three HD-GL2 genes in maize (Ingram et al., 1999; Ingram et al., 2000).
This conserved two-step expression pattern between angiosperm and gymnosperm HD-GL2 genes points to an ancient molecular process directing a protoderm-specific expression of HD-GL2 genes during embryo development in seed plants.
The *anl2* mutant is affected in the radial pattern of the primary root; roots produce extra cells (intervening cells) that are located between the cortical and epidermal layers (Kubo et al., 1999). Thus *ANL2* is probably involved in patterning the subepidermal layers of the roots (Kubo *et al,* 1999). The spatial expression pattern of *ANL2* is unknown at present but the *anl2* root phenotype suggests a similar gene activity localized in the cortical layers of the primary root as described for *PaHB2*.
The *ZmOCL2* gene was detected by RT-PCR in the shoot and root meristem regions of the seedling (Ingram et al., 2000). In embryonic, vegetative and floral apical meristems, *ZmOCL2* was mainly restricted to the subepidermal layer (Ingram et al., 2000).
Taken together, these data suggest an ancestral subepidermal-specific expression of HD-GL2 genes related to the *PaHB2* clade.
The conservation of layer-specific expression of HD-GL2 genes in highly divergent groups, in conifers and in angiosperms, suggests an important role in establishing and/or maintaining the radial pattern in seed plants.

Ectopic expression of *PaHB1* blocks somatic embryo development in Norway spruce (I)

Ectopic expression of *PaHB1* was obtained in one Norway spruce embryogenic subline. The phenotype of the PaHB1-transformed subline and the corresponding control cell line were compared upon transfer to a maturation medium. In the control line, a typical maturing somatic embryo features a protruding embryonal mass with a smooth surface supported by a suspensor (figure 5a, paper I). In the PaHB1-transformed somatic embryos, the embryonal mass lacked a smooth surface (figure 5b, paper I). In all cases, the PaHB1-transgenic embryos degenerated and no organs were formed, suggesting a developmental block occurring early during embryo development. The ectopic expression of *PaHB1* leads the maintenance of *PaHB1* transcripts throughout the somatic embryos even when the switch in expression of the native *PaHB1* gene takes place.

The drastic effects observed on embryo development using our transgenic approach is reminiscent of what has been described in two embryo-specific (*emb*) mutants *emb*-8518* and *emb*-8521* in maize (Elster et al., 2000). Unlike wild-type development, the expression of *ZmOCL1*, a cognate gene of *PaHB1*, is retained in the inner cells of the two mutant embryos. Consequently, the radial pattern of the abnormal embryos is incomplete, leading to an early developmental arrest in the proembryo/early embryo transition stages before entering the organogenic phase of embryogenesis.

Taken together, these data suggest that *PaHB1* must be restricted to the protoderm layer before entering the organogenic phase of embryo development.

Although the transgenic somatic embryos had an abnormal phenotype they still expressed the Norway spruce *viviparous* gene (*Pavp1*) (data not shown). Furthermore, these transgenic embryos also accumulated storage material (figure 5b, paper I). This suggests that the embryo pattern formation program is independent of the maturation process.

Molecular cues of a centripetal embryo radial patterning (III)

Two gymnosperm genes *PaHB1*, encoding a HD-GL2 homeodomain protein and *Pa18*, encoding a putative LTP, are specifically expressed in the protoderm-layer during somatic embryo development in Norway spruce (paper I; Sabala et al., 2000). An additional HD-GL2 gene, *PaHB2* was also isolated. *PaHB2* expression is specifically detected in the cortical cell layers (manuscript II). The expression data of these three genes during somatic embryo development is summarized in figure 2. In proliferating cultures, the three genes are expressed in the proembryogenic masses. In early somatic embryos, the expression of *PaHB1*, *PaHB2* and *Pa18* were mainly localized in the embryonal mass.

In maturing embryos, the expression of *PaHB1* and *Pa18* switch from a ubiquitous expression to a protoderm-specific localization. Ectopic expression of *PaHB1* or *Pa18* in somatic embryos maintaining their expression in the inner layers, leads to an abnormal embryo development (paper I; Sabala et al., 2000) suggesting that the inner layers of the embryos must be devoid of *PaHB1* and *Pa18* transcripts at this stage. These dramatic changes in *PaHB1* and *Pa18* expression pattern indicate a critical development decision taken during early embryo development. However we cannot rule out whether the protoderm-specific expression of *PaHB1* and *Pa18* correspond to a unique or two distinct developmental decisions. In maize, a similar situation has been seen where two genes *ZmOCL1*, a cognate HD-GL2 gene of *PaHB1* (Ingram et al., 1999) and *LTP2*, belonging to the LTP family (Sossountzov et al., 1991) have overlapping protoderm-specific expressions. The comparisons of *LTP2* and *ZmOCL1* expression patterns in wild type and two embryo-specific mutants provided evidences, that the tissue-specific expression of these genes marks two independent developmental cues (Elster et al., 2000).
When the tissue-specific expression of *Pa18* and *PaHB1* is established in maturing somatic embryos, *PaHB2* transcripts are not detected. Later when all the primary tissues are formed and the root/shoot axis is delineated, *PaHB1* and *Pa18* expression remain in the protodermal layer and *PaHB2* is specifically expressed in the cortical cell layers, mainly in the outermost cortical layer. The expression pattern of these three genes suggests that the expression of *PaHB1* and *Pa18* must be positioned in the protoderm layer before the cortex-specific expression of *PaHB2* is set up. It further suggests that the primary tissues are defined centripetally during embryo development in Norway spruce.

Is protoderm-specific expression of HD-GL2 genes equivalently operated in Norway spruce and *Arabidopsis*? (III)

The retention of protoderm-specific expression in the HD-GL2 and LTP gene families in angiosperms and conifers raises an interesting question as to the spatial restriction of expression of these genes is achieved by similar molecular mechanisms.
Approximately 1 kb of the 5'-flanking regions of two protoderm-specific genes *PaHB1* and *Pa18* were isolated by inverse PCR (Siebert et al., 1995; Rosati et al., 1999). Similarly, about 1kb of the upstream region of the cortex-specific gene *PaHB2* was cloned.
Computer analysis of the *PaHB1* promoter allowed us to identify potential cis-regulatory elements (Higo et al., 1999; Rombauts et al., 1999) (figure 2, manuscript III). A sucrose-responsive element (Sucrose-RE) (Grierson et al., 1994) was detected at position –828. Putative Myb-responsive elements (Myb-RE) (Urao et al., 1993; Solano et al., 1995) were identified at four different positions. Several potential Myb-RE elements are also present in the region of the *GLABRA2* (*GL2*) promoter that are essential for the specific expression in specific

cells of the leaf, hypocotyl and root epidermis (Szymanski et al., 1998; Hung et al., 1998). Genetic and molecular studies strongly suggested that at least four MYB transcription factors (Wada et al., 1997; Szymanski et al., 1998; Lee and Schiefelbein, 1999; Kirik et al., 2001) directly participate in the regulation of *GL2* expression. An analogous situation might occur for the protodermal expression of *PaHB1* in the embryos.

An 8-bp cis-acting motif called the L1 box (Abe et al., 2001) was recently identified in promoter regions of four L1-layer specific *Arabidopsis* genes, notably *AtML1* (Sessions et al., 1999) and *LTP1* (Thoma et al., 1994). A visual inspection of the *PaHB1* promoter revealed the presence of a putative L1 box at position –646 (figure 2) and in the 950-bp *PaHB2* upstream region. A similar motif was detected in the 947-bp *Pa18* upstream region but with a less strict consensus sequence. The presence of the L1 box in *PaHB1* and *Pa18* promoters suggests that these genes are specifically expressed in the epidermal layer. The detection of an L1 box in *PaHB2* promoter is more intriguing as *PaHB2* expression is clearly restricted to the cortex in the embryo and the seedling root.

The *SCARECROW* (*SCR*) gene has an endoderm-specific expression gene during *Arabidopsis* embryo development, but postembryonically the *SCR* expression is primarily detected in the L1 layer of the shoot apical meristem and in most cell layers of the leaf primordia (Wysocka-Diller et al., 2000). As the leaf primordium expands, *SCR* expression becomes progressively restricted to the bundle sheath cells, adjacent to the vascular strands. A homologue of *PaHB2*, *ANL2* is involved in root patterning and also in anthocyanin accumulation in subepidermal layers of leaves (Kubo et al., 1999). Taken together, *PaHB2* expression might be transiently present in the epidermal layer in the shoot region and later confined to the subepidermal layers of the needles.

To detect additional putative evolutionarily conserved motifs in promoter regions of protoderm-specific genes, we compared a 200-bp sequence in the *AtML1* promoter-proximal region that is sufficient for protodermal/epidermal-specific expression (Sessions et al., 1999) with the isolated *PaHB1* promoter fragment. In our analysis using a DNA matrix method (Pustell and Kafatos, 1984), only one motif (BOX 1) in the *PaHB1* promoter is homologous in the *AtML1* promoter region (figure 3a).

Similarly, an about 1.1-kb sequence in the *Arabidopsis thaliana LTP1* promoter was able to direct a protoderm-specific expresion (Thoma et al., 1994; Vroemen et al;, 1996). A similar comparative analysis was carried out with *Pa18* and *LTP1* promoters. Four nearly identical motifs (BOX 2, 3, 4 and 5, figure 3b) were identified in both promoters. No similar motifs were selected from the comparison of *PaHB1* and *PaHB2* promoters. The sequences corresponding to the four BOX motifs do not correspond to any known cis-regulatory motifs in plant promoters.

To determine whether protoderm-specific transcriptional regulatory elements in *PaHB1* and *AtML1* promoters were equivalent, we used two transformation vectors in which the *uidA* reporter gene (Jefferson et al., 1987) is transcriptionally fused to either *AtML1* promoter sequence (*ML1::GUS3*, Sessions et al., 1999) or

to *PaHB1* promoter sequence (*PaHB1::GUS*). The *ML1::GUS3* construct was transferred to Norway spruce embryogenic cells by particle bombardment (Clapham et al., 2000) and the *PaHB1::GUS* construct to *Arabidopsis* by *Agrobacterium*-mediated vacuum infiltration (Bechtold et al., 1993). Fifty spruce sublines and eight independent T2 transgenic *Arabidopsis* plants, carrying the heterologous promoter GUS fusion, have been obtained.
The accumulation of *GUS* mRNA in spruce and *Arabidopsis* transformants will be tested using *in situ* hybridization. In parallel, the GUS staining pattern will be assessed in both kinds of plants. These two experiments will provide indications if the protoderm/epiderm gene expression in the HD-GL2 family represents a general mechanism that has been conserved during the evolution of seed plants.

Isolation of the Norway spruce *viviparous1* gene (*Pavp1*) (IV)

Using degenerate primers based on a nucleotidic sequence alignment of the region encoding for the B3 DNA-binding domain of angiosperms *viviparous* (*vp1*)/*aba insensitive3* (*abi3*) genes and RT-PCR, a cDNA clone called *Pavp1* (*Picea abies viviparous1*) was isolated. The predicted PAVP1 protein features the four conserved domains (A1, B1, B2 and B3) identified in angiosperm VP1 proteins (Giraudat et al., 1992; McCarty et al., 1991) and in the gymnopserm yellow cedar CNVP1 protein (Accession number AJ131113) (figure 2, manuscript IV). The B3 domain of PAVP1 shares extensive sequence similarities with the corresponding region of cedar VP1 and angiosperm VP1 proteins. The other domains of PAVP1 show moderate sequence conservation with the angiosperm homologues (figure 2, manuscript IV). In contrast, striking sequence conservation remains in all the domains of PAVP1 and CNVP1 proteins. Low sequence similarity is found in protein regions located outside the domains.
The *Pavp1* gene structure features all the conserved intron positions previously defined in the *vp1* angiosperm genes (figure 2, manuscript IV). A gel blot containing genomic DNA cleaved by two different restriction enzymes, was hybridized with a *Pavp1* fragment corresponding to the B3 domain and washed under stringent conditions. One main genomic restriction fragment was obtained in each lane indicating that *Pavp1* represents a single-copy gene (figure 3, manuscript IV).
The *Pavp1* expression was analyzed by RT-PCR during embryonic and postembryonic development. The expression of *Pavp1* was detected throughout somatic embryo development and in dormant organs (dormant buds, pollen grains) (figure 4, manuscript IV).
Expression of *Pavp1* was rapidly induced when proliferating embryogenic cells were transferred to an ABA-containing medium (figure 6, manuscript IV). *Pavp1* expression peaked at the early cotyledonary stage approximately 14 days after transfer to the maturation medium (figure 7, manuscript IV). Thereafter the expression decreased. In addition, *Pavp1* expression gradually declined as the desiccation process is completed (figure 8, manuscript IV).

Taken together, these data suggest that a single-copy *vp1*-like gene regulated upon maturation, desiccation and quiescence processes was present in the last ancestor of conifers and angiosperms, about 300 million years ago (Stewart and Roth well, 1993).

Future perspectives

The *PaHB* homeobox genes are expressed in very specific tissues during Norway spruce embryo development. The *PaHB1* gene has a protoderm-specific expression and the *PaHB2* gene a cortex-specific expression suggesting that they are involved in radial patterning events. Studies on *Arabidopsis* homologues suggest similar tissue-specificities. The activity of these tissue-specifically-expressed genes would therefore be a characteristic of seed plants.

The conservation of such particular expressions suggests common but not necessarily identical molecular mechanisms regulating embryo formation in angiosperms and gymnosperms.

One pressing question is how their tissue-specificity is orchestrated. The approach undertaken with the *PaHB1* and *AtML1* promoter *GUS* fusions will provide hints.

Another interesting prospect is to understand the function of these genes. At present mutants for *AtML1* and *ANL2* are available. One standard experiment to assess the gene function is to try to complement the mutant defect in the heterologous system by ectopic expression of the spruce gene. However, it does not tell us about the role of the gene in the spruce context.

One alternative would be to generate transgenic spruce embryos that express *PaHB1* and *PaHB2* under the control of the cortex-specific *PaHB2* and protoderm-specific *PaHB1* respectively. Experiments using chimerical constructs have been recently described in *Arabidopsis* and *Antirrhinum* (Efremova et al., 2001).

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