Signal molecules in embryogenesis of Norway spruce

Malgorzata Wiweger
Department of Plant Biology and Forest Genetics
Uppsala

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Abstract

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Signaling molecules regulating embryo development have been described in angiosperms, but very little is known about how embryogenesis is controlled in gymnosperms. In this work we show that lipophilic low molecular weight molecule(s) with GlcNAc residues that are sensitive to chitinase are secreted by embryogenic cultures of Norway spruce. These data indicate that lipo-chitooligosaccharides (LCOs), homologous with rhizobial Nod factors, are present in plants. Interestingly, developmentally blocked lines secrete more LCOs than normally developing lines do. Endogenous LCOs from spruce and rhizobial Nod factors suppress programmed cell death (PCD) and stimulate proliferation of proembryogenic masses and somatic embryo formation but not further embryo development. LCOs are known to be degraded by chitinases. The *Chia4-Pa1* gene, encoding for class IV chitinase, was isolated and characterised. The *Chia4-Pa1* gene belongs to a small family with highly similar members. The expression of *Chia4-Pa* genes increases significantly after withdrawal of plant growth regulators, i.e. during a treatment that triggers PCD and stimulates the switch from proliferation of proembryogenic masses to somatic embryo differentiation. Based on the spatial expression pattern of *Chia4-Pa*, I propose that chitinase-expressing cells have a megagametophyte signaling function. The localisation of the CHIA4-Pa proteins does not correspond to the expression pattern of the encoding genes. I suggest that the CHIA4-Pa proteins are targeted to places where the substrates are localised. Furthermore, chitinases might act on arabinogalactan proteins (AGPs) thereby causing cell wall loosening and cell elongation. In our laboratory, work is being carried out to identify markers specific for different developmental stages of embryo development in Norway spruce. The level of endogenous LCOs secreted to a medium might be used as an indicator of the embrogenic potential of the proliferating cultures, while the increased level *of Chia4-Pa* transcript coincides with massive PCD and differentiation of somatic embryos. In addition, the *PaHB2* gene, a member of the sub-group of the HD-GL2 family with subepiderm- and protoderm/epiderm-specificity, was isolated and characterised.

Keywords: AGP, chitinase, gymnosperms, homeobox, LCO, markers, *Picea abies*, signaling molecule, somatic embryogenesis.

Author's address: Malgorzata Wiweger, Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences, Box 7080, SE-75007 Uppsala, Sweden. E-mail: Malgorzata.Wiweger@vbsg.slu.se.

Appendix

Papers I-IV

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

I. Dyachok J., Wiweger M., Kenne L. and von Arnold S. (2002). Endogenous Nod-Factor-Like Signal Molecules Suppress Cell Death and Promote Early Somatic Embryo Development in Norway spruce. Plant Physiol. 128: 523-533.

II. Wiweger M., Dyachok J., Gohil S., Kenne L. and von Arnold S. The impact of endogenous lipophilic chitooligosaccharides on embryo development in Norway spruce. (manuscript).

III. Wiweger M., Farbos I., Ingouff M., Lagercrantz U. and von Arnold S. Expression of Chia4-Pa chitinase genes during somatic and zygotic embryo development in Norway spruce (*Picea abies*): similarities and differences between gymnosperm and angiosperm class IV chitinases. J. Exp. Bot.54: (*in press*).

IV. Ingouff M., Farbos I., Wiweger M and von Arnold S. (2003). The molecular characterization of *PaHB2*, a homeobox gene of the HD-GL2 family expressed during embryo development in Norway spruce. J. Exp. Bot. 54: 1343-1350.

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Abbreviations

AGP	Arabinogalactan protein
ABA	Abscisic acid
BA	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
GlcN	N-glucosamine
GlcNAc	N-acetylglucosamine
HD-GL2	Homeodomain-Glabra2
IAA	Indole-3-acetic acid
IDGF	Imaginal disks growth factor
LCO	Lipo-chitooligosaccharide
LTP	Lipid transfer protein
Nod factor	Nodulation factor
NPA	Naphthylphthalamic acid
OG	Oligogalacturonide
ORF	Open reading frame
PCD	Programmed cell death
PEM	Proembryogenic mass
PGR	Plant growth regulator
Rt	Retention time
SE	Somatic embryo
TIBA	2,3,5-triiodobenzoic acid
TLC	Thin layer chromatography
UTR	Untranslated region
ZE	Zygotic embryo

Introduction

Introduction to embryogenesis: From zygote to mature embryo

Early stages of embryogenesis

Zygotic embryogenesis starts from the fertilised egg cell. In the majority of angiosperms, the first division of the zygote is asymmetric and gives rise to a small apical cell and a large basal cell. In *Arabidopsis*, three rounds of divisions of the apical cell results in formation of the octant stage which is organised as two tiers that give rise to the major part of the embryo (Jurgens, 1994). The basal cell forms the suspensor and the very basal end of the embryo. The fates of the apical and basal cells are clearly distinct. Deviation in cell fate, that occur in the early stages of development, result in the accumulation of errors and formation of abnormal embryos (Dunn *et al*., 1997).

In most gymnosperms, the nucleus in the zygote divides so that four free nuclei are formed, which become arranged in a tier (Singh, 1978). After several divisions, the proembryo becomes cellularised. In Norway spruce (*Picea abies*) the 16-cell stage is organised in four distinct tiers, of which two tiers constitute embryonal-tiers which give rise to the embryonal mass and to the secondary suspensor, one suspensor-tier that elongates and forms the primary suspensor, and one upper-tier that degenerates.

Attainment of the radial pattern

In angiosperms, already during the early stages of embryogenesis, a radial pattern with three primordial tissues (protoderm, procambium and ground meristem cells) is established. The protoderm is formed as a result of periclinal division of the early globular embryo. Establishment of this tissue is essential for restriction of cell expansion and thereby co-ordination of further embryo development.

The embryonal tiers in gymnosperms continue to divide, creating the embryonal mass and the secondary suspensor. The outer layer of cells in the embryonal mass divides periclinally, but also anticlinally, thereby not permitting the differentiation of the classical protoderm (Singh, 1978). However, the outer cell layer of the embryonal mass in Norway spruce possesses similar functions as the protoderm in *Arabidopsis* (Ingouff *et al*., 2001; Sabala *et al*., 2000).

Establishment of plant axis

One of the major steps during plant embryogenesis is the establishment of the plant axis. First, the apical root meristem is formed. Later, the shoot apical meristem and cotyledon primordia are organised at the distal part of the embryo proper. Once both meristems are delineated, the plant axis becomes established. In *Arabidopsis*, two colyledon primordia are formed at the time of the transition from the globular to the heart-shaped stage. Hence, the establishment of the shoot-root axis coincides with the switch from radial to bilateral symmetry. Norway spruce embryos form a ring with several cotyledon primordia.

The mechanism controlling the switch from radial to (bi)lateral symmetry is not fully known, although polar auxin transport seems be the key player involved in the establishment of the plant axis. This conclusion is based on results from experiments where embryos treated with auxin transport inhibitors either could not proceed beyond the globular stage (Schiavone and Cooke, 1987) or continued development but showed severe abnormalities (Fischer *et al*., 1997; Liu *et al*., 1993). Moreover, treatment of globular embryos with auxin blocked the attainment of bilateral symmetry (Fischer and Neuhaus, 1996). The occurrence of the radial growth phenotype increased with higher amounts of auxin, although the response was also dependent on the developmental stage of the treated embryos (Fischer and Neuhaus, 1996). Fischer and Neuhaus (1996) suggested that during early embryogenesis in monocots, auxin is being synthesised in an area located in the lower part of the embryo proper, near the suspensor. Thereupon, auxin is transported polarly along the longitudinal axis towards the area where the scutellum will differentiate and laterally towards the area where the promeristem will be initiated (Fischer and Neuhaus, 1996). Fischer and Neuhaus (1996) also proposed that non-homogeneous distribution of auxin within the embryo proper at the globular and early transition stage or 'auxin gradients' might play a major role in the embryonic polarity.

The suspensor is the first differentiated structure produced during plant embryogenesis (Schwartz *et al*., 1994). Previously, the suspensor was thought to play a passive role in embryo development by holding the embryo proper in a fixed position within the seed. However, it is now clear that the suspensor also plays an active role during early development by promoting continued growth of the embryo proper (Yeung and Meinke, 1993). For example, Friml and coauthors (2002) suggested that auxin homeostasis may be regulated via the suspensor cells. Normal development of the suspensor during early embryo development is followed by programmed cell death (PCD) prior to seed maturation (Cansonni *et al*., 2003; Filonova *et al*., 2002; Giuliani *et al*., 2002). Several lines of evidence indicate that, in higher plants, the embryo proper restricts further growth of the suspensor (Schwartz *et al*., 1994). Abnormal suspensors not only proliferate, when released from control by the embryo proper, but also acquire characteristics normally restricted to cells of the embryo proper. The abnormal suspensor phenotype illustrates the importance of establishing normal communication between the embryo proper and the suspensor during early embryogenesis (Schwartz *et al*., 1994; Yadegari *et al*., 1994).

Maturation and germination

When most of the morphogenic changes are completed, embryogenesis ends and the developmental programme switches from pattern formation to accumulation of storage products. Vastly increased rates of synthesis and deposition of storage proteins, lipids and starch result in cell expansion. Reserves are accumulated in the megagametophyte (gymnosperms) or in the endosperm or cotyledons (angiosperms) (Dodeman *et al*., 1997). In both gymnosperms and angiosperms, seeds are designed to supply the embryo with nutrients and signaling molecules, as well as to protect the embryo from different stresses and premature germination.

However, the gymnosperm embryos are surrounded by the megagametophyte (haploid maternal tissue), while in angiosperm embryos are surrounded by the endosperm (triploid tissues arising as a result of double fertilisation).
The mature seeds are classified as orthodox or recalcitrant (Engelmann, 1991). The embryos of orthodox seeds undergo maturation drying while recalcitrant seeds do not and are generally desiccation intolerant. The majority of angiosperm and gymnosperm seeds are of the orthodox type. At the end of the maturation phase, seeds of the orthodox type enter dormancy, including that physiological processes stop and the water content rapidly decreases (Goldberg *et al*., 1989).

Somatic embryogenesis

Plant regeneration via somatic embryogenesis includes four major steps: (i) initation of embryogenic cultures from a primary explant, (ii) proliferation of embryogenic cultures, (iii) maturation of somatic embryos and (iv) regeneration of plants. In contrast to zygotic embryogenesis, somatic embryogenesis is a non-sexual propagation process where somatic cells differentiate somatic embryos. Somatic embryos develop in a similar way as zygotic embryos. Therefore, somatic embryos can be used for studying the regulation of embryo development. One advantage with somatic embryos is that the developmental process can be controlled and synchronised so that sufficient quantities of developmentally homogeneous tissue can be collected at specific stages. However, the greatest interest of somatic embryos is based on its practical application for large scale vegetative propagation. Embryogenic cultures are also an attractive target for gene transformation.

Carrot and Norway spruce are two model plants commonly used for studies of developmental pathways of somatic embryogenesis and the molecular mechanisms underlying somatic embryo development in angiosperms and gymnosperms respectively.

Somatic embryogenesis in carrot

In vitro cell-cultures of carrot are usually initiated from hypocotyls (Zimmerman, 1993), but any other explants can be induced to proliferate and form embryogenic callus (Toonen and de Vries, 1996). High auxin concentration stimulates proliferation and induces embryogenesis. However, only 1-2% cells become embryogenic (de Vries *et al*., 1988). In the presence of auxin, proliferating embryonic cells that are small and cytoplasm-rich tend to aggregate and form proembryogenic masses (PEMs). These PEMs probably correspond to the pre-globular stage of zygotic embryos (Emons, 1994). The embryogenic cultures consist of single cells, PEMs and loosely aggregated single cells (Emons, 1994). Somatic embryos develop from different kind of single cells with different frequencies. The first division can be either symmetric or asymmetric (Toonen and de Vries, 1996). Withdrawal of auxin from the medium triggers further embryo development.

Somatic embryogenesis in Norway spruce

Embryogenic cell-cultures of Norway spruce are established from zygotic embryos. In the presence of auxin and cytokinin (PGRs) proembryogenic masses (PEMs) proliferate, passing through three different stages (PEMI-III) distinguished by cellular organisation and cell number (Filonova *et al*., 2000b). In contrast to the carrot system, the first divisions leading to formation of the proembryo stage has not been described in embryogenic cell-cultures of Norway spruce. However, later stages, corresponding to early and late embryogeny, were identified and characterised (Filonova *et al*., 2000b).

Withdrawal of PGRs stimulates transition from PEM proliferation to somatic embryo formation. The transition from PEM to somatic embryo is a key developmental switch that determines the yield and quality of mature somatic embryos in Norway spruce (Bozhkov *et al*., 2002). Pre-treatment in PGR-free medium synchronises development of the cultures. Hence, at the time when maturation treatment is given, the somatic embryos are at a developmental stage corresponding to early embryogeny. The embryonal masses are connected with suspensors via tube cells. Therefore the term "suspensor" was used to denote the structure that is formed during early embryogeny, but according to classic embryogeny it is the "secondary suspensor". At the end of early embryogeny, the outer layer of the embryonal mass becomes smooth and starts to resemble the protoderm. However, some genotypes deviate from normal embryo pattern formation, exhibiting developmental arrest at certain stages (Egertsdotter and von Arnold, 1995; Filonova *et al*., 2000b). Arrested cell lines are classified as B-type, while those that are not arrested are classified as A-type. The end of early embryogeny is the first stage at which development of the somatic embryo starts to resemble the basal plan of zygotic embryogeny in Pinaceae (Singh, 1978).

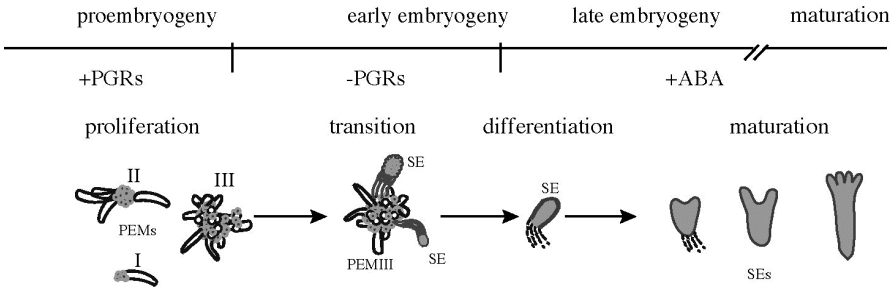


Figure 1. Schematic overview on the developmental pathway of somatic embryogenesis in Norway spruce (adapted from Filonova *et al*., 2000b). Proliferation of embryogenic cultures of Norway spruce is maintained in the presence of PGRs (auxin and cytokinin). Transition from PEMIII-to-SE is stimulated by pre-treatment for one week in PGR-free medium. Further development and maturation of SEs requires the presence of ABA.

Once embryo pattern formation is completed, both types of embryo are ready to mature (Filonova *et al*., 2000b; von Arnold and Hakman, 1988). However, maturation of somatic embryos has to be stimulated by treatment with abscisic

acid (ABA). Only A-type cell-lines and not B-type respond to ABA-treatment by developing into normal mature embryos which, after partial desiccation, germinate and develop into somatic embryo plants. For schematic overview on somatic embryogenesis of Norway spruce see figure 1.

Markers for different stages of embryo development

Different stages of embryo development are a consequence of a series of morphological changes, e.g. division into the apical and basal cells, cell positioning, switch from radial to lateral symmetry, differentiation of tissues. This requires the correct timing of cell division, cell-fate commitment and differentiation. Therefore each of those events is under strict control. Microarrays are used for studying overall changes in gene expression during different processes. By using microarray analysis it was shown that 495 genes out of 9280 were differentially expressed during somatic embryogenesis in soybean (Thibaud-Nissen *et al*., 2003) and 35 genes out of 373 were differentially expressed specifically during normal somatic embryogenesis in Norway spruce (van Zyl *et al*., 2003). Microarrray analysis of differentially expressed genes is often followed by detailed studies of single genes. In our laboratory, work is being carried out to identify markers specific for different developmental stages of embryo development in Norway spruce. By using well-controlled model systems, based on somatic embryos, we shall in the future gain insight into the regulation of embryo development. Here I describe a few examples of markers, well known for angiosperms embryogenesis, that are also common to embryogenesis in Norway spruce, a gymnosperm.

Radial pattern formation

The elucidation of the mechanism of pattern formation and the subsequent determination of cell fate is one of the important objectives of developmental biology (Ito *et al*., 2002). The radial pattern is characterised by a concentric tissue layer arrangement consisting of protoderm (epiderm), ground and conductive tissues. Protoderm differentiation is considered to be the earliest event of radial pattern formation in plant embryogenesis (West and Harada, 1993).

Lipid transfer proteins (LTPs) have been isolated from animals, fungi, plants and bacteria and are characterized by their ability to catalyse the exchange of lipid molecules between membranes (Kader, 1996). The expression pattern of genes encoding LTPs are complex and, in many cases temporally and spatially controlled. However, it was shown that for normal embryo development in angiosperms, expression of the *ltp* genes must be restricted to the protoderm cells (Sabala *et al*., 2000 and references therein). Therefore, the *ltp* genes could serve as a marker for radial pattern formation and/or the globular stage. In gymnosperms, the outer cell layer of embryo is less delineated. However, Sabala and others (2000) have shown that *Pa18*, a putative *ltp* gene, is expressed during embryogenesis in Norway spruce in a tissue-specific manner. Moreover, the expression of *Pa18* must be restricted to the outer cell layer, suggesting that the outer cell layer in gymnosperms functions as protoderm in angiosperms.

Homeobox genes are universal transcription-regulating factors carried by all eukaryotes (Gehring *et al*., 1994; Ingouff *et al*., 2001 and references therein). In animals, homeobox genes are involved in the determination of cell fate and specification of the body plan (Gehring *et al*., 1994). In plants, the tissue-specific expression of the homeobox genes belonging to the HD-GL2 family and mutant analysis suggests that the HD-GL2 is involved in the regulation of epidermal and subepidermal cell fate (Ingouff *et al*., 2001; Ito *et al*., 2002 and references therein). The *PaHB1* gene from Norway spruce is homologous to genes from the HD-GL2 family that are expressed during embryogenesis. The expression of *PaHB1* switches from a ubiquitous expression in proembryogenic masses to an outer cell layer-specific localisation during somatic embryo development (Ingouff *et al*., 2001). Overexpression of *PaHB1* gene results in disturbance of the smooth surface of the embryonal mass leading to developmental blockage.

Programmed cell death

Programmed cell death (PCD), in which a cell guides its own destruction, is a part of the quality control mechanism. PCD controls the number and type of cells at certain location (Consonni *et al*., 2003; Filonova *et al*., 2002; He and Kermode, 2003; Wan *et al*., 2002) and the number of embryos in polyembryonic seeds (Filonova *et al*., 2002). During embryogenesis, the suspensor, but not the embryonal mass, undergoes DNA fragmentation (Filonova *et al*., 2000a; Filonova *et al*., 2002; Giuliani *et al*., 2002). However, in the case of polyembryonic seeds of pine, all but one embryo are eliminated by gradual PCD, starting in the most basally situated cells within the embryonal mass and proceeding towards the apical region of the subordinate embryos (Filonova *et al*., 2002). During somatic embryogenesis in Norway spruce, the A-type of cell line that develops into normal somatic embryo plants, has a 7-20-fold higher level of PCD than a B-line (Smertenko *et al*., 2003). Considering that PCD regulates proper embryogenesis, it is not surprising that embryo development is blocked in B-lines.

The $p34^{cdc2}$ protein kinase genes are involved in cell cycle progression and apoptosis (Flower *et al*., 1998; Shi *et al*., 1994). The *cdc2Pa* gene encodes a Norway spruce $p34^{cdc2}$ protein kinase homologue (Footitt *et al*., 2003). The expression of *cdc2Pa* during somatic embryogenesis of Norway spruce increased during pre-maturation treatment in PGR-free medium, so it correlates with the PEM-to-SE switch and increased PCD. The second peak of expression occurs during early embryo maturation coinciding with the second wave of PCD that eliminates embryo suspensors. The third peak in expression of *cdc2Pa* occurs during germination.

Cytoskeleton reorganisation

The reorganisation of the cytoskeleton, both microtubules and F-actin, is important for embryogenesis. The microtubule arrays appear normal in the embryonal mass cells, but the microtubule network is partially disorganised in the embryonal tube cells and the microtubules disrupted in the suspensor cells. MAP-65, a microtubule-associated protein, binds only to organised microtubules. However, in developmentally arrested lines, MAP-65 does not bind the cortical

microtubules. In embryos, the organisation of F-actin gradually changes from a fine network in the embryonal mass cells to thick cables in the suspensor cells. Depolymerisation of the F-actin abolishes the normal embronic pattern formation and associated PCD in the suspensor, strongly suggesting that the actin network is vital in this PCD pathway (Smertenko *et al*., 2003).

pH

One possible marker of differentiation and cell activation is the cellular pH. A modification in cytoplasmic pH was found to be required for the control of the cell cycle, cell division and growth (Pasternak *et al*., 2002). The pH values in the vacuoles as well as in the chloroplasts may serve as an indicator of the cell type (embryogenic or non-embryogenic) (Pasternak *et al*., 2002). Increased cytoplasmic pH correlates with cell division, although it is not known whether cytoplasmic alkalisation serves as a mitotic signal or is a consequence of cell activation (Pasternak *et al*., 2002). Auxin increases proton export, resulting in reduction of pH. Low cellular pH is proposed to be involved in the cell wall-loosening process required for directed cell elongation during embryo development (Rober-Kleber *et al*., 2003).

Buffering of the pH in a medium abolishes establishment of the cellular pH gradient (Pasternak *et al*., 2002). This results in the formation of elongated and vacuolated cells instead of the proliferation of small cells with dense cytoplasm. Changes in cell division and cell morphology are correlated with different timing of accumulation of endogenous IAA (Pasternak *et al*., 2002). The pH of the medium was also found to influence somatic embryo induction and development in plants (Pasternak *et al*., 2002). In carrot somatic embryogenesis, low pH (4-4,5) in PGR-free medium can substitute for 2,4-D in its ability to sustain multiplication of embryogenic cells without permitting development into later embryo stages (Smith and Krikorian, 1990). However, pH 4,5 and higher was needed to allow further embryo development (Smith and Krikorian, 1990). PEM-to-SE transition in cell-cultures of Norway spruce is associated with a drop in the pH of the medium and increased PCD (Bozhkov *et al*., 2002). However, cultures maintained in a medium with buffered pH (either high or low pH, pH 5.8 and 4.5 respectively), are suppressed in SE differentiation (Bozhkov *et al*., 2002).

Viviparous

The *Viviparous* genes from the VP1/ABI3 gene family control the expression of embryo maturation genes, the acquisition of desiccation tolerance and dormancy (Wobus and Weber, 1999). *Pavp1* is the Norway spruce homologue of the angiosperm *Viviparous 1* genes (Footitt *et al*., 2003). The expression of *Pavp1* increases after only 1 hour of maturation treatment, and peaks after 3 weeks of maturation.

Signal molecules regulating embryogenesis

Endosperm and megagametophyte are known to function as source of nutrients, but is that all? *In vitro* cell cultures, which are supplemented with nutrients, still

require the presence of plant growth regulators or "conditioning factors". Recently it has been hypothesised that the endosperm has functions critical for embryo development (Berger, 1999). The endosperm and the embryo probably interact during their development, although failure of endosperm development usually results in embryo abortion (Kinoshita *et al*., 1999; Kiyosue *et al*., 1999). The interplay of several signaling pathways co-ordinates and regulates the proliferation, elongation and differentiation resulting in embryo body formation. Filonova and coworkers (2002) suggested that the female gametophyte signals PCD in subordinate embryos in a pine seed.
Several molecules have been shown to signal embryo development, only a few of which are presented below.

Plant growth regulators

Plant growth regulators (PGRs) play an important role as signals and regulators of growth and development in plants. Auxin is produced in pollen and in developing seeds (both in the endosperm and embryo). It was suggested that the endosperm supplies auxin during the first stage of fruit growth, and the developing embryo is a main auxin producer during the latter stages (Fischer and Neuhaus, 1996). Auxin provides positional information for co-ordination of correct cellular patterning from the globular stage onwards (Fischer and Neuhaus, 1996). An endogenous auxin pulse is one of the first signals leading to the induction of somatic embryogenesis. (Thomas *et al*., 2002). Endogenous levels of PGRs vary over the course of embryo development with the highest level of free IAA at the globular stage (Ribnicky *et al*., 1996). The same variation in the level of IAA occurs during zygotic and somatic embryo development (Ribnicky *et al*., 2002). Moreover, a transient increase in cellular IAA concentration was observed under both embryogenic and non-embryogenic conditions, although the timing of the IAA accumulation differs between embryogenic and non-embryogenic cultures.
Two distinct auxin transport streams have been described: polar transport (unique for auxin) and a non-polar transport system via the phloem. Polar transport of the auxin controls many aspects of plant growth and development. Polar auxin transport is essential for the establishment of bilateral symmetry during early plant embryogenesis (Liu *et al*., 1993) and for lateral root formation (Sussex *et al*., 1995) and for root nodule formation (Mathesius *et al*., 1998). A number of synthetic compounds e.g. 2,3,5-triiodobenzoic acid (TIBA) and naphthyl-phthalamic acid (NPA) were successfully used as inhibitors of the auxin efflux carrier complex. It was suggested that endogenous auxin transport inhibitors act in a similar way as the synthetic compounds (Liu *et al*., 1993). Flavonoids and chitin oligosaccharides were suggested as possible candidates for endogenous regulators of auxin transport (Mathesius, 2001; Mathesius *et al*., 1998).
Auxin regulates cell divisions, differentiation and elongation. A mechanism of action was suggested in which auxin acts at the plasma membrane or within the cell. In response, auxin increase the H^+-ATPase expression level required to augment the capacity of the membranes for proton export, resulting in a lowered pH (Rober-Kleber *et al*., 2003). Thereupon, lower pH activates cell-wall-loosening enzymes that promote the breakage of key cell wall bonds, increasing wall extensibility. In addition, auxin is known to increase activity of enzymes involved

in wall polysaccharide synthesis (Vissenberg *et al*., 2001). It was suggested that calcium and ABA suppress auxin response and down-regulate the putative auxin influx and efflux carriers (Swarup *et al*., 2002; Vissenberg *et al*., 2001).

Cytokinins are plant growth regulators that together with auxin induce cell divisions in plant cells. When applied alone, cytokinins act antagonistically to auxins and determine cell fate by promoting organogenesis (Frank and Schmulling., 1999). High concentrations of cytokinins block cell proliferation and induce PCD, although cytokinin-induced PCD can be abolished by auxin. Interestingly, cell cultures of different ages had different sensitivities to the cytokinin treatment (Carimi *et al*., 2003). Several cytokinin mutants were described in *Arabidopsis*. The *pas1* mutant, which has an increased cytokinin signaling, shows a strong phenotype including ectopic cell proliferation in the cotyledons, extra cell layers in the hypocotyl and an abnormal apical meristem (Vittorioso *et al*., 1998). The *ckr* mutant, which is resistant to cytokinin, has longer roots than the wild type but shorter root hairs, suggesting that endogenous cytokinin acts to inhibit root growth and stimulate root hair elongation (Estelle and Klee, 1994). Furthermore, pulse treatment with a high concentration of cytokinin stimulates adventitious bud formation (von Arnold and Eriksson, 1985 and references therein). Similarly, when globular embryos were grown on medium containing a synthetic auxin polar transport inhibitor (NPA) multiple shoot and root meristems were formed, and the abnormal development was restricted to the embryo proper and did not affect the suspensor (Fischer *et al*., 1997).
The level of active cytokinin can be reduced through oxidative breakdown (by cytokinin oxidase) or by glucosylation (Carimi *et al*., 2003). Moreover, cytokinins have been implicated in anthocyanin production.

Flavonoids

Flavonoids are a large group of secondary metabolites categorised as phenolics or polyphenols. They are widely distributed in the plant kingdom and prokaryotes. In higher plants flavonoids are involved in numerous functions such as: UV filtration, symbiotic nitrogen fixation, floral pigmentation, pollen germination, cell division and differentiation (for review see Buslig and Manthey, 2002). It was suggested that flavonoids affect gene expression or protein activity (Woo *et al*., 2002) and polar transport of auxin (Brown *et al*., 2001; Jacobs and Rubery, 1988; Rubery and Jacobs, 1990). The composition of flavonoids varies in different organs (Woo *et al*., 2002). Some flavonoids are known to be involved in the degradation of IAA, but there are also some which inhibit degradation of IAA (Mathesius, 2001). Flavonoids were shown to be involved in the metabolism of IAA (for review see Buslig and Manthey, 2002). Furthermore, endogenous flavonoids regulate auxin efflux from cells during polar transport from the shoot tip to the root tip. However, to regulate auxin transport through the plasma membrane, flavonoids must be localised on the plasma membrane (Murphy *et al*., 2000). The co-localisation of flavonoids and aminopeptidases (enzymes that hydrolyse NPA) in the zone below the cotyledonary node, the hypocotyl-root transition zone and the root elongation zone suggests that these regions may be sites where auxin efflux is regulated (Murphy *et al*., 2000). Certain flavonoids e.g.

quercetin have been found to compete with NPA for binding sites and also block polar auxin efflux and stimulate local auxin accumulation (Murphy *et al*., 2000). Embryos treated with quercetin or NPA develop multiple meristem and multiple organ phenotypes, although the occurrence of specific abnormal phenotypes depended on the concentration of NPA or quercetin added as well as on the developmental stage of the isolated embryo (Fischer *et al*., 1997). The early-to-late globular embryos were most sensitive to auxin transport inhibitors. Less sensitive were the embryos at the globular-to-early transition stages. However, in both cases, abnormal development was restricted to the embryo proper and did not affect suspensors. When NPA or flavonoid (quercetin) treatment was given to the isolated bilateral embryos the majority of them underwent normal *in vitro* development (Fischer *et al*., 1997). Interestingly, globular embryos treated with TIBA, an auxin transport inhibitor that does not belong to phytotropins represented by NPA and flavonoids, did not differentiate into polyembryos but generated an abnormal overall embryonic symmetry (Fischer *et al*., 1997). TIBA and NPA have different binding sites on the auxin efflux carrier while flavonoids compete for the same receptors as NPA (Jacobs and Rubery, 1988; Murphy *et al*., 2000; Rubery and Jacobs, 1990; Thomson *et al*., 1973).

Flavonoid aglycones were suggested as endogenous signal molecules that mediate the effect of lipo-chitooligosacchrides (LCOs) on auxin transport (Mathesius *et al*., 1998).

Sugars

Recent studies indicate that sugars have a dual function as a nutrient and as signaling molecules that control gene expression and developmental processes in plants in a similar manner as classical PGRs (Etzler, 1998; Geurts and Bisseling, 2002; Rolland *et al*., 2002; Sheen *et al*., 1999, Baldan *et al*., 2003). Sugars probably act as morphogens, providing positional information to the cell cycle machinery and different developmental programs (for a review see Rolland *et al*., 2002). Hexose signaling was suggested to control the balance between the auxin and cytokinins. Transgenic plants lacking hexose signaling showed a strong phenotype: malformed embryos and multi-apical shoot meristems. Sucrose can have the same effect as hexose, although in many cases sucrose is not the direct signaling molecule (for review see Sheen *et al*., 1999). Oligoglucosides, oligogalacturonides, xyloglucans, oligogalacturonides, chitin oligosaccharides, chitosan oligosaccharides and nodulation factors (Nod factors) are known as oligosaccharins, biologically active oligosaccharides (Etzler, 1998; Spiro *et al*., 1998). They stimulate plant defence responses, and influence plant growth and development (for review see Etzler, 1998; Spiro *et al*., 1998). Oligosaccharins are either synthesised *de novo* (Nod factors) or they are released from the cell walls as a product of enzymatic degradation (oligogalacturonides, OGs). Irrespective of the origin of these molecules the degree of polymerisation (>10 for galactosyluronic acid, 9-18 for OGs, >4 for oligochitin, >7 for oligochitosan) influences biological activity. Although modifications at the reducing end also play an important role in signaling (Spiro *et al*., 1998). Plant cells respond to nanomolar concentrations of added chitooligosaccharides by rapid alkalisation of the culture medium (Baier *et*

al., 1999; Bakkers *et al*., 1997; Staehelin *et al*., 1994a) and protein phosphorylation (Felix *et al*., 1991).

Nod factors are produced by bacteria belonging to the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* in response to plant flavonoids (for review see Spaink, 1996). Different rhizobia produce different sets of Nod factors with specific modifications, and these appear to determine host specificity (Staehelin *et al*., 1994b). However, Nod factors uniformly consist of an oligosaccharide backbone of β-1,4-linked N-acetylglucosamine (GlcNAc) tri-, tetra- or pentasaccharide, with an N-linked fatty acid moiety replacing the N-acetyl group on the nonreducing end. The length of the oligosaccharide chain, the acetylation at the nonreducing end and the sulfatation at the reducing end of the LCO, influence the stability of the molecule against degradation by chitinases (Staehelin *et al*., 1994b). Nod factors are known to induce cell divisions in the root cortex of the host legume, leading to formation of nodules (Schultze and Kondorosi, 1996; Spaink, 1996). Rhizobial LCOs and chitin oligosaccharides stimulate the earliest stages of nodulation probably by perturbing the auxin flow in the root, and this auxin transport inhibition is probably mediated by endogenous flavonoids (Mathesius *et al*., 1998). A number of studies have shown that Nod factors influence the embryo development of non-leguminous plants (De Jong *et al*., 1993; Dyachok *et al*., 2000; Egertsdotter and von Arnold, 1998).

Recently, Baldan and coworkers (2003) described the OG-induced changes in the developmental pattern of somatic embryos in carrot. The response to OGs was strictly dependent on the developmental stage of the treated embryos (Baldan *et al*., 2003). Treatment of embryos at the globular stage resulted in the inhibition of the elongation of the axis and the formation of a multiple shoot apex. This is another example where treatment of embryos at early stages of development results in severe abnormalities during later development.

Peptides

Peptide signaling molecules (<100 amino acid residues) occur widely in animals. In contrast, only a few signaling peptides have been identified in plants. In both kingdoms, signaling peptides regulate a broad range of physiological processes. During the last decade, plant peptides were recognised as critical factors in defence signaling (systemins and cyclotides), self incompatibility (S-locus cysteine-rich protein, SCR), cell division (early nodulin, ENOD40), cell proliferation (rapid alkalisation factor, RALF; phytosulfokinase, PSK; CLAVATA, CLV3), meristem organisation (PSK, CLV3) and root nodulation (PSK) (for reviews see Franssen and Bisseling, 2001; Jennings *et al*., 2001; Ryan and Pearce, 2001; Ryan *et al*., 2002). It was suggested that CLV3 is a ligand for receptor kinase (CLV1). CLV3 together with CLV1 regulate cell proliferation and differentiation in the apical meristem region (Ryan *et al*., 2002 and references therein) Another signaling peptide, the PKS, induces cell proliferation although it requires the presence of auxin or cytokinin (Ryan and Pearce, 2001; Ryan *et al*., 2002 and references therein). PKS were also found to promote organogenesis in roots, buds and embryos. Peptides encoded by the early nodulation gene, *ENOD40*, induce cortical

cell divisions in nodulating roots, but no biological activity has been directly associated with the polypeptide *in vivo* (Ryan *et al*., 2002 and references therein)

Chitinases

Chitinases are enzymes that hydrolyse β-1,4-N-acetyl-D-glucosamine (GlcNAc) linkages. Those with lysozyme activity also cleave β-1,4 linkages between GlcNAc and N-acetylmuramic acid. Apart from chitin, the main substrate, which is not present in plants, chitinases can hydrolyse arabinogalactan protein (AGPs) (van Hengel *et al*., 2001), Rhizobial Nod factors (Staehelin *et al*., 1994a; Staehelin *et al*., 1994b) and other LCOs (Brunner *et al*., 1998). According to their primary structure, chitinases are divided into seven classes (class I-VII) (Collinge *et al*., 1993; Gomez *et al*., 2002; Neuhaus *et al*., 1996) and two families (18 and 19) of glycohydrolases (E.C.3.2.1.14). The primary structure of plant chitinases is characterised by the presence of a signal peptide at the N-terminus and catalytic domain at the C-terminal end. In addition, class I, IV, VI chitinases have a cysteine-reach domain that is believed to be a chitin-binding domain followed by a variable hinge region. Class I, II, IV, VI and VII of plant chitinases share a high amino acid sequence identity within their catalytic domain. Class IV and VII resemble class I and II respectively, but they are significantly smaller owing to some deletions. Class VI chitinase has high similarity to class I but it has a significantly longer proline-rich hinge region. The catalytic domain of plant class III chitinase shows no sequence similarity to enzymes in class I, II, IV and VII. Class V chitinases show over 50% amino acid identity with lectin precursor. Family 18 contains chitinases from bacteria, fungi, viruses and animals and some class III and V chitinases from plants (Watanabe *et al*., 1999). Family 19 contains plant class I, II and IV chitinases and chitinases C from *S.griseus* (Watanabe *et al*., 1999). Chitinases within one family share similar three-dimensional structure and the same mechanism of the hydrolytic action (Iseli *et al*., 1996). However, family 18 chitinases have an (α/β) eight-barrel fold and hydrolyse the glycosidic bond with retention of the anomeric configuration, whereas family 19 chitinasese have a different protein structure with an α-helical fold and hydrolyse with inversion. Moreover, family 18 chitinases are sensitive to inhibition by allosamidin, unlike the family 19 chitinases.

Plant chitinases and lysozymes are likely to have arisen from one coancestor by divergent evolution (Monzingo *et al*., 1996). The protein genealogy of chitinases shows that classes I and class II chitinase genes evolved from the same ancestral gene (Araki and Torikata, 1995; Shinshi *et al*., 1990) Moreover, a basic class II chitinase is a putative ancestor of basic class I and acidic class II chitinase genes (Ohme-Takagi *et al*., 1998). It was also proposed that genes of low molecular weight class VII (formerly class II-L) and class IV chitinases evolved from the high molecular weight genes (class I and II chitinases) by four deletions in the coding sequence (Araki and Torikata, 1995; Gomez *et al*., 2002; Hamel *et al*., 1997). Hamel and collaborators (1997) suggested that the derivation of the class IV lineage from a common ancestral sequence would have occurred before the separation of monocots and dicots, estimated to take place around 200 million years ago. The class III proteins appeared to be derived from an ancestral sequence

different from that of classes I, II and IV plant chitinases, but similar to a type of chitinase found in yeast cells. Therefore, the origin of class III chitinases preceded the divergence between fungi and plants (Hamel *et al*., 1997).

Chitinases exist as multiple structural isoforms that differ in their size, isoelectric point, primary structure, cellular localisation and pattern of regulation (Petruzzelli *et al*., 1999). One single plant produces several different chitinase isoforms. For example, in *Arabidopsis*, there are twenty chitinase genes: nine class IV chitinases, nine class V chitinases and single members representing classes I and III, but not all of them code for functional proteins (Passarinho and de Vries, 2002). Chitinases can inhibit the fungal or bacterial growth by causing dissolution of their cell walls, but are also capable of releasing chitin oligomers, which elicit the series of defence reactions (Kurosaki *et al*., 1988; Nishizawa *et al*., 1999). However, chitinases without enzymatic activity might still show antimicrobial activity (Van Damme *et al*., 1999). Expression of chitinase genes can also be influenced by different stresses (Chlan and Bourgeois, 2001; Margis-Pinheiro *et al*., 1994; Petruzzelli *et al*., 1999; Pittock *et al*., 1997; Regalado *et al*., 2000) or plant growth regulators (Shinshi *et al*., 1987). There are several reports of developmentally regulated chitinase expression (Passarinho and de Vries, 2002 and references therein). It was shown that chitinase can stimulate embryo- (De Jong *et al*., 1992; Egertsdotter and von Arnold, 1998, Baldan *et al*., 1997; Kragh *et al*., 1996) and fruit development (Petruzzelli *et al*., 1999; Swegle *et al*., 1992; Van Damme *et al*., 1999; Yeboah *et al*., 1998).

Arabinogalactan proteins

Arabinogalactan proteins (AGPs) are a family of glycosylated hydroxyproline-rich glycoproteins analogous to animal proteoglycans (Showalter, 2001). AGPs are widely distributed in the plant kingdom, mainly attached to the plasma membrane or in cell walls. However, AGPs are also present in plant secretions. AGPs are implicated in three fundamental cellular processes: cell proliferation, cell expansion and cell differentiation (Steele-King *et al*., 2000). Furthermore, various AGPs play an important role in plant embryogenesis (Chapman *et al*., 2000; Egertsdotter and von Arnold, 1995; Kreuger and van Holst, 1995; Steele-King *et al*., 2000; Toonen *et al*., 1997; van Hengel *et al*., 2001; van Hengel *et al*., 2002). The presence of AGPs stimulating somatic embryogenesis and sensitive to chitinase treatment was reported in carrot (van Hengel *et al*., 2002) and Carribean pine (Domon *et al*., 2000). Interestingly, it was shown that when AGPs are hydrolysed by chitinases, LCO-like molecules are released (van Hengel *et al*., 2001). In addition, van Hengel et al. (2001) presented evidence that AGP side chains with intact arabinogalactan carbohydrate moieties are essential for the effect on somatic embryogenesis, whereas hydrolytic activation with endochitinases appears essential for full embryo-forming activity of the AGPs.

The protein core of AGPs is decorated by arabinose and galactose-rich polysaccharide units. Different carbohydrate epitopes on AGPs are used to design antibodies that can recognise certain AGP-epitopes. AGPs are known to interact with the Yariv reagent. The majority of reports on a role of APGs for plant growth

and development are based on their spatial or temporal pattern of expression and/or modification. AGPs can be used as markers of cellular identity or cell fate (Showalter, 2001; Stacey *et al*., 1995). Some AGPs, e.g. the JIM8 epitope, can be localised in gametes, anthers, ovules, and in the early embryos. Other AGPs, such as the MAC207 epitope, are absent in cells involved in sexual reproduction as well as in early zygotic embryos but reappear after the embryos have reached the heart stage (Toonen and de Vries, 1996).

Results and discussion

Endogenous lipo-chitooligosaccharides in Norway spruce (paper I, II and unpublished)

Nod factors are a group of LCOs, secreted by rhizobia prior to nodule formation in a host-plant. When externally applied in nanomolar concentrations, Nod factors provoke different responses, e.g. reduction of auxin transport capacity, changes in the microtubular cytoskeleton, and cortical cell division in host and non-host plants (Boot *et al*., 1999; Mathesius *et al*., 1998; Timmers *et al*., 1998). Nod factors can also promote somatic embryogenesis in plants (De Jong *et al*., 1993; Dyachok *et al*., 2000; Egertsdotter and von Arnold, 1998 and paper I). In *Rhizobium*, the backbone of Nod factors is synthesised by three enzymes, *NodA*, *NodB* and *NodC*. Genes homologous to *NodC* are present in animals (Semino and Robbins, 1995; Semino *et al*., 1996). Interestingly, Nod-like chitin oligosaccharides are synthesised by embryos of zebrafish, carp and *Xenopus* (Bakkers *et al*., 1997; Semino *et al*., 1996) and they are biologically active when applied to cultures of *Cataranthus roseus* (Bakkers *et al*., 1997).

Based on the previous results (Dyachok *et al*., 2000) it was shown that rhizobial Nod factors stimulate PEM proliferation in embryogenic cultures of Norway spruce. In this work the question was addressed whether LCOs homologous with Nod factors are present in embryogenic cultures of Norway spruce and if so, how do they influence embryo development?

Purification, detection and quantification of LCOs

Nod factors are LCOs with a basic structure consisting of β-1,4-linked N-acetylglucosamine (GlcNAc) tri-, tetra- or pentasaccharide, with an N-linked fatty acid moiety replacing the N-acetyl group on the non-reducing end. We screened Norway spruce cultures for β-1,4-GlcNAc-lipophilic compounds by using a similar approach to that used previously to isolate rhizobial Nod factors (Spaink *et al*., 1991; Truchet *et al*., 1991). Lipophilic compounds present in a conditioned medium were purified on a reverse-phase cartridge (C18, Chromabond), eluted with methanol and screened for the presence of GlcNAc molecules. GlcNAc-containing compounds are commonly detected by the Morgan-Elson assay. Nanomolar concentration of Morgan-Elson positive compounds were detected in the lipophilic extracts from embryogenic cultures. In addition, higher levels of

GlcNAc were detected in B-type cultures, which are unable to form mature embryos, while A-type cultures, with more advanced development than B-type, had lower levels of GlcNAc. Interestingly, no GlcNAc was detected in the extracts from non-embryogenic cultures.

Embryogenic cultures were labelled with sodium [1-^{14}C]acetate or N-acetyl-D-[1-^{14}C]glucosamine. Lipophilic compounds present in the conditioned medium were extracted on Chromabond C18 cartridge and eluted with different concentrations of methanol in water. The highest radioactivity was found in fractions eluted with 80% methanol. Therefore, the 80% methanol fraction was used for further analysis. The 80% methanol fraction, radiolabelled with sodium [1-^{14}C]acetate, was separated by thin layer chromatography (TLC) on Silica Gel 60 thin-layer plates. Three distinct bands were detected in all samples. The results did not reveal any obvious differences in the composition of the 80% methanol fraction from A and B-lines. However, TLC analysis may not be sensitive enough to detect small changes in amount or type of Morgan-Elson-positive lipophilic compounds. For further analysis of the biological activity, lipophilic compounds were extracted from cell-line B1, which has a high content of GlcNAc. The 80% methanol fraction radiolabelled with N-acetyl-D-[1-^{14}C]glucosamine was subjected to the HPLC separation. The fractions with retention time 0-1 min and 13-17 min contained the majority of ^{14}C-labelled compounds. The non-radiolabelled 80% methanol fractions separated by HPLC were assayed for the presence of Morgan-Elson-positive compounds. The same fractions with retention time 0-1 min and 13-17 min were shown to contain GlcNAc molecules. Furthermore, GC-MS analysis for constituent monosaccharides revealed that the peak corresponding to GlcN is present only in three fractions: fraction A (Rt 0-4 min), fraction B (Rt 5-9 min) and fraction C (13-17 min). Additional analysis of the MALDI-TOF mass spectra revealed that fraction C consists of low MW compound(s). Nod factors, and other chitin-oligomers, are rapidly degraded by chitinases (Staehelin *et al*., 1994b). In fact, when the B-line of Norway spruce was grown in the presence of allosamidin, the chitinase inhibitor, there was significantly more GlcNAc present in the 80% methanol fraction. Furthermore, the degradation of the 80% methanol fraction by chitinase from *S.griseus* was used to test whether the lipophilic fraction contains chitin derivatives. This method allowed for preliminary identification of at least one fraction, fraction C (Rt 13-17 min) in which the GlcN content decreased after chitinase treatment. In conclusion, the isolated fraction C contains lipophilic low molecular weight molecule(s) with GlcNAc residues that are sensitive to chitinase. Therefore, we assumed that fraction C contains Nod-factor-like molecule(s).

Mo and coauthors (1996) showed that embryogenic cultures of Norway spruce secrete chitinases and that there is a close correlation between the presence of specific chitinases and the developmental stage of PEMs and SEs. The total extracellular chitinase activity was measured using 4-methylumbeliferyl β-D-N, N' diacetylchitobioside (Sigma) as a substrate. Proteins secreted by A-lines showed more chitinase activity than those that were secreted by B-lines (Wiweger, unpublished). The chitinase activity decreased after withdrawal of PGRs in A-lines but not in the B1-line (Table 1). These data together with results presented in

Table 1. *Total chitinase activity in cultures of Norway spruce. Embryogenic cultures of A21-line (normally developing A-type of cell-line) and B1-line (developmentally blocked B-type cell-line) were grown for seven days in a medium with (+PGRs) and without (-PGRs) PGRs. The total chitinase activity was measured using 4-methylumbeliferyl b-D-N, N' diacetylchitobioside (Sigma) as a substrate. Values of the chitinase activity, in arbitrary units per mg protein, are means ± SE of three to four independent analysis.*

	A21-line	B1-line
+PGRs	49.25±7.7	12.30±3.4
-PGRs	19.33±3.0	12.00±2.1

paper III show that there are several differentially regulated chitinases present in embryogenic cultures of Norway spruce. At present we do not know how chitinase activity is regulated in Norway and which chitinases are involved in degradation and which are involved in production of LCOs.

Biological activity of endogenous LCOs

Rhizobial Nod factors were shown to stimulate protoplast divisions, PEM proliferation and somatic embryo formation but not further embryo development (Dyachok *et al*., 2000). Therefore, isolated Nod-factor-like molecules, 80% methanol fraction (LCO-total), fraction A (LCO-A) and fraction C (LCO-C) were further tested for their biological activity on somatic embryogenesis. LCO-A and LCO-C stimulated PEM proliferation in a similar way as the non-separated extracts. In addition, pre-treatment of LCO-C with chitinases from *S.griseus* resulted in loss of the ability to stimulate PEM proliferation. Therefore we concluded that biological activity of LCO-C is related to the chitinase sensitive lipophilic chitooligosaccharide(s). To date, biologically active oligosaccharins have been obtained by enzymatic degradation of cell wall polysaccharides but their presence in planta is still questionable. However, our finding of endogenous LCOs in embryogenic cultures of Norway spruce supports the hypothesis that plants produce analogues to rhizobial Nod factors.

The developmental pathways of somatic embryogenesis in Norway spruce involves the proliferation of PEMs, PEM-to-SE transition, maturation and germination of SEs (Filonova *et al*., 2000b). PGRs (auxin and cytokinin) are necessary to maintain PEM proliferation, while withdrawal of PGRs stimulates PEM-to-SE transition and differentiation of SEs (Bozhkov *et al*., 2002). In paper I we showed that the amount of extracellular LCOs (LCO-total and LCO-C) is higher in developmentally blocked B-lines and lower in lines with more advanced structures (A-lines). Therefore, the question arose if the concentration of LCOs secreted to a medium changes during embryo development. The radioactivity of the metabolically labelled LCOs revealed significant differences in the amounts of LCO-total extracted from media conditioned by cultures grown with or without PGRs. In A21 and B41-lines, lower radioactivity was detected in LCOs extracted from PGR-free medium, while the opposite was found in the B1-line. However, the lowest level of LCOs in B-lines was still higher than the highest level in A-

lines. These data indicate that high level of LCOs might be one of the components causing developmental blockage in B-lines.

It was suggested that PCD in PEMs and PEM-to-SE transition are closely interlinked processes, stimulated upon withdrawal of plant growth regulators (PGRs) (Filonova *et al*., 2000a). The signal pathway that triggers PCD following withdrawal of PGRs is normally kept suppressed by a constant supply of signal molecules (Jacobson *et al*., 1997; Raff, 1992). LCO-total and LCO-C suppressed PCD induced by withdrawal of auxin. Furthermore, LCO-total and LCO-C stimulated PEM-to-SE transition and differentiation of SEs. Therefore, PGRs and LCOs have an opposite effect on the differentiation of somatic embryos.

Pre-treatment of the A-lines for one week in the PGR-free medium before maturation treatment synchronises the culture at the early embryogeny stage. Subsequently, five weeks ABA-treatment is sufficient to accomplish late embryogeny and embryo maturation. Addition of LCO-total during the pre-treatment in PGR-free medium abolished the effect of pre-treatment, i.e. proliferation proceeded in the same way as when the cultures were exposed to PGRs during the pre-treatment. Therefore, LCO-total had a similar effect as PGRs on proliferation and embryo development, whereas LCO-C did not have any effect. The effect of LCO-total might be explained by the PGRs contamination present in the extract (10^{-6}M BA and $4x10^{-8}$M 2,4-D). However, since LCO-total had an opposite effect to PGRs during PEM-to-SE transition, other components of LCO-total might be biologically active. At present, we know that LCO-total contains LCO-C and PGRs. Preliminary data have shown presence of chitinases related to CH4 from sugar beet, AGPs and probably flavonoids. Further studies will show how the different compounds affect embryogenesis.

Conclusions

Nod-factor-like molecules are present in embryogenic cultures of the gymnosperm, Norway spruce. We have identified an LCO that influences early stages of embryo development, i.e. suppresses PCD and stimulates PEM proliferation. This LCO also promotes PEM-to-SE transition, but does not influence later development. The amount of LCO is developmentally regulated. High concentrations of LCO together with low chitinase activity present in B-lines might be one of the factors causing developmental blockage.

Chitinases in embryogenic cultures of Norway spruce (paper III and unpublished)

Chia4-Pa1 chitinase

The *Chia4-Pa1* sequence was isolated from cDNA from proliferating embryogenic cultures of Norway spruce (Genebank, accession number AY270018). The multi-band pattern detected in the southern blot suggests that *Chia4-Pa1* belongs to a small gene family. Partial sequencing confirmed the presence of a few highly similar chitinase genes e.g. gene with accession number AY270019. The *Chia4-Pa1* gene could not be distinguished from other members of the Chia4-Pa family

either by mRNA *in situ* hybridisation or Northern blot analysis, therefore the conclusion about the expression pattern of the *Chia4-Pa1* gene had to be expanded to the Chia4-Pa family. However, at this stage, the possibility that the Chia4-Pa family includes different alleles of the same gene cannot be excluded. Similarly, a multigene family consisting of nearly identical chitinase genes was reported for *Pschi4* from pine (Wu *et al*., 1997), *EP3* from carrot (van Hengel *et al*., 1998), *AtEP3/AtchitIV* from *Arabidopsis* (Passarinho and de Vries, 2002) and *OsChia1;175* from rice (Takakura *et al*., 2000).

The predicted CHIA4-Pa1 protein is organized into regions that include a signal peptide, a chitin-binding domain, a hinge region and a catalytic domain with the pI value and molecular weight typical for chitinases belonging to a basic chitinase class IV in family 19 of glycosyl hydrolases (Figure 2A). Furthermore, the catalytic domain of CHIA4-Pa1 shows around 50% identity with other class IV chitinases. In order to study the enzymatic activity of CHIA4-Pa1, different deletion constructs of CHIA4-Pa1 were prepared (Figure 2B) and cloned into three expression vectors: pGEX-5x-2 (Pharmacia), pQE30 (Clontech) and pBAD/Thio-TOPO (Invitrogen). Recombinant CHIA4-Pa1 proteins with Glutathione S-transferase (GST), histidine (6xHis) and thioredoxin (Trx) tags were expressed in *E.coli*. The CHIA4-Pa1-6xHis proteins appeared to be lethal for *E.coli* while the

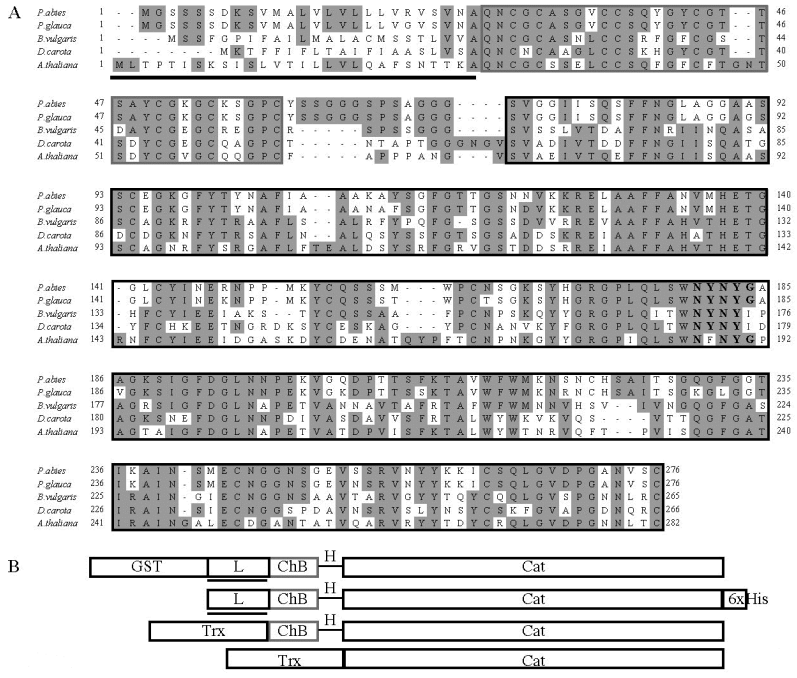


Figure 2. The CHIA4-Pa1 protein.
A) Alignment of class IV chitinases from gymnosperms and angiosperms. The deduced amino acid sequence of CHIA4-Pa1 from *P.abies* is compared with PIACHI from *P.glauca* (Dong and Dunstan, 1997), EP3 from *D.carrota* (Kragh *et al*., 1996), CH4 from *B.vulgaris* (Mikkelsen *et al*., 1992) and AtEP3/AtCHIV from *A.thaliana* (Passarinho *et*

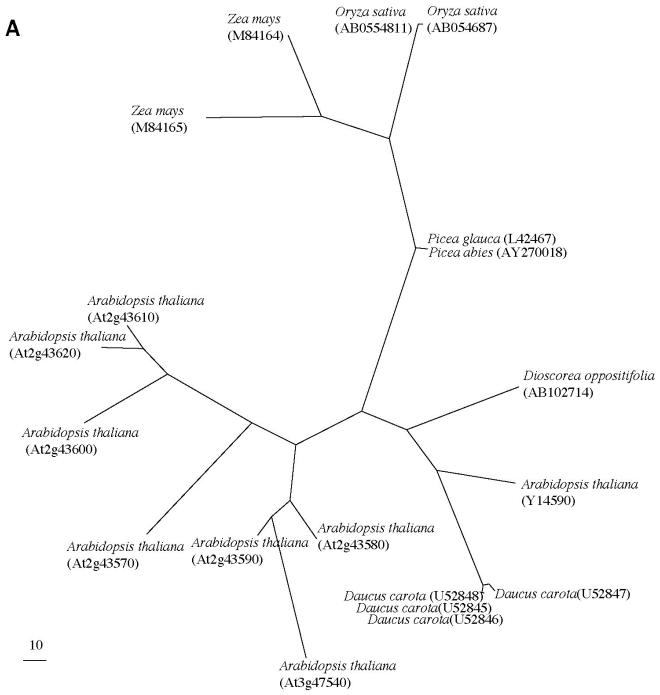
al., 2001). Identical amino acids are shaded in grey when found in at least three out of five proteins. The sequences corresponding to a signal peptide are underlined, a chitin-binding domain is in grey boxes and the catalytic domain is in black boxes. The region between the chitin-binding domain and the catalytic domain belongs to the hinge region. The active site with its conserved NYNYG motive is marked in bold letters. The putative glycosylation sites are indicated by asterisks. B) Schematic representation of the domain organisation in different protein-expression constructs. Elements of the Chia4-Pa1 protein: catalytic domain (Cat), chitin-binding domain (ChB), hinge region (H) and signal peptide (L). Protein tags: Glutathione S-transferase (GST), histidine (6xHis) and thioredoxin (Trx).

GST- and the Trx-fusion chitinase did not show any toxicity. GST-CHIA-Pa and Trx-CHIA4-Pa proteins were mainly secreted as inclusion bodies. However, a small part of the recombinant proteins were expressed in a soluble form and these soluble fractions were subjected to further purification using sepharose, superdex and diethylaminoethyl cellulose based ion-exchange columns. Fusion proteins could not be eluted under conditions that would preserve the native form of the CHIA4-Pa. The same strong binding of the recombinant protein was observed for all tags and all deletion constructs. Hence, I concluded that the catalytic domain of the CHIA4-Pa is responsible for the tight binding to the matrix. Western blot analysis of the recombinant protein revealed that CHIA4-Pa1 is serologically related to chitinase 4 (CH4). The CH4 antibody, which was raised against a basic class IV chitinase from sugar beet (CH4), recognises CHIA4-Pa1 and angiosperm basic (Mikkelsen *et al*., 1992; Nielsen *et al*., 1996) and acidic (De Jong *et al*., 1993; Nielsen *et al*., 1994; Passarinho *et al*., 2001) class IV chitinases. This suggests that CHIA4-Pa1, EP3 and CH4 have similar protein structures enabling similar biochemical functions.

Evolution of class IV chitinases in plants

To analyse the genetic relationships of plant chitinases, phylogenetic trees were constructed. Class I, II, IV and VII chitinase genes were used and class III and V genes were excluded from the comparison because of their pronouncedly different primary structure. The phylogenetic analysis revealed the presence of several subgroups. The *Chia4-Pa1* chitinase clustered together with a highly supported subclass comprising class IV and VII chitinases. Grouping of class I and II chitinases suggest that they are ancestral to the short chitinases (class IV and class VII). However, it is not clear whether class IV chitinases evolved from class I or class II chitinases. Ohme-Takagi and coauthors (1998) suggested that plants have either class IV or acidic class II chitinase. Nowadays this hypothesis can be ruled out. Co-existence of both classes of chitinase in one plant was verified in *Arabidopsis* (AB81807, AAF29390.1) and rice (AB054687, L40336) and sugar

A



B

accession number	plant name	intron1 boundary		intron2 boundary		intron3 boundary	
		5'	3'	5'	3'	5'	3'
*	*Picea glauca*						
AY270016	*Picea abies*	-	-	HETG	GLCY	QLSW	NYNY
M84164	*Zea mays*	-	-	*HGGT*	*EVEG*	*QISW*	*NYNY*
M84165	*Zea mays*	-	-	*HETG*	*HFCY*	*QISW*	*NYNY*
AB054811	*Oryza sativa*	-	-	HETG	HMCY	*QISW*	*NFNY*
AB054687	*Oryza sativa*	-	-	HETG	HMCY	*QISW*	*NFNY*
AB102714	*Dioscorea oppositifolia*	-	-	HETG	HLCY	*QLSW*	*NYNY*
Y14590	*Arabidopsis thaliana*	-	-	HETG	RNFC	*QLSW*	*NFNY*
U52847	*Daucus carota*	-	-	HETG	YFCH	*QLTW*	*NYNY*
At2g43580	*Arabidopsis thaliana*	-	-	YETG	HFCY	*LLSW*	*NFNY*
At 3g47540	*Arabidopsis thaliana*	-	-	HETG	HFCY	-	-
At2g43590	*Arabidopsis thaliana*	-	-	HETG	HFCY	*QLSW*	*NYNY*
At2g43570	*Arabidopsis thaliana*	-	-	QETG	FMCY	*QLSW*	*NYNY*
At2g43600	*Arabidopsis thaliana*	-	-	YGSK	SFCY	-	-
At2g43620	*Arabidopsis thaliana*	-	-	HESG	SFCY	*QITW*	*NYNY*
At2g43610	*Arabidopsis thaliana*	-	-	HESG	SFCY	*QITW*	*NYNY*

Figure 3. Phylogenetic analysis of *Chia4-Pa*.

A) Phylogenetic unrooted tree showing the relationships between class IV chitinases. The tree presented here is a maximum parasimony tree based on nucleotide sequence alignment. Branch lengths are proportional to the number of nucleotide substitutions. Gene accession numbers and chitinase class numbers are indicated in brackets. B) Exon-intron organisation of class IV chitinase genes. Boundaries: the amino acid sequences surrounding introns at their 5'- and 3'- end, respectively. Numbers: 1, 2 and 3 indicate the intron positions; italic, boundary is present even though the intron is absent; -, indicates lack of intron and intron boundaries; asterisk, no genomic sequence available. The AY271253 and AY270016 are genomic sequences corresponding to the U52847 and AY270018 cDNA clones, respectively.

beet (Mikkelsen *et al*., 1992). Hamel and coautors (1997) proposed that the derivation of the class IV lineage from a common ancestral sequence would have occurred before the separation of monocots and dicots, estimated to take place around 200 million years ago. According to our results, which are in agreement with work by Gomez et al., (2002), class IV chitinases probably evolved from class I or II chitinases before the separation of angiosperms and gymnosperm i.e. more than 300 million years ago. In order to study relationships among different class IV chitinases, an unrooted tree was constructed (Figure 3A). Phylogenetic analysis of class IV chitinases indicated that *Chia4-Pa1* and other gymnosperm chitinase gene are more closely related to the chitinase genes from monocots than to chitinases from dicots.

Sequencing of the genomic fragment of the *Chia4-Pa1* gene revealed the presence of two introns within the coding sequence. It has previously been shown that the phylogenetic classification of some proteins is supported by the exon-intron structures of the corresponding genes (Ingouff *et al*., 2001, paper IV). Therefore, the position of the introns in the *Chia4-Pa* was compared to that from other known plant chitinases. Most of chitinases revealed the presence of two highly conserved 5'- and 3'- boundaries, identical with those in *Chia4-Pa1*. Interestingly, all of the angiosperm class IV genes, despite the presence of conserved boundaries, lack the intron at position number 3. An intron pattern identical to that of the *Chia4-Pa* genes exists in some members of class I and II chitinases. Since an intron at position number 3 is present in evolutionarily older sequences (class I, II and gymnosperm class IV chitinases) but not in the angiosperm class IV chitinases, it is most likely that intron number 3 was lost during evolution. In contrast, intron number 1, present in angiosperm class VII chitinase, was probably gained during evolution. It is remarkable, that the analysis of *Arabidopsis* class IV chitinase genes revealed that all of them lack introns at positions 1 and 3 (Figure 3B). The phylogenetic analysis revealed that the *Chia4-Pa* genes belong to a highly supported subclass including other class IV chitinases. However, the intron-exon structure suggests that they might be two sister groups: gymnosperm and angiosperm class IV chitinases. In this respect, *Chia4-Pa1* is more similar to class I and II chitinases than to angiosperm class IV and VII chitinases indicating that gymnosperm class IV chitinases derived from gymnosperm class II chitinase with two introns within the coding sequence. However, more information from other gymnosperm chitinase genes is required to support our hypothesis.

Expression of the Chia4-Pa *as a marker for PEM-to-SE transition*

In proliferating embryogenic cultures of Norway spruce, the *Chia4-Pa* was expressed at a low level in all the tested lines. After withdrawal of PGRs the expression of *Chia4-Pa* increased significantly in A-lines and in most B-lines. Withdrawal of PGRs (pre-treatment) stimulates PEM-to-SE transition and concomitant activation of PCD. However, the B1-line, which as the only one, does not respond to pre-treatment by forming somatic embryos, had a low and constant level of the *Chia4-Pa* transcript (data not shown) and low level of PCD (Smertenko *et al*., 2003). We suggest that CHIA4-Pa together with other chitinases might influence PCD, thereby controlling PEM-to-SE. In accordance,

the EP3 and AtEP3/AtchitIV chitinase were proposed to be involved in the regulation of PCD in carrot and Arabidopsis (Passarinho *et al*., 2001; van Hengel *et al*., 1998).

Proteins secreted by proliferating embryogenic suspension cultures of Norway spruce were subjected to SDS-PAGE and western blot analysis. The CH4 antibody recognised proteins with the size of 26 kDa and 28kDa which is in accordance with results reported previously (Egertsdotter and von Arnold, 1998; Mo *et al*., 1996). The putative size of Chia4-Pa1 is 26kDa, which is analogous to the size of Chia4-Pa1 expressed in bacteria. However, since the *Chia4-Pa1* gene contains putative glycosylation sites, and glycosylation does not occur in E.coli, it is most likely that 26/28kDa proteins detected by the CH4 antibody, correspond to non-gycosylated and glycosylated forms of CHIA4-Pa1. The amount of the 26/28kDa chitinases varies between lines. A-lines had higher concentrations of 26/28kDa chitinases than B-lines. However, in all tested lines, the amount of extracellular 26/28kDa chitinases increased after pre-treatment in PGR-free medium. Hence, the expression of the 26/28kDa proteins correlates with the expression pattern of *Chia4-Pa* genes.

Localisation of the CHIA4-Pa proteins does not correspond to the expression pattern of the encoding genes

In order to test if localisation of the CHIA4-Pa proteins correlates with the expression pattern of the *Chia4-Pa* genes, an *in situ* immuno-localisation of CH4 related proteins was compared with *in situ* mRNA analysis of the corresponding genes. In whole-mount, unfixed material, the CHIA4-Pa proteins were detected on the surface of embryonal masses. In contrast, no signal could be detected in fixed material. Therefore, we assume that CHIA4-Pa is bound to the surface by weak ionic forces. In accordance, a basic chitinase-like protein, ionically bounded to the surface of preglobular embryos in Carribian pine, was reported by Domon and coauthors (2000).

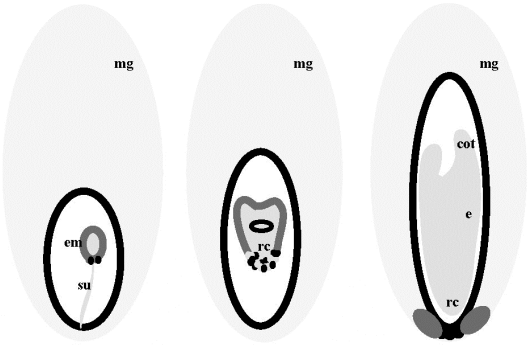
The *Chia4-Pa* genes were expressed in a sub-population of cells in proliferating embryonic cultures, and during embryo development in cells at the base of the embryonal mass. Expression of chitinase genes in cells close to the developing embryo proper but not in the embryo proper itself was also reported for *EP3* and *AtEP3/AtchiIV* (Passarinho *et al*., 2001, van Hengel *et al*., 1998). All this suggests that, at the early stages of embryo development, expression of *Chia4-Pa*, *EP3* and *AtEP3/AtchiIV* is very specific and restricted to cells which themselves do not develop into embryos.

During late embryogeny and maturation, the *Chia4-Pa* transcript was detected in root cap and cortex tissue, unlike those of *EP3* and *AtEP3/AtchiIV* which were not expressed in embryos (Passarinho *et al*., 2001, van Hengel *et al*., 1998). The CHIA4-Pa proteins were localised at the surface of the embryonal mass but only in the apical part of the maturing embryo. In mature SEs, CHIA4-Pa signal became limited to small patchy areas and finally declined. AGPs are the plant cell wall components that contain GlcNAc and Glc groups in a form that can be hydrolysed

by chitinases (van Hengel *et al*., 1998). Furthermore, it has been shown that the identity of cells or tissues might be reflected by the AGPs present in the cellular matrix (Nothnagel, 1997). It was previously showed that AGPs are involved in the regulation of somatic embryogenesis in Norway spruce (Egertsdotter and von Arnold, 1995). The identity and localisation of the AGPs, the putative substrate for CHIA4-Pa chitinases, remain unknown. However, it is likely that CHIA4-Pa chitinases migrate towards the place where a substrate is localised. The covering film of CHIA4-Pa bound to the surface of embryonal masses might act on AGPs present in epidermal cell walls and thereby cause loosening of the cell walls and/or protection of the embryonal masses from signaling molecules, e.g. LCOs.

In seeds the *Chia4-Pa* and *EP3* chitinases were expressed in non-dividing cells in the inner layer of the megagametophyte/endosperm (van Hengel *et al*., 1998; paper III). Moreover, the *Chia4-Pa* transcript was also detected in the region of the micropylar megagametophyte of Norway spruce. Chitinase expression in the micropylar region was also observed in germinating seeds of tomato (Wu *et al*., 2001).

An hypothetical model of the localisation of the *Chia4-Pa* transcripts and CHIA4-Pa protein in developing seeds of Norway spruce is presented in figure 4.



	Before pollination	Early embryogeny		Late embryogeny		Maturation		Germination	
	ZE	SE	ZE	SE	ZE	SE	ZE	SE	ZE
gene	+	+	+	+	na	+	+	+	+
protein	+	+	na	+	na	+	+	+	+

Figure 4. Hypothetical model of the localisation of the *Chia4-Pa* mRNA and protein in developing seeds of Norway spruce. The model is based on the results from both somatic and zygotic embryos. Black color indicates localisation of the mRNA; dark grey color indicates localisation of the CHIA4-Pa protein; cot, cotyledons; e, embryo; mg, megagametophyte; rm, root meristem; roc, root organisation centrum; sm, shoot meristem; su, suspensor. +, presence of the signal; -, absence of the signal detected in somatic embryos (SE) or in seeds (ZE); na, not analysed.

"Nurse cells" express Chia4-Pa *genes*

In Norway spruce, *Chia4-Pa* is expressed in ovules, but not in the immature female strobili, or in germinating pollen (Figure 5). Furthermore, the *in situ* analysis showed that *Chia4-Pa* transcript is predominantly present in the megagametophyte, in the single zone surrounding the corrosion cavity. Our data are in agreement with *EP3* in carrot (van Hengel *et al*., 1998) where *EP3* mRNA was detected in a few inner layers of the integuments (early stages) and in the endosperm (late stages). In contrast the *Arabidopsis AtEP3/AtchiIV*, an ortholog of *EP3*, is not expressed in the integuments or in the endosperm (Passarinho *et al*., 2001). It is worth mentioning that *Arabidopsis* has only debris of endosperm because it might explain lack of expression of *AtEP3/AtchiIV* in seeds. It is tempting to conclude that the inner layer of the megagametophyte and the inner integument cells, or middle zone of the endosperm cells surrounding the corrosion cavity are analogous tissues. Recently, it has been hypothesised that endosperm and embryo interact during their development (Berger, 1999). The importance of an interaction between the embryo and the megagametophyte has been shown in pine (Filonova *et al*., 2002). It was suggested that PCD affecting the female gametophyte is likely triggered by a signal given by rapidly growing embryos, and its developmental roles are to make room for the dominant embryo and to prepare cell corpses to store nutrients (Filonova *et al*., 2002). Failure of endosperm development usually results in embryo abortion (Birchler, 1993). In *Arabidopsis,* the maternal *MEA* allele is required for proper endosperm and embryo development. Mutation in *MEA* causes precocious endosperm formation before fertilisation and prolonged endosperm nuclear proliferation after fertilisation (Kiyosue *et al*., 1999). Defective function of endosperm causes, at least to some extent, *mea* embryo abortion (Kinoshita *et al*., 1999).

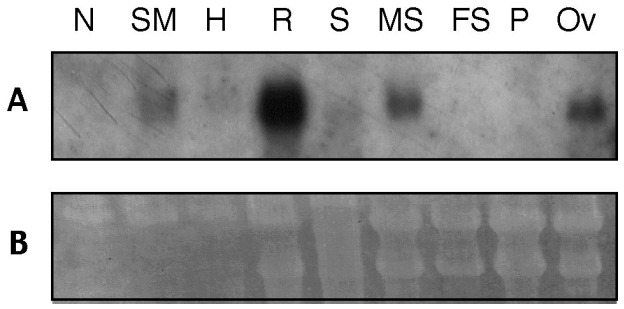


Figure 5. Northern blot analysis of Chia4-Pa expression in different tissues. FS, immature female strobili; H, hypocotyls; MS, immature male strobili; N, needles; Ov, ovules; P, germinating pollen; R, roots; S, seedlings; SM, shoot meristems. In each lane, 15 mg of total RNA was loaded. Filter was probed with Chia4-Pa1 (A) and washed (0.1xSSC, 0.5% SDS at 65°C), or stained with ethidium bromide (B).

Chitinases that promote somatic embryogenesis in carrot (*EP3)* are mainly expressed in the endosperm during seed development. This suggests that cells other than those in the embryos produce molecules that influence embryo

development (Passarinho *et al*., 2001; van Hengel *et al*., 1998; van Hengel *et al*., 2001) or as explained by van Hengel and coworkers (1998) the EP3 chitinase has a nurse function. Moreover, since the expression pattern is similar in a gymnosperm (Norway spruce) and in an angiosperm (carrot) it suggests that this function is evolutionarily conserved. In addition, in *Drosophila*, the IDGF (for imaginal disks growth factor) transcripts are detected in the oocyte, but are absent from follicle cells. The fat body may therefore be an important source of growth factors that support peripheral tissue growth during insect development (Kawamura *et al*., 1999).

A transgenic approach for studying a single member of the Chia4-Pa family.

In order to study a single member of the Chia4-Pa family, the 0.8 kB upstream region of *Chia4-Pa1* was isolated and sequenced (Figure 6). Computer analysis of the isolated fragment revealed the presence of several putative responsive elements. Northern blot and mRNA *in situ* analysis revealed that the *Chia4-Pa* genes are differentially expressed during embryo development and withdrawal of PGRs stimulated increased expression of the *Chia4-P*a in Norway spruce. Hence, it is not surprising that the isolated fragment of the *Chia4-Pa1* promoter contains the NtBBF1 and SEF3 (for soybean embryo factors 3) motives. The NtBBF1 is required for tissue-specific and auxin-regulated expression of the rolB oncogene in plants (Baumann *et al*., 1999). The binding activity of SEF3 has been shown to increase during mid stage of embryo development and decrease at seed maturity (Allen *et al*., 1989). Interestingly, the isolated fragment of the *Chia4-Pa1* promoter is organised into two overlapping tandem-repeats with two repeats of NtBBF1 followed by single SEF3. This kind of long tandem-repeat has not previously been described in other chitinase promoters.

The *pChia4-Pa1::uidA* construct (PCH6pCB308) was prepared and used for transformation of Norway spruce and *Arabidopsis*. Preliminary experiments with transient expression of *uidA* showed that, 24 hours after transformation, GUS expression could be detected in proliferating embryogenic cultures of Norway spruce and in *Arabidopsis* roots using GUS histochemical staining. Therefore, embryogenic cultures of Norway spruce were transformed with *pChia4-Pa1::uidA* constructs. Eight transgenic sub-lines of Norway spruce carrying *pChia4-Pa1::uidA* were chosen for further studies. Histochemical staining of proliferating embryogenic cells or maturing somatic embryos did not show any GUS staining in any of the tested transgenic sub-lines. However, when a more sensitive method was used (fluorimetric method), weak GUS activity was detected in all tested sub-lines maintained under proliferating conditions in the presence of PGRs. After withdrawal of PGRs the GUS activity increased significantly in all sub-lines transformed with PCH6pCB308. In contrast, the mother lines and sub-lines transformed with *uidA* driven under pUbi or pEu-CAD promoters did not show any significant increase in GUS activity after withdrawal of PGRs (Table 2). The expression of *pChia4-Pa1::uidA* is in agreement with our results from Northern and western blot analysis of *Chia4-Pa*. However the weak GUS activity in transgenic sub-lines did not allow for verification of the localisation of the *Chia4-*

Pa1 transcript. The length of the chitinase promoter used in this study was relatively short, so the PCH6pCB308 construct might lack some of the regulatory elements, resulting in a weak expression of the *uidA* transcript. However, the first 200bp of the *Pschi4* promoter, 600bp of P4-ch 5' flanking DNA and the DNA region between –788 and –345 from the start site of transcription in *CHN50* were sufficient for response to different stimuli (Fukuda and Shinshi, 1994; Margis-Pinheiro *et al*., 1994; Wu *et al*., 1999). Worth noticing is that, judging by GUS histochemical staining, the transient GUS activity (24 hours after transformation)

Figure 6. Nucleotide sequence of the upstream region of *Chia4-Pa1*. Positions of the putative regulatory elements are boxed: SEF3 in grey and NtBBF1 in black. References for the described motifs: SEF3 (Allen *et al*., 1989), NtBBF1 (Baumann *et al*., 1999). The putative TATA-box is bolded and underlined, putative transcription start is indicated by bigger font.

Table 2. *GUS-specific activity in transformed embryogenic cultures of Norway spruce. Embryogenic cultures of Norway spruce were transformed with A, PCH6pCB308 (Wiweger, unpublished); B, pEu-CAD-GUS and C, pUbi BAR (Clapham et al., 2000). Presence of the* uidA *gene was verified by PCR. +, presence; -, absence of expected band. Two non-transgenic mother-lines are indicated in bold. Proteins were extracted from cultures grown with (+PGRs) and without (-PGRs) PGRs. Level of expression of the* uidA *gene was estimated by fluorometric assay of GUS activity. Values of the GUS activity are means of four to six independent analysis.*

	GUS activity (pmol 4-MU/ min^{-1}/ml^{-1}/mg protein)		plasmid	*uidA*
	+PGRs	-PGRs		
A22	0.065	0.079	-	-
G88-5	0.077	0.108 [a]	A	+
G88-9	0.060	0.109 [a]	A	+
G88-11	0.053	0.128 [a]	A	+
A75-9	0.078	0.046	C	-
A21	0.055	0.064	-	-
G95-1	0.068	0.095 [a]	A	+
G95-5	0.079	0.091	A	+
G95-8	0.072	0.095 [a]	A	+
A49	4.073	3.342 [a]	B+C	+

[a] Significantly different from the +PGRs, estimated using paired t-test ($P \leq 0.05$).

was at a much higher level than that found in stable transformants. It was shown that if the promoter construct contains even short stretches of the corresponding gene, there is an increased probability for gene silencing. In fact, the PCH6pCB308 construct contains the 15bp long 5'-UTR of the *Chia4-Pa1* gene. Therefore, it is probable that gene silencing and not the short length of *Chia4-Pa1*promoter is the reason for the low GUS activity in transgenics carrying the PCH6pCB308 construct.

Conclusions

The *Chia4-Pa* genes from Norway spruce encode basic class IV chitinases. The phylogenetic classification of chitinases supported by the exon-intron structures of the corresponding genes suggests that class IV chitinase evolved from class I more than 300 million years ago, before the angiosperms separated from gymnosperms. The *Chia4-Pa1* is closely related to other class IV chitinases. However, *Chia4-Pa1* together with other gymnosperm class IV genes forms a separate subgroup with an intron-exon pattern identical to class I and II chitinase. I suggest that gymnosperm (CHIA4-Pa) and angiosperm (EP3) class IV chitinases represent protein families similar in their origin and in biochemical functions. Based on the spatial expression pattern of *Chia4-Pa* I propose that chitinase-expressing cells have a megagametophyte signaling function and that CHIA4-Pa takes part in the regulation of the PEM-to-SE transition, probably by controlling PCD. Localisation of the CHIA4-Pa proteins does not correspond to the expression

pattern of the encoding genes. The *Chia4-Pa* genes are expressed in nursing cells but the CHIA4-Pa1 proteins might act on AGPs causing cell wall loosening and elongation.

Localisation of AGPs during embryogenesis of Norway spruce (unpublished).

Immunolocalisation of different AGP epitopes

AGPs are known to stimulate somatic embryo development in angiosperms and gymnosperms (Chapman *et al*., 2000; Egertsdotter and von Arnold, 1995; Kreuger and van Holst, 1995; Steele-King *et al*., 2000; Toonen *et al*., 1997; van Hengel *et al*., 2001; van Hengel *et al*., 2002). However, the majority of reports on the spatial or temporal pattern of expression and/or modification of APGs during plant growth and development come from angiosperm species (Showalter, 2001). In order to learn more about AGPs during embryogenesis of gymnosperms, I followed the localisation of JIM4, JIM8, JIM13, MAC207 and ZUM18-epitopes in seeds of Norway spruce (Figure 7).

In Norway spruce, JIM4 epitope was restricted to the apical part of the maturing embryo. No signal was detected in the root cap. In mature embryos, weak signal became restricted to the epidermal layer. In the megagametophyte the JIM4 epitope was restricted to the most outer layer and to the thin cell layer surrounding the corrosion cavity. However, during maturation the JIM4 epitope was also detected in the zone that degrades prior to enlargement of the corrosion cavity during embryo maturation (the zone in front of the embryo). Developmental regulation of JIM4-epitope was reported in carrot (Stacey *et al*., 1990). Immunolocalisation of the JIM4-epitope revealed the presence of the signal specific for epiderm and provascular tissue (Stacey *et al*., 1990). Interestingly, abnormalities in somatic embryo development of carrot were concomitant with loss of JIM4-epitope from the epiderm (Stacey *et al*., 1990).

JIM8 epitope was mostly localised to the outer layers of cells of the maturing embryo. No signal was detected in the embryo proper or in the root cap of the maturing embryo. In mature embryos, weak signal was equally distributed all over the embryo. Most of the megagametophyte was decorated by the JIM8-epitope with the exception of the outermost layer. Interestingly, the zone that degrades prior to enlargement of the corrosion cavity during embryo maturation (the zone in front of the embryo) was also JIM8-free. In angiosperms, JIM8-epitope was detected in gametes, anthers, ovules, and in the early embryo (Coimbra and Salems, 1997; Pennell *et al*., 1991; Toonen *et al*., 1996). The JIM8-epitope that was suggested as a marker for embryogenic competence of somatic cells is present in "nursing cells" (Coimbra and Salems, 1997; Toonen *et al*., 1996).

JIM13 was uniformly distributed within the maturing embryo, mature SE and most of the megagametophyte. However, no JIM13-signal was detected in the outermost layer of the megagametophyte. In angiosperms, JIM13 epitope is

localised mostly in xylem, floem, and epidermal cells, but deposition of this epitope differs among different species (Stacey *et al*., 1990).

The MAC207-epitope was localised beneath the emerging cotyledons and in the provascular tissues and below the root meristem in mature embryos but not in early embryos or in the megagametophyte. In agreement, MAC 207 epitope was absent in cells involved in sexual reproduction as well as in early zygotic embryos before they reach the heart stages (Coimbra and Salems, 1997; Pennell *et al*., 1989) and present in bilateral embryos (Pennell *et al*., 1991).

ZUM18 epitope was restricted to the epidermal layer in cotyledons of the mature zygotic and somatic embryos. However weak signal could be detected all over the embryo. No signal was present in the megagametophyte. van Hengel and coworkers (2002) showed that ZUM18 epitope is relatively poorly represented in

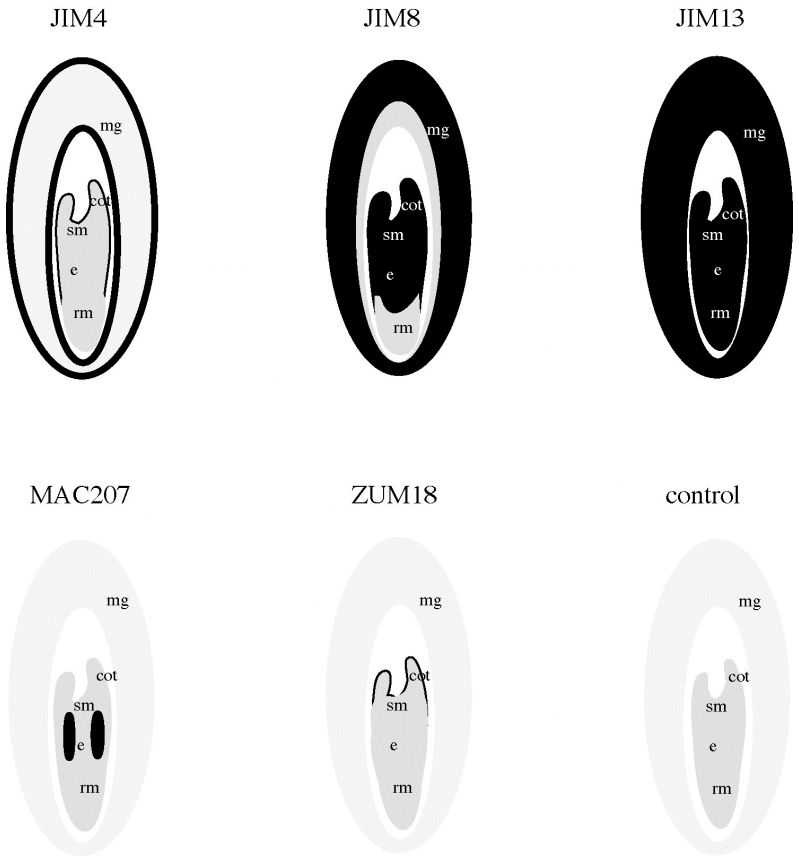


Figure 7. Schematic overview on localisation of different AGP-epitopes in seeds of Norway spruce. Localisation of AGPs has been visualised by using antibodies against different AGP-epitopes. cot, cotyledons; e, embryo; mg, megagametophyte; rm, root meristem; sm, shoot meristem. Localisation of an AGP-epitope is indicated in black.

the total pool of proteins. However, when added to *in vitro* cultures, it stimulates embryo development (Kreuger and van Holst, 1995).

Relations between AGPs, chitinases and LCOs

AGPs and LCOs as well as chitinases (EP3 and CH4) can stimulate somatic embryogenesis in Norway spruce (Dyachok *et al*., 2000; Egertsdotter and von Arnold, 1995; Egertsdotter and von Arnold, 1998 and paper I) and in carrot (De Jong *et al*., 1995; Kragh *et al*., 1996). It has been suggested that LCOs are parts of AGPs that are released by chitinases. Recently, van Hengel and coworkers (2001) showed that LCO-like molecules are released from AGPs after they have been hydrolysed by chitinases. Moreover, the susceptibility of AGPs, for endochitinase activity changes during embryo development (van Hengel *et al*., 2001). The *Chia4-Pa1* gene (whole ORF) as well as different chitinase domains were cloned and expressed in *E.coli* but the active protein could not be purified. Therefore I have not been able to check if a specific AGP-epitope act as a substrate for CHIA4-Pa.

Our second approach was to use the βGlc Yariv reagent, which has emerged as a novel class of synthetic plant growth inhibitor (Steele-King *et al*., 2000). Yariv reagent is known to bind specifically to AGPs, however the mechanism of this recognition is not clear. Treatment with βGlc Yariv reagent caused slight acidification of the media, stimulated PCD and inhibited proliferation in cultures of rose (Steele-King *et al*., 2000 and references therein). Our preliminary data suggest that addition of Yariv reagent to embryogenic cultures of Norway spruce also stimulates PCD (Wiwger, unpublished). Consequently, Yariv reagent and LCO-C have opposite effects on PCD. In addition, an *in vitro* assay indicated that Yariv reagent binds to the LCO-C. Therefore, I suggest that Yariv reagent binds to the LCO-part of AGP and thereby protects the whole molecule from degradation by chitinase, resulting in the reduced amount of LCOs secreted to a medium.

Conclusions

In Norway spruce, the localisation of the CHIA4-Pa proteins does not correspond to the expression pattern of the encoding genes. I have suggested that the CHIA4-Pa proteins might be targeted to places where the substrate(s) e.g. AGPs are localised. Therefore the question arose, if any AGP-epitope co-localise with CHIA4-Pa chitinase? Localisation of AGPs in Norway spruce was similar but not identical with that reported in angiosperms. None of the tested AGP-epitopes had an identical localisation pattern to this that was found for CHIA4-Pa chitinase. However, epidermal localisation of the JIM4 and JIM8-epitopes and cortex-specific localisation of the MAC207-epitope agrees with the localisation of CHIA4-Pa.

Markers for embryo development (paper IV)

The PaHB2 *gene as molecular marker for cortex*

The basic plant body plan can be characterised by a root/shoot axis and by a radial organisation of primary tissue layers. In angiosperms, the epidermis derives from the protoderm, the cortex and endodermis derive from the ground tissue, and the

pericycle and vascular tissues derive from the procambium. The embryonal mass of gymnosperms (analogous to the embryo proper in angiosperms) is surrounded by a surface layer of cells that functions as a protoderm layer even though it is formed as result of both periclinal and anticlinal divisions (Rombeger *et al*., 1993). Late embryogeny in gymnosperms corresponds to the "post-globular embryo" development in angiosperms (Rombeger *et al*., 1993; Singh, 1978) and as in angiospems, comprises the establishment of the root/shoot axis, inner primary tissues, and cotyledons (Rombeger *et al*., 1993). Although the general plan of embryo development in both angiosperms and gymnosperms is similar, it is not known if similar molecular mechanisms operate during embryo pattern formation in these two groups.

The homeodomain proteins are typically involved in the determination of cell fate during animal and plant development (Chan *et al*., 1998; Gehring *et al*., 1994; Kappen, 2000). The Homeodomain-Glabra2 (HD-GL2) is a plant-specific family (Lu *et al*., 1996). The members of HD-GL2 are characterised by an amino-terminal homeodomain linked to a putative leucine zipper (HD-ZIP) followed by a sterol/lipid-binding START domain (Ponting and Aravind, 1999; Tsujishita and Hurley, 2000). The HD-GL2 family is represented by O39 from orchid, ZmOCL (1-5) from maize (Ingram *et al*., 2000; Ingram *et al*., 1999), HAHR1 from *Helianthus*, GHGL2 from *Gossypium* and ANL2, ATLM1, GL2 and PDF2 from *Arabidopsis*. The *AtML1* gene and three related genes in maize *ZmOCL* are essentially protodermal/epidermal-specific in angiosperms. In addition, *PaHB1*, an evolutionarily conserved HD-GL2 homeobox gene with protoderm-specific expression during somatic embryo development in Norway spruce, was recently isolated in our laboratory (Ingouff *et al*., 2001). A phylogenetic analysis revealed that the HD-GL2 family consists of at least three distinct subgroups. The *AtML1*, *PaHB1* and *ZmOCL5* genes form one group while most of the *ZmOCL* genes together with *ANL2* comprise another group. The third group of HD-GL2, is the only group that contain genes that lack conserved intron position number 6 (Ingouff *et al*., 2001; paper IV). *Arabidopsis* and maize have HD-GL2 genes representing all three sister-groups, so it is most likely that a similar situation occurs in other plants.

In this work we aimed to identify genes, representatives of different sister-groups of HD-GL2, which are expressed during somatic embryogenesis in Norway spruce. Using degenerate primers binding to the homeodomain, two main products were amplified by RT-PCR. One PCR product was identical to *PaHB1*, while the other one, with similar but not identical sequence, was designated *PaHB2*. The *PaHB2* gene encodes for a typical predicted HD-GL2 protein with N-terminal homeodomain linked to a non-canonical leucine zipper domain, and a putative StAR-related lipid transfer (START) domain. The N-terminal region of PAHB2 comprises a short stretch rich in aspartate and glycine, which is reminiscent of the N-terminal regions of HD-GL2 proteins. Phylogenetic reconstructions and intron pattern analyses revealed that PAHB2 clearly belongs to the well-supported subgroup comprising two Arabidopsis proteins ANL2 and GL2-1, and three maize proteins, OCL1, OCL2 and OCL3, but not PAHB1. Therefore, PAHB proteins fall within two distinct clades comprising highly similar angiosperm homologues,

which suggests that at least two distinct HD-GL2 genes already existed in the last common ancestor of angiosperms and gymnosperms

The *PaHB2* gene is expressed in roots, needles, hypocotyls, reproductive organs, proliferating embryonic cells and mature somatic embryos. The spatial expression pattern of the *PaHB2* gene was investigated by mRNA *in situ* analysis. During somatic embryogenesis, the *PaHB2* gene was uniformly expressed in proembryogenic masses and in early somatic embryos but it was not detectably transcribed at the beginning of late embryogeny. Later, in mature embryos, the *PaHB2* gene exhibited a spatial expression localised to the root cap and the cortical layers of both the primary root and the hypocotyl. Histological studies of embryos from conifers have generally been interpreted to imply that cortex specification occurs early during late embryogeny (Filonova *et al*., 2000b; Rombeger *et al*., 1993). However, *PaHB2* expression was not detected at this stage. Therefore, involvement of PAHB2 in the specification and/or maintenance of the cortex identity remains to be verified. As mentioned before, PAHB2 is homologous to the *Arabidopsis* ANL2 protein that was suggested to be functional in the sub-epidermal layers of the roots. Therefore, despite the evolutionary distance between angiosperms and gymnosperms, the *PaHB2* and *ANL2* homologues share a highly conserved gene structure, protein sequence and probably gene expression pattern in the root.

Conclusions

The HD-GL2 family consists of at least three groups of closely related proteins. The PAHB proteins fall within two distinct subclasses comprising highly similar angiosperm homologues. The PAHB1 groups with protoderm/epiderm-specific HD-GL2, while PAHB2 clusters with subepidermal- and protoderm/epiderm-specific members of HD-GL2. We have therefore shown that expression of the HD-GL2 genes can be used as a molecular marker to study establishment of the radial pattern in both angiosperms and gymnosperms.

Future perspectives

We have shown that LCOs, chitinases and AGPs are involved in the regulation of somatic embryogenesis in Norway spruce. However, the mechanism of their action needs to be characterised.

The chemical composition of LCO-C should be elucidated. However, embryogenic cultures of Norway spruce secrete only nanomolar concentrations of LCOs, so it is difficult to obtain relatively large amounts of pure compounds that are required for chemical analysis. This problem can be solved either by establishing more efficient methods of purification or by manipulating the culture so that more LCOs are produced.

At present, we do not know how the amount of LCOs is regulated in Norway spruce. We showed that inhibition of chitinase activity by allosamidin results in an increase of the amount of LCO present in the medium. However, *in vitro* assays showed that LCO-C is also degradable by chitinase from *S.griseus*. Different chitinases might be involved in degradation and/or production of LCOs. Hence, more studies aimed at describing the function of chitinases and the origin of LCOs are needed.

I have suggested that CHIA4-Pa1 is transported towards the place where a substrate is localised. Two main questions remain to be answered: (i) where and how is the CHIA4-Pa1 protein transported? (ii) what are the substrates for CHIA4-Pa1? *In vitro* transcription and translation of the *Chia4-Pa* chitinases could allow for production of labelled and active chitinase that after being added to the embryogenic cultures of Norway spruce should be able to compete with native CHIA4-Pa1 for recognition and binding to a substrate. However, more experiments e.g. protein-protein interactions would be needed in order to identify the substrate(s) for CHIA4-Pa1.

Chitinases might act on AGPs resulting in release of LCOs. Yariv reagent is known to bind specifically to AGPs, however the mechanism of this recognition is not clear. Our preliminary data suggested that addition of Yariv reagent induces PCD, thereby opposing the action of LCO-C. In addition, an *in vitro* assay indicated that Yariv reagent binds to the LCO-C. Therefore, I suggest that Yariv reagent binds to the LCO-part of AGP and thereby protects the AGP-molecule from degradation by chitinase. However, this hypothesis needs to be tested.

At present, we know that LCO-total contains LCO-C, PGRs and other putative signaling molecules i.e. AGPs, protein(s) serologically related to sugar beet CH4 chitinase and probably flavonoids. However, different components of LCO-total need to be separated and assayed for their biological activity.

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