

Endogenous Nod-Factor-Like Signal Molecules Promote Early Somatic Embryo Development in Norway Spruce¹

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Embryogenic cultures of Norway spruce (*Picea abies*) are composed of pro-embryogenic masses (PEMs) and somatic embryos of various developmental stages. Auxin is important for PEM formation and proliferation. In this report we show that depletion of auxin blocks PEM development and causes large-scale cell death. Extracts of the media conditioned by embryogenic cultures stimulate development of PEM aggregates in auxin-deficient cultures. Partial characterization of the conditioning factor shows that it is a lipophilic, low-molecular-weight molecule, which is sensitive to chitinase and contains GlcNAc residues. On the basis of this information, we propose that the factor is a lipophilic chitin oligosaccharide (LCO). The amount of LCO correlates to the developmental stages of PEMs and embryos, with the highest level in the media conditioned by developmentally blocked cultures. LCO is not present in nonembryogenic cultures. Cell death, induced by withdrawal of auxin, is suppressed by extra supply of endogenous LCO or Nod factor from *Rhizobium* sp. NGR234. The effect can be mimicked by a chitotetraose or chitinase from *Streptomyces griseus*. Taken together, our data suggest that endogenous LCO acts as a signal molecule stimulating PEM and early embryo development in Norway spruce.

The involvement of extracellular signal molecules in somatic embryogenesis has been reported in several plant species. Already in 1980 it was shown that when nonembryogenic cultures were treated with growth medium conditioned by highly embryogenic cultures, the cultures became embryogenic (Hari, 1980). Several components in the conditioned growth medium have been found to promote somatic embryogenesis. These components include chitinases (De Jong et al., 1992; Egertsdotter et al., 1993) and arabinogalactan proteins (AGPs; Kreuger and Van Holst, 1993, 1995; Egertsdotter and von Arnold, 1995; Thompson and Knox, 1998; Chapman et al., 2000). It has been suggested that oligosaccharides released from AGPs by a chitinase act as signal molecules stimulating somatic embryogenesis (Van Hengel et al., 2001).

Oligosaccharides with signaling functions, oligosaccharins (Darvill et al., 1992), are involved in the regulation of the plant growth and development (for review, see Spiro et al., 1998). Oligosaccharins of endogenous nature are suggested as activating factors in differentiation of tracheary elements in zinnia (*Zinnia elegans*; Roberts et al., 1997; Groover and

Jones, 1999), *Fucus* sp. embryo patterning (Bouget et al., 1998) and regeneration of roots or flowers in thin-cell-layer explants of tobacco (*Nicotiana tabacum*; Eberhard et al., 1989). In addition, endogenous oligosaccharins (McCabe et al., 1997b; Van Hengel et al., 2001) and Nod factors, oligosaccharins isolated from *Rhizobium*, promote embryogenesis in plants (De Jong et al., 1993; Egertsdotter and von Arnold, 1998; Dyachok et al., 2000).

Nod factors are a family of lipo-chitooligosaccharide (LCO) signals uniformly consisting of an oligosaccharide backbone of β -1,4-linked GlcNAc residues varying in length between three and five sugar units, with an *N*-linked fatty acid moiety replacing the *N*-acetyl group on the nonreducing end. Because of their lipophilicity, Nod factors are generally isolated by the reverse-phase extraction, followed by purification by reverse-phase thin-layer chromatography or HPLC (Spaink et al., 1991; Price et al., 1992). In the absence of a suitable chromophore, LCOs are often labeled by metabolic incorporation of D -[1-¹⁴C] glucosamine into the chitin oligomeric backbone (Price and Carlson, 1995). Sensitivity to chitinases and a positive reaction to the modified Morgan-Elson assay are further evidences for the presence of a β -1,4-linked GlcNAc backbone. In addition, LCOs are further defined by their ability to induce certain morphological responses on plants.

It has long been known that Nod factors produced by rhizobia induce cell divisions in the root cortex of the host legume, leading to the formation of nodules (Spaink et al., 1991; Truchet et al., 1991). Furthermore, in Norway spruce (*Picea abies*), Nod factors can substitute for auxin and cytokinin to promote cell division (Dyachok et al., 2000). They also promote the

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development of pro-embryogenic masses (PEMs) from small cell aggregates in Norway spruce (Egertsdotter and von Arnold, 1998; Dyachok et al., 2000). In carrot (*Daucus carota*), Nod factors stimulate somatic embryos to proceed to the late globular stage (De Jong et al., 1993). Both in carrot and Norway spruce embryogenic systems, bacterial Nod factors can substitute for chitinases in their effect on early somatic embryo development (De Jong et al., 1992; Egertsdotter and von Arnold, 1998).

A homolog of the early nodulin gene *ENOD40*, *OsENOD40*, has been isolated from rice (*Oryza sativa*; Kouchi et al., 1999). In transgenic soybean (*Glycine max*), the *OsENOD40* is expressed in peripheral nodule cells, suggesting that *OsENOD40* and legume *ENOD40* have similar functions in plants. Furthermore, the expression of another early nodulin gene from legume, *ENOD12*, in transgenic rice is stimulated by rhizobial Nod factors (Reddy et al., 1998). This demonstrates that the perception and transduction machinery required for the activation of this leguminous promoter by Nod factors is present in a non-legume.

Somatic embryogenesis in Norway spruce is a multistep regeneration process, which starts with development of PEMs, followed by somatic embryo formation, maturation, desiccation, and plant regeneration. Embryogenic cultures contain a large number of PEMs that have reached various developmental stages, as well as early somatic embryos. Plant growth regulators (PGRs) auxin and cytokinin are required for the proliferation of PEMs, which includes subsequent development of PEMI through PEMII into PEMIII (Fig. 1). At stages of PEMI and PEMII the cell aggregates lack the organization and

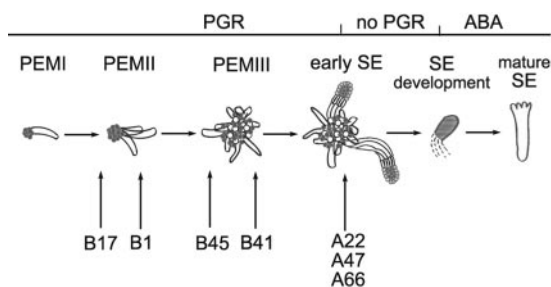


Figure 1. Schematic overview of somatic embryogenesis in Norway spruce (adapted from Filonova et al., 2000b). Proliferating cultures supplemented with the PGR cytokinin and auxin contain PEMs and early somatic embryos (SEs). PGRs are required for proliferation of PEMs, which includes subsequent development of PEMI through PEMII into PEMIII. Trans-differentiation of SEs from PEMIII is stimulated by withdrawal of PGRs. Abscisic acid (ABA) is required for development of mature SEs. Arrows under the developmental path indicate the most developed stage present in different cell lines under proliferation conditions in the presence of PGRs. In normal cell lines (A22, A47, and A66) SEs start to differentiate in the presence of PGRs. In cell lines B41 and B45, no SEs are formed but PEMIII are present. Cell line B41 contains more PEMIII than B45. Cell lines B1 and B17 contain only PEMI and PEMII. Cell line B1 contains more PEMII than B17.

size that are needed for the differentiation of somatic embryos, whereas PEMIII is the stage when somatic embryos differentiate. Depletion of PGRs stimulates differentiation of somatic embryos from PEMIII (Filonova et al., 2000b). The rhizobial Nod factor and chitinases stimulate PEM development but not further embryo development (Egertsdotter and von Arnold, 1998; Dyachok et al., 2000).

In this work, we describe a biologically active Nod-factor-like compound that is present in conditioned medium from embryogenic cultures of Norway spruce. We show that endogenous LCO and rhizobial Nod factor stimulate early stages of somatic embryogenesis in Norway spruce. We also suggest a possible mechanism of LCO's action by demonstrating that LCOs suppress death of embryogenic cells.

RESULTS

Isolation of Extracellular Lipophilic Chitoooligosaccharides from Embryogenic Cultures of Norway Spruce

When fractionated embryogenic cells were cultured in medium supplemented with the culture filtrate from embryogenic suspension cultures, the proliferation of PEMs was stimulated (data not shown). Based on previous work (Dyachok et al., 2000), we assumed that the stimulatory activity was related to LCO(s).

Lipophilic compounds were extracted from culture filtrates of embryogenic suspensions of different cell lines. Extracts were analyzed for the presence of GlcNAc-containing compounds using Morgan-Elson assay. Morgan-Elson positive compounds were found in lipophilic extracts from embryogenic suspension cultures but not in lipophilic extracts from nonembryogenic cultures (Fig. 2). The amount of Morgan-Elson positive compounds present in embryogenic cultures correlated to the developmental stage of PEMs and somatic embryos present in the cell line (Fig. 1). The content was higher in cell lines consisting of only PEMI and PEMII (B17 and B1), and lower in cell lines with developed somatic embryos (A66, A22, and A47). Addition of the chitinase inhibitor, allosamidin, at 10^{-6} M to the cell line B17 increased the content of Morgan-Elson positive compounds significantly from 1.1×10^{-8} M to 1.6×10^{-8} M (estimated using Student *t* test at $P \leq 0.05$).

The lipophilic extract from embryogenic cultures of cell line B1 grown in the presence of sodium [^{14}C]acetate was fractionated using the elution of the reverse-phase cartridge by successively increasing concentrations of methanol in water. The highest radioactivity was found in a fraction eluted by 80% (v/v) methanol (Fig. 3A). Eluates from the reverse-phase cartridge upon extraction of nonradiolabeled cultures (2 L) were further tested for their effect on PEM proliferation. In the absence of auxin, 9% of the PEMs proliferated into PEM aggregates as compared

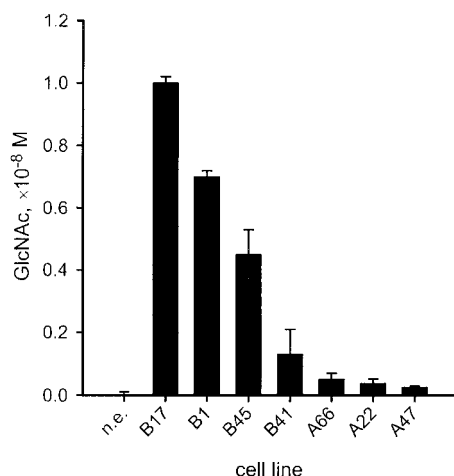


Figure 2. Content of GlcNAc in Norway spruce suspension cultures. A nonembryogenic (n.e.) cell line and cell lines of group B (B17, B1, B45, and B41) and A (A66, A22, and A47) were cultured for 2 weeks. Lipophilic compounds were extracted from 500 mL of culture filtrates, and the amount of Morgan-Elson positive compounds was determined in the extracts. The data represent means \pm SE of two to three independent measurements per cell line.

with 18% for those cultured with 2,4-D (Fig. 3B). Addition of the NGR234 Nod factor stimulated PEM proliferation. Similar stimulation was obtained when the nonseparated 100% (v/v) methanol extract or the 80% (v/v) methanol fraction was added.

The 80% (v/v) methanol fraction from B1 cultures grown in the presence of *N*-acetyl-D-[1- 14 C]glucosamine was further separated using HPLC (Fig. 4A). The HPLC separation was repeated three times with different culture filtrates. In all cases, the fractions with the retention times (Rt) 13 to 22 min contained 14 C-labeled compounds. However, most radioactivity of the culture filtrate extract was found in the injection peak (Rt 0–1 min). In some culture filtrates there was also incorporation of radioactivity from *N*-acetyl-D-[1- 14 C]glucosamine into fractions with Rt 25, 27 to 28, 30 to 31, and 35 to 39 min (data not shown). A large volume of nonradiolabeled culture filtrate was separated using HPLC and assayed for Morgan-Elson positive compounds (Fig. 4B). Morgan-Elson positive compounds were detected in fractions with Rt 0 to 1 min and 13 to 22 min (Fig. 4B). Fractions that contained Morgan-Elson positive compounds and fractions labeled with *N*-acetyl-D-[1- 14 C]glucosamine had the same Rt (Fig. 4, A and B). The experiment was repeated twice with similar results.

Culture filtrate fractionated by HPLC was analyzed by gas chromatography-mass spectrometry (GC-MS) for constituent monosaccharides. A GC peak corresponding to GlcN was obtained in fractions with the Rt 0 to 4 min (fraction A), 5 to 9 min (fraction B), and 13 to 17 min (fraction C), but not in the later fractions (Table I). The experiment was repeated three times with similar results. HPLC fractions were further tested for their ability to stimulate

PEM proliferation. Fractions A and C stimulated PEM proliferation in a similar way as the nonseparated extract (Table I). MALDI-TOF mass spectra of the isolated fraction C revealed low- M_r compound(s) (M_r approximately 700) (data not shown).

To test if the biological activity of fraction C is related to chitin containing compounds, we assayed the sensitivity to treatment with chitinase from *Streptomyces griseus* (Table II). In cultures supplemented with concentrated compounds from fraction C of the nontreated extract, the frequency of proliferating PEMs was significantly higher compared with the control (Table II). Pretreatment of the extract with chitinase significantly decreased the amount of GlcN in fraction C and resulted in the loss of the stimulatory effect of fraction C on PEM proliferation (Table II).

We thus found that embryogenic cultures of spruce produce a compound that stimulates PEM proliferation in a similar way as rhizobial Nod factor. The biologically active compound is lipophilic, contains GlcNAc, is sensitive to chitinase, and has a low M_r .

Influence of Chitin Oligosaccharides and Chitinase on Embryogenic Cultures

The 80- to 160- μ m fraction of the cell line A22 consists of single cells and small PEMs (PEMI and PEMII; Fig. 5A). When cultured in medium containing benzyladenine (BA) and 2,4-D for 5 d, some PEMs proliferated while others died (Fig. 5C and E). Proliferating PEMs consisted of two types of cells, small meristematic cells and vacuolated cells (Fig. 5C). Most of the cells in proliferating PEMs were fluorescein diacetate (FDA)-positive, which indicated their viability (data not shown). Very few TUNEL-positive cells were present in proliferating PEMs (Fig. 5D). On the contrary, dead structures consisted of cells expressing morphological features of cell death, such as condensed and shrunken cytoplasm, and small and condensed nucleus (Fig. 5E). Most of the dead cells excluded FDA but were TUNEL-positive (Fig. 5F). Fractionated cultures proliferated and formed large cell aggregates consisting of PEMIII (>600 μ m) and somatic embryos after 3 weeks (Fig. 5, B and G). The newly formed embryos could be distinguished by the presence of the embryonal mass consisting of densely packed small meristematic cells and the suspensor formed by the long vacuolated cells (Fig. 5, G and H).

Three-day-old cultures were fractionated using nylon mesh, and the 80- to 160- μ m cell fraction was transferred to medium supplemented with BA but free from 2,4-D. Cell death was assayed in cultures 5 d after fractionating. In the control cultures 70% of the cell aggregates were dead (Table III). Addition of 2,4-D significantly decreased the number of dead cell aggregates to 25%. The absence of 2,4-D could be

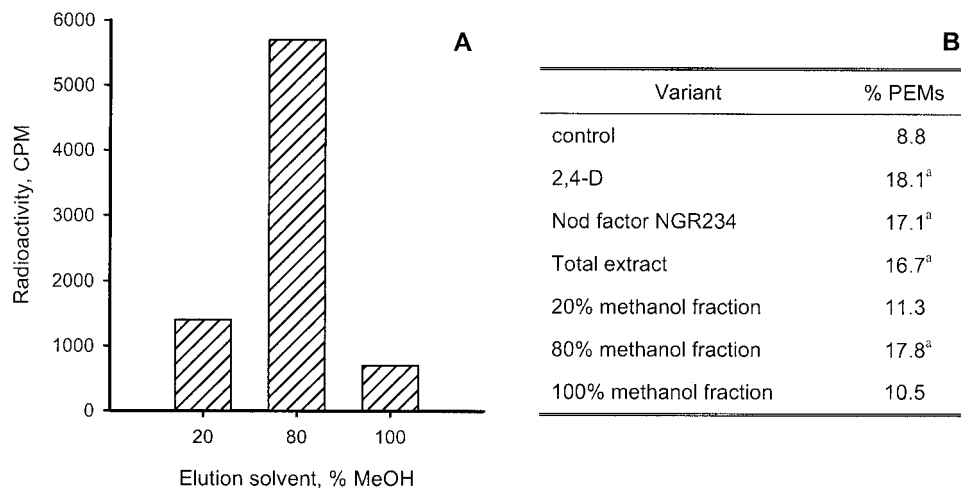


Figure 3. Metabolic labeling and biological activity of lipophilic compounds in culture filtrates from embryogenic cultures of Norway spruce. Suspension cultures (20 mL) of cell line B1 were grown in the presence of sodium [^{14}C] acetate for 7 d. A larger volume (2 L) of this cell line also was cultured without radiolabeled precursor. Lipophilic compounds extracted by reverse-phase cartridge were eluted in one portion with 100% (v/v) methanol (total extract) or in sequential fractions with 20% (v/v), 80% (v/v), and 100% (v/v) methanol in water. A, Fractions of radiolabeled extract assayed for ^{14}C radioactivity. B, Effect of different fractions on PEM proliferation. Total extract (amount corresponded to 10^{-8} M GlcNAc) or equivalent aliquots of fractions were added to the 80- to 160- μm fraction of A22 cultures. As an alternative, 9×10^{-6} M 2,4-D or 10^{-8} M Nod factor NGR234 were added to the fractionated cell cultures. The frequency of proliferating PEMs was determined after 3 weeks. The data are based on 2,000 PEMs per trial. ^aSignificantly different from the control as estimated using Z-test ($P \leq 0.05$).

compensated for by addition of fraction C, Nod factor, or chitotetraose. The effect could be mimicked by addition of chitinase. In contrast, addition of chitobiose did not significantly influence the number of dead cell aggregates.

After 3 weeks of growth, fractionated cultures were analyzed for proliferation and presence of somatic embryos. In the control cultures, 8% of small PEMs had proliferated (Table III). However, proliferation frequency increased to 21% when 2,4-D was added to the medium. In the absence of 2,4-D, proliferation was enhanced significantly by the addition of fraction C, Nod factor, chitotetraose, or chitinase but not by chitobiose (Table III). Regression analysis revealed negative correlation between the frequency of dead structures and the percentage of proliferating PEMs ($r^2 = 0.951$).

The formation of somatic embryos in control cultures was poor (Table III). However, somatic embryo formation was stimulated when 2,4-D was added to the medium. Addition of fraction C, Nod factor, chitotetraose, or chitinase significantly enhanced formation of somatic embryos in the absence of 2,4-D (Table III). Addition of chitobiose did not significantly influence formation of somatic embryos. A positive correlation ($r^2 = 0.836$) was seen between the frequency of proliferating PEMs and the number of somatic embryos.

We thus found that the frequency of cell death decreases upon addition of 2,4-D with simultaneous increase in PEM and embryo formation, and that addition of fraction C of culture filtrate, Nod factor,

chitotetraose, or chitinase, but not chitobiose, could substitute for 2,4-D suppressing cell death and promoting PEM and embryo formation.

DISCUSSION

Embryogenic Cultures of Norway Spruce Produce LCO

To identify endogenous LCO in embryogenic cultures of Norway spruce, we screened cultures for β -1,4-GlcNAc-containing lipophilic compounds with pronounced biological activities. A similar approach was previously used to isolate LCO Nod factors from *Rhizobium* spp. (Spaink et al., 1991; Truchet et al., 1991). Reverse-phase extracts of the media conditioned by embryogenic cultures contain several lipophilic compounds. This is reflected by the presence of several absorbance peaks in the HPLC profile (not shown). We used degradation of the crude extract by chitinase in combination with the bioassay to test whether biological activity in the lipophilic fractions is related to chitin derivatives (Price et al., 1992). This method allowed preliminary identification of at least one fraction, fraction C (Rt 13–17 min), in which the decrease in GlcN content was accompanied by the loss of the ability to stimulate PEM formation. This fraction was also labeled by *N*-acetyl-D-[^{14}C]glucosamine (Price and Carlson, 1995) and was positive in the Morgan-Elson assay for *N*-acetylated aminosugars (Chaplin, 1994). Further-

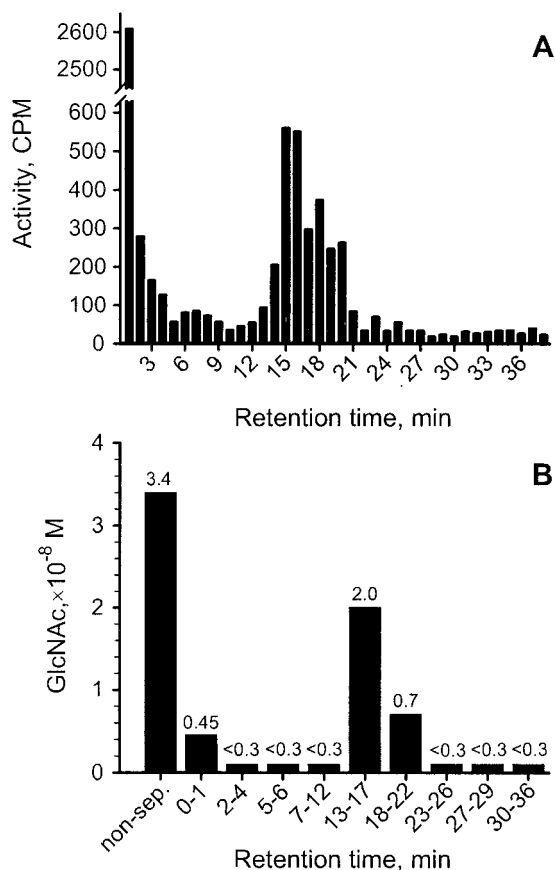


Figure 4. HPLC separation of lipophilic compounds from embryonic cultures of Norway spruce. Suspension cultures (20 mL) of cell line B1 were grown in the presence of *N*-acetyl-D-[1-¹⁴C] glucosamine for 14 d. A larger volume (2 L) of this cell line was also cultured without radiolabeled precursor. Lipophilic compounds were extracted by reverse-phase cartridge and separated by reverse-phase HPLC. A, HPLC fractions of radiolabeled extract assayed for ¹⁴C radioactivity. B, Amount of GlcNAc measured in extract before separation (non-sep), and in aliquot of fractions with Rts: 0 to 1, 2 to 4, 5 to 6, 7 to 12, 13 to 17, 18 to 22, 23 to 26, 27 to 29, and 30 to 36 min.

more, sugar analysis revealed the presence of GlcN. Mass-spectra analysis identified a low- M_r compound. These data indicate that the biological activity of the compound in fraction C with Rt 13 to 17 min is related to LCO.

Oligosaccharide isolated from the conditioned medium of embryonic cultures of Norway spruce can be assigned to a group of oligosaccharins, the oligosaccharides with signaling function (Darvill et al., 1992). To date, biologically active oligosaccharins have been obtained by enzymatic degradation of cell wall polysaccharides, and their presence in planta is still questionable. It has been suggested that plants produce an endogenous oligosaccharin similar in structure to rhizobial Nod factors (Staehelin et al., 1994). Finding endogenous LCO in embryonic cultures of Norway spruce supports this possibility.

The Content of LCO Is Developmentally Regulated

Embryonic suspension cultures of Norway spruce contain PEMs, at different developmental stages, as well as early somatic embryos (Fig. 1). PEMs and embryos of a certain degree of development predominate in each of the cell lines we used in this study. Type B cell lines with only PEMI and PEMII have the highest content of extracellular LCOs, corresponding to 10^{-8} M GlcNAc. In type A cultures, which also contain PEMIII and somatic embryos, the extracellular LCO concentration is significantly lower. It is interesting that LCOs were not detected in nonembryonic cultures. These findings indicate that LCOs are stimulating early processes during somatic embryogenesis. In consistence, *Rhizobium* sp. NGR234 Nod factor was previously found to stimulate PEM formation but not further embryo development (Egertsdotter and von Arnold, 1998; Dyachok et al., 2000). Inhibiting chitinase with allosamidin increases LCO content in embryonic cultures. We have previously shown that cells in embryonic cultures secrete chitinases and that there is a close correlation between the presence of specific chitinase and the developmental stage of PEMs and somatic embryos (Mo et al., 1996). At present we do not know if secreted chitinases can degrade LCOs in a similar way as plant chitinases hydrolyze the rhizobial Nod factors (Staehelin et al., 1994, 1995). However, it is tempting to assume that the 28-kD chitinase secreted in type A cultures but not in type B cultures degrades LCOs with the result that the LCO content is lower in type A cultures.

Other data suggest that chitinases are involved in the production of plant signal molecules, similar to the rhizobial Nod factors. The effect of EP3 endochiti-

Table 1. Effect of GlcN-positive lipophilic compounds on PEM proliferation

Lipophilic extracts from culture filtrates of cell line B1 were separated by HPLC. The content of GlcN was measured by GC-MS in the nonseparated 80% (v/v) methanol extract and in fractions with retention times (min): fraction A, 0 to 4; fraction B, 5 to 9; fraction C, 13 to 17; fraction D, 18 to 26. Equivalent aliquots of the nonseparated extract or fractions A, B, C, and D were added to the 80- to 160- μ m fraction of A22 cultures. As an alternative, 9×10^{-6} M 2,4-D was added to the fractionated cultures. The frequency of proliferating PEMs was determined after 3 weeks. The data are based on 2,000 PEMs per trial.

Variant	GlcN $\times 10^{-8}$ M	PEMs %
Control		8.5
2,4-D		17.6 ^a
80% (v/v) methanol extract, nonseparated	2.0	14.7 ^a
Fraction A	0.5	13.6 ^a
Fraction B	0.2	10.8
Fraction C	0.8	15.9 ^a
Fraction D	<0.1	7.5

^a Significantly different from the control as estimated using Z-test ($P \leq 0.05$).

Table II. Effect of chitinase treatment on lipophilic compounds from embryogenic cell line B1 of Norway spruce

Equivalent aliquots of the nonseparated 80% (v/v) methanol extract were subjected to incubation without (–) or with (+) chitinase from *S. griseus* and thereafter separated by HPLC. Radiolabeled compounds were used as markers for HPLC separation. The GlcN content of the fractions coeluting with ¹⁴C radioactivity was measured by GC-MS. Equivalent aliquots of the nonseparated extract, fraction C of the non-digested extract, or fraction C of the extract treated with chitinase were added to the 80- to 160- μ m fraction of A22 cultures. As an alternative, 9×10^{-6} M 2,4-D or 10^{-8} M Nod factor NGR234 was added to the fractionated cell cultures. The frequency of proliferating PEMs was determined after 3 weeks. The data are based on 2,000 PEMs per trial.

Variant	Pretreatment with Chitinase	GlcN $\times 10^{-8}$ M	PEMs %
Control			8.00
2,4-D			18.20 ^a
Nod factor NGR234			14.10 ^a
80% (v/v) methanol extract, nonseparated	–	1.6	17.50 ^a
Fraction C	–	0.7	16.15 ^a
Fraction C	+	<0.1	6.90

^a Significantly different from the control as estimated using Z-test ($P \leq 0.05$).

nase promoting somatic embryo development in carrot could be mimicked by rhizobial LCOs (De Jong et al., 1993). The EP3 chitinase colocalizes with AGPs in developing seeds, and it was shown to cleave AGPs in vitro (Van Hengel et al., 2001). Extracellular AGPs stimulate somatic embryogenesis in carrot. The stimulatory effect is enhanced if AGPs are first treated with EP3 (Van Hengel et al., 2001). These results suggest that AGPs are a substrate for EP3. This is supported by the finding that the nurse cells in carrot embryogenic cultures have specific AGP epitopes and release an embryogenesis promoting carbohydrate compound into the medium (McCabe et al., 1997b). Furthermore, in this study we show that chitinase from *S. griseus* stimulates PEM growth in a similar way as endogenous LCO. In accordance, endochitinase of class IV from sugar beet was found to stimulate early embryo development in Norway spruce in a similar way as LCO Nod factors from *Rhizobium* (Egertsdotter and von Arnold, 1998; Dyachok et al., 2000). Taken together these data indicate that different chitinases regulate embryogenesis in different ways. Some chitinases, such as the 28-kD chitinase specifically secreted by type A cultures, degrade LCOs, whereas others, such as the *S. griseus* chitinase and EP3, are involved in the formation of LCOs. It has been previously shown that enzymes that form and degrade oligosaccharins are largely responsible for when and where oligosaccharins are active in plant tissues (Albersheim et al., 1994). Chitinases might therefore be part of such a regulatory mechanism involving production and degradation of LCO oligosaccharins.

LCOs and Chitinase Stimulate Survival and Growth of PEMs

Embryogenic capacity of PEMs is closely related to the stage of development. According to their mor-

phology and size, PEMs were divided into three groups, I, II, and III (Filonova et al., 2000b). PEMIII have an average size greater than 600 μ m and after withdrawal of PGR give rise to somatic embryos. PEMs of smaller sizes, PEMI and PEMII, cannot differentiate somatic embryos. We used fractionated cultures consisting of PEMI and PEMII for the bioassay to examine specifically the effect of endogenous LCO and chitinase from *S. griseus* on early PEM development. Nod factor from *Rhizobium* sp. NGR234 and chitin fragments were tested for reference. Our data show that in Norway spruce endogenous and rhizobial LCOs, chitotetraose, and chitinase from *S. griseus* stimulate proliferation of PEMs. These results are consistent with previous data showing that Nod factors from *Rhizobium* and endochitinase from sugar beet stimulate the early stages of somatic embryogenesis in Norway spruce by promoting division of embryogenic cells (Egertsdotter and von Arnold, 1998; Dyachok et al., 2000). Stimulated PEM proliferation results in formation of PEMIII, large-sized PEMs giving rise to somatic embryos. We therefore propose that the increased number of embryos is a result of stimulating cell division and subsequent growth of PEMIII in cultures.

Chitotetraose but not chitobiose mimics the promotive effect of LCOs on PEM development. This indicates that the size of the chitin oligosaccharide is crucial. In accordance, synthetic chitin oligosaccharides inducing cortical cell division in a host plant always contain a carbohydrate core of four or more GlcNAc residues (Schlaman et al., 1997). Chitin oligosaccharides higher than trisaccharides are necessary to induce alkalization response in cultures of tomato (Baureithel et al., 1994). The lipid part of the molecule does not appear to be required for stimulating somatic embryo development in Norway spruce. Similarly, chitin pentase induces transient expression of the early nodulin gene *ENOD40* in

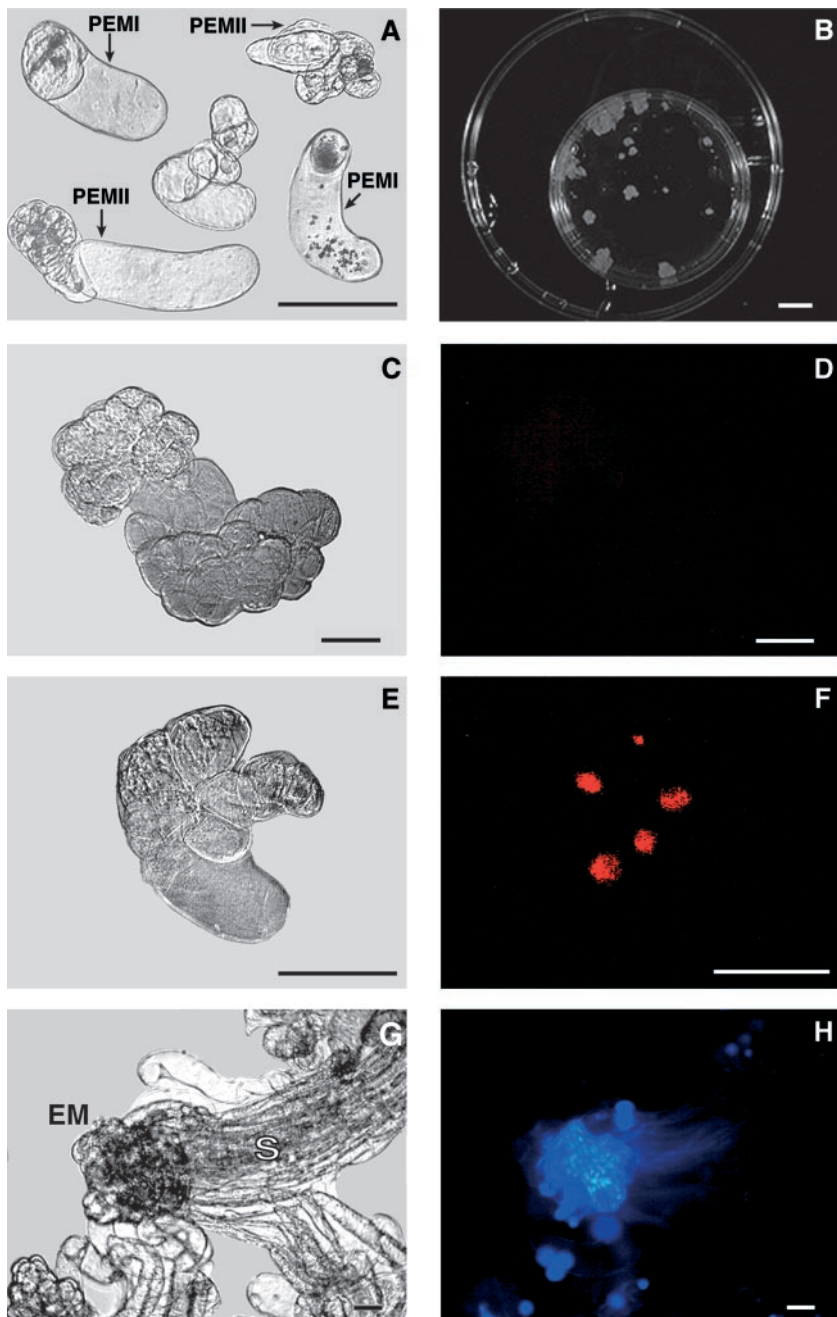


Figure 5. Development of PEMs in proliferation medium. Cell suspensions of cell line A22 were fractionated, and the 80- to 160- μm fraction consisting of single cells and small PEMs (PEMI and PEMII) was transferred to the medium supplemented with 9×10^{-6} M 2,4-D and 4.4×10^{-6} M BA. A, Day 0 (light microscopy). B, Examples of PEMI and PEMII at d 21. C to F, Examples of PEM aggregates after 5 d. Examples of proliferating (C and D) and dead (E and F) PEMs. C and E, Light microscopy. D and F, Labeling with TUNEL. Notice poor labeling in proliferating PEM and bright fluorescence in dead PEM. G and H, d 21. Example of a somatic embryo. G, Light microscopy. EM, Embryonal mass. S, Suspensor. H, Labeling with 4,6-diamidino-2-phenylindole. Notice bright fluorescence in the EM region consisting of small, densely packed cells. Bars, 100 μm in A and in C to H; 1 cm in B.

soybean roots when applied externally (Minami et al., 1996), and the chitin core devoid of the lipid part is sufficient to induce the mitogenic response once the molecule is delivered inside the cell (Schlaman et al., 1997). This indicates the crucial role of the chitin core for the activity of chitin oligosaccharides. However, the lipid moiety may still be important for the signaling function of oligosaccharins in planta.

Differentiation of somatic embryos from PEMIII in Norway spruce is accompanied by a large-scale programmed cell death (PCD; Filonova et al., 2000a). PCD is induced in embryogenic cultures of Norway spruce by withdrawal of PGRs (Filonova et al.,

2000a). In this study we have shown that withdrawal of auxin induces PCD in PEMI and PEMII. Addition of LCOs, chitotetraose, or chitinase suppresses PCD. Similarly, carrot cells cultured at low density activate a PCD pathway that can be prevented by addition of cell free conditioned medium (McCabe et al., 1997a). It has previously been postulated that PCD occurs by default unless a constant supply of signal molecules released by the other cells keep it suppressed (Raff, 1992; Jacobson et al., 1997). β -D-glucosyl Yariv phenylglycoside, a chemical that specifically binds AGPs, inhibited growth of suspension-cultured cells of rose (Serpe and Nothnagel, 1994) and Arabidopsis

Table III. Effects of 2,4-D, Nod factor NGR234, LCO fraction, chitin oligosaccharides, and chitinase on survival and proliferation of PEMs and on differentiation of somatic embryos

Lipophilic compounds were extracted from culture filtrates of 14-d-old cultures of the B1 cell line. Extract was separated by reverse-phase HPLC, and the fraction corresponding to the retention times 13 to 17 min (fraction C) was collected. The GlcN content of the fraction was measured by GC-MS. 2,4-D (9×10^{-6} M), Nod factor (10^{-8} M), fraction C (10^{-8} M GlcN), chitobiose (10^{-8} M), chitotetraose (10^{-8} M), or chitinase from *S. griseus* (5.7×10^{-3} units mL^{-1}) were added to the 80- to 160- μm fraction of A22 cultures. The frequency of dead cell aggregates was determined after 5 d. The frequency of proliferating PEMs and somatic embryos was determined after 3 weeks. The data are based on 2,000 PEMs per trial.

Variant	Dead Aggregates %	PEMs	Embryos per 100 str
Control	70.7	7.8	18.8
2,4-D	25.5 ^a	20.9 ^a	37.5 ^a
Nod factor NGR234	37.0 ^a	15.7 ^a	26.8 ^a
Fraction C	40.0 ^a	16.3 ^a	26.6 ^a
Chitobiose	64.8	8.5	16.2
Chitotetraose	44.7 ^a	16.7 ^a	25.0 ^a
Chitinase	27.4 ^a	22.1 ^a	47.0 ^a

^a Significantly different from corresponding controls as estimated using Z-test ($P \leq 0.05$).

(Gao and Showalter, 1999), implicating AGPs or their derivatives as signaling molecules suppressing cell death. Similarly, we showed that chitin oligosaccharides and chitinase substitute for auxin in suppressing cell death in embryogenic cultures of Norway spruce.

In conclusion, our findings support the hypothesis that LCOs analogous to the rhizobial Nod factors do occur in plants. We have identified such an LCO in embryogenic cultures of Norway spruce. The amount of LCO is developmentally regulated. The endogenous LCO suppresses cell death in embryogenic cultures of Norway spruce in a similar manner as the bacterial Nod factors do. The future work will address the precise structure of endogenous compounds and their role in plant morphogenesis.

MATERIALS AND METHODS

Embryogenic and Nonembryogenic Cultures

Embryogenic suspension cultures of Norway spruce (*Picea abies* [L.] Karst) were maintained in LP medium as described earlier (Egertsdotter and von Arnold, 1993). Cell lines A22, A47, A66, B1, B17, B41, and B45 were used in these studies. The A cell lines were subcultured into fresh one-half-strength LP medium weekly, and the B cell lines were subcultured at 2-week intervals.

Somatic embryos were present in group A cell lines A22, A47, and A66, whereas group B cell lines B41, B45, B1, and B17 contained PEMs only (Fig. 1). The B cell lines were rated in order of their degree of development based on the size and organization of PEMs: B41 > B45 > B1 > B17. Cell line B41 contains large PEMs corresponding to PEMIII,

whereas cell line B17 contains small PEMs corresponding to PEMI and PEMII (Fig. 1; Filonova et al., 2000b).

Nonembryogenic cultures were maintained as described earlier (Egertsdotter and von Arnold, 1995). The cell line NE1 was used in this study. The nonembryogenic suspension cultures were subcultured weekly.

Identification of LCO in Embryogenic Cultures of Norway Spruce

Radiolabeling and Extraction of LCO Fraction

Embryogenic cultures of cell line B1 (20 mL) were labeled with *N*-acetyl-D-[1-¹⁴C]glucosamine or sodium [1-¹⁴C]acetate (1 $\mu\text{Ci mL}^{-1}$), for 2 weeks.

Extraction of the LCO fraction was achieved by passing culture filtrates through a C18 reverse-phase silica cartridge (Chromabond, Chromos Express Ltd., Macclesfield, Cheshire, UK). After washing the cartridge with five volumes of water, compounds bound to the cartridge were eluted with 3-mL volume of 100% (v/v) methanol. As an alternative, cartridge bound compounds were eluted sequentially with 3-mL volumes of 20% (v/v) methanol in water, 80% (v/v) methanol in water, and 100% (v/v) methanol. Methanol fractions were evaporated to dryness under an airflow. The radioactivity in each fraction was assayed by scintillation counting (liquid scintillation counter 1209 Rackbeta, LKB, Uppsala, Sweden).

HPLC

The radiolabeled samples of culture filtrate extract were redissolved in aqueous 20% (v/v) acetonitrile. Aliquots of radiolabeled extract (20 μL , 5–10 nCi) were separated on an analytical C18 reverse-phase LiChrospher column (4 \times 125 mm) using isocratic conditions of aqueous 20% (v/v) acetonitrile for 10 min, followed by a linear gradient to 100% (v/v) acetonitrile within 30 min at a flow rate of 2 mL min^{-1} . The eluate was monitored at 206 nm, and the radioactivity in fractions was measured by scintillation counting.

Morgan-Elson Assay

Culture filtrates (0.5–2.0 L) were extracted by passing through a reverse-phase cartridge. After washing the cartridge with 5 volumes of water, bound compounds were eluted with a volume of 100% (v/v) methanol. The free GlcNAc content of the extract was measured by the Morgan-Elson reaction, using the procedure of Chaplin (1994). The glycosidic linkages in GlcNAc chains were hydrolyzed with trifluoroacetic acid:acetic acid:water (1:15:4, v/v) for 2 h at 100°C before Morgan-Elson analysis. Hydrolyzed samples were evaporated to dryness, redissolved in water, and assayed for free GlcNAc.

Compositional and Structural Analysis of LCO

MALDI-TOF mass spectra were recorded in positive detection mode on a Linear LDI 1700 XS mass spectrom-

ter, using a dihydroxybenzoic acid matrix (100 mM dihydroxybenzoic acid in 50% [v/v] methanol in water). GC-MS was performed on a Hewlett-Packard 5989B instrument (Hewlett-Packard, Palo Alto, CA). Samples were initially hydrolyzed with trifluoroacetic acid (2 M, 0.5 mL, 1 h, 121°C). Hydrolyzed sugars were reduced with 3 mg of sodium borohydride in 300 μ L of 1 M ammonium hydroxide. After 1 h at room temperature the reaction was stopped by the drop-wise addition of acetic acid. Peracetylation of the alditols was completed by heating with 0.2 mL of pyridine and 0.2 mL of acetic anhydride (121°C, 30 min). The alditol acetates were then extracted into chloroform. For the quantitative estimation of GlcN, the instrument was calibrated using GlcNAc (Sigma, St. Louis). The GlcNAc content of the samples was determined from the areas of peaks co-eluting with an GlcNAc standard (Rt 29.6 min) and having the electron impact mass spectral fragmentation pattern predicted for GlcNAc alditol acetate. These data were then recalculated to give the initial GlcN content.

Manipulating Content of Morgan-Elson Positive Compounds by the Specific Chitinase Inhibitor Allosamidin

Allosamidin from *Streptomyces* sp. 1713 (Sakuda et al., 1986) was a kind gift of Dr. S. Sakuda (Dept. Applied Biological Chemistry, University of Tokyo). Allosamidin was previously reported to inhibit endochitinase activities in *Pinus sylvestris* roots (Hodge et al., 1996). Allosamidin was dissolved in 0.1 M acetic acid, diluted with growth media, filter sterilized, and added to the suspension cultures of Norway spruce at 10^{-6} M. After 1 week, the GlcNAc content of culture filtrates was determined by Morgan-Elson assay.

Isolation of Partially Purified LCO from Culture Filtrate of Embryogenic Cultures of Norway Spruce

Preparative scale volumes (up to 100 L) of culture filtrates were extracted by passing through C18 reverse-phase silica cartridges. The culture filtrate was extracted in portions of 2.5 to 3.0 L per cartridge. The cartridges were then washed, as described above for the radiolabeled cultures, with 5 volumes of water and eluted sequentially with volumes of 20% (v/v), 80% (v/v), and 100% methanol in water. The methanol phases from all cartridges were pooled and evaporated under an airflow. The residue was redissolved in aqueous 20% (v/v) acetonitrile. Radiolabeled compounds were used as markers for the isolation procedures. Aliquots of extract (370 μ L) were separated by analytical reverse-phase HPLC as described in the "HPLC" section. Further separation was achieved using 20% (v/v) acetonitrile in an aqueous 20 mM ammonium acetate buffer (pH 5.8) for 10 min, followed by a linear gradient to 60% (v/v) acetonitrile within 15 min at a flow rate of 2 mL min^{-1} . The amount of GlcNAc or GlcN in fractions co-eluting with radiolabeled compounds was determined by the Morgan-Elson reaction or by GC-MS, respectively. The nonseparated reverse-phase extract and fractions contain-

ing Morgan-Elson- or GlcN-positive compounds were used in bioassays.

Somatic Embryogenesis Bioassay

The bioassay was established to test the effect of conditioning factors on somatic embryo development in Norway spruce. The bioassay makes it possible to follow the development of small PEMs (PEMI and PEMII). The survival of PEMs was analyzed in parallel with the proliferation of PEMs and embryo formation. Suspension cultures of cell line A22 were fractionated by sequential sieving through nylon meshes with pore sizes of 160 and 80 μ m. The fraction from 80 to 160 μ m in size (80- to 160- μ m fraction) was collected. This fraction consisted of single cells, PEMI and PEMII (Fig. 5A).

The recovered cells were washed thoroughly in liquid one-half-strength LP medium containing 4.4×10^{-6} M BA and then resuspended in the same medium. The number of PEMI and PEMII per mL of the 80- to 160- μ m fraction was counted microscopically and adjusted to approximately 800 PEMs mL^{-1} . Aliquots of 2.5 mL of the suspensions were mixed 1:1 with the medium containing the nonseparated reverse-phase extract or its fractions, Nod factor from *Rhizobium* sp. NGR234, tetra-*N*-acetyl-chitotetraose (chitotetraose, Sigma), *N,N'*-diacetylchitobiose (chitobiose, Sigma), or chitinase from *Streptomyces griseus* (Sigma). Purified broad host range Nod factor NGR234 was a kind gift from Prof. W.J. Broughton (Universite de Geneve, Switzerland). All compounds tested were dissolved in 80% (v/v) methanol in water, except for the chitinase, which was dissolved in water and added to a final volume of 20 μ L per assay. Controls were supplied with 20 μ L of corresponding solvent. As an alternative, 2.5 mL of the suspensions were mixed 1:1 with the medium supplemented with 2,4-D at 1.8×10^{-5} M.

Cultures were plated in agarose medium for the analysis of PEM formation, or grown in liquid medium for the analysis of the cell death and embryo formation. For the analysis of PEM development, the suspensions were mixed 1:1 with the medium containing 1.2% (w/v) low temperature melting agarose, 2-mL aliquots were plated in 60-mm Petri dishes. Cultures were kept under high relative humidity by placing the Petri dishes with cultures within 90-mm Petri dishes containing 5 mL of sterile water. The external dishes were sealed with plastic tape and kept in the dark at 22°C. After 3 to 4 weeks without subculturing, proliferating PEMs had protruded through the agarose layer and formed aggregates of PEMs on the surface of the solid medium (Fig. 5B). During this period, cultures were analyzed for changes in morphology. The frequency of proliferating PEMs was determined as the proportion of the initial PEMI and PEMII that proliferated and formed PEM aggregates (>600 μ m) 3 to 4 weeks after plating.

For the in situ detection of the cell death associated with DNA fragmentation (TUNEL assay) and for the analysis of somatic embryo development, the suspensions containing tested compounds were mixed 1:1 with the liquid medium and cultured in 25-mL Erlenmeyer flasks. The suspensions

(5 mL) were sampled for TUNEL assay after 5 d. The preparations were fixed in 4% (w/v) paraformaldehyde as previously described (Filonova et al., 2000a) and labeled with the in situ cell death detection kit, tetramethylrhodamine-dUTP (TMR)-red (Roche, Basel). As an alternative, the suspensions (5 mL) were sampled for staining with FDA (Sigma) and 4,6-diamidino-2-phenylindole (Boehringer Mannheim, Basel) at d 6, 10, 14, 17 and 21. Samples were examined using a Microphot FXA fluorescent microscope (Nikon, Tokyo).

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