Plant Cell Rep (2001) 20:596–603
DOI 10.1007/s002990100360

M. Elfstrand · C.G. Fossdal · F. Sitbon · O. Olsson
A. Lönneborg · S. von Arnold

Overexpression of the endogenous peroxidase-like gene *spi 2* in transgenic Norway spruce plants results in increased total peroxidase activity and reduced growth

Received: 11 December 2000 / Revision received: 1 April 2001 / Accepted: 9 May 2001 / Published online: 5 September 2001

Abstract Peroxidases constitute a large family of proteins found in all higher plants. Owing to the complexity of the peroxidase isoenzyme family it has been difficult to assess the precise function of individual peroxidase enzymes. In this work we have studied the effects of an endogenous peroxidase-like gene from Norway spruce [*Picea abies* (L.) Karst], *spi 2*, on the development and growth of Norway spruce somatic embryo plants. Embryogenic cells of Norway spruce transformed with *spi 2* under control of the maize *ubi-1* promoter showed up to 40 times higher total peroxidase activity than the control cells; regenerated plants overexpressing *spi 2* showed an increased total peroxidase activity. Based on these results and the overall sequence similarity with cationic peroxidases we conclude that *spi 2* encodes a peroxidase. Overexpression of *spi 2* resulted in increased sensitivity to stress, leading to a reduction in epicotyl formation and in height growth compared with control plants. The plants overexpressing *spi 2* also showed a deeper phloroglucinol staining but similar levels of Klason lignin.

Keywords Peroxidase · Norway spruce · Transformation

Communicated by K. Glimelius

M. Elfstrand · S. von Arnold (✉)
Department of Forest Genetics,
Swedish University of Agricultural Sciences,
P.O. Box 7027, 750 07 Uppsala, Sweden
e-mail: Sara.von.Arnold@sgen.slu.se
Fax: +46-18-673279

C.G. Fossdal · A. Lönneborg
Norwegian Forest Research Institute (NISK),
Högskoleveien 12, 1432 Ås-NLH, Norway

F. Sitbon
Department of Plant Biology,
Swedish University of Agricultural Sciences,
P.O. Box 7080, 750 07 Uppsala, Sweden

O. Olsson
Department of Cell and Molecular Biology,
Göteborg University, Box 462, 405 30 Göteborg, Sweden

Abbreviations *ABA*: Abscisic acid · *BA*: N^6-Benzyl adenine · *2,4-D*: 2,4-Dichlorophenoxyacetic acid

Introduction

Plant peroxidases are a large group of enzymes that are believed to be involved in a number of biological processes – such as lignification (Lagrimini et al. 1997a), cross-linking of the cell-wall proteins (Bradley et al. 1992), auxin catabolism (Lagrimini et al. 1997b), production of oxygen radicals (Bolwell and Wojtaszek 1997) – and to be up-regulated after pathogen attack (Svalheim and Robertsen 1990; Young et al. 1995; Smit and Dubery 1997). Over 40 peroxidase genes have been identified in *Arabidopsis* (Østergaard et al. 1998), and the number of genes is likely to be high in other plant species also. Consequently, dissecting the role of individual peroxidase enzymes is a complicated process. A study of the regulation of 21 rice EST clones displaying functions similar to those of peroxidases showed unique expression patterns among the clones, suggesting distinct functions of individual isozymes (Hiraga et al. 2000). In accordance with this observation, transformation of tobacco and tomato plants with a number of different peroxidase genes (Lagrimini et al. 1990; Kristensen et al. 1997; Kazan et al. 1998; Amaya et al. 1999; El Mansouri et al. 1999) has resulted in various phenotypes. For instance, the overexpression of an endogenous anionic peroxidase in tobacco results in a wilty phenotype, possibly caused by an underdeveloped root system (Lagrimini et al. 1997b). The heterologous expression of peroxidase genes in tobacco also results in changes, such as improved germination under osmotic stress (Amaya et al. 1999) and improved resistance to pathogen attack (Kazan et al. 1998). In tomato, overexpression of the *tpx-1* gene, which encodes a cationic peroxidase, results in significantly increased lignification (El Mansouri et al. 1999).

Our present knowledge of the function of individual peroxidase genes in conifers is limited. There are several

reports on the induction of peroxidase activity in Norway spruce by biotic and abiotic factors (Asiegbu et al. 1993; Baumbusch et al. 1998; Mensen et al. 1998) and on the association of peroxidases with lignification in the needles (Polle et al. 1994). Recently, a cDNA clone, *spi 2*, encoding a putative cationic peroxidase with a predicted molecular mass of 34 kDa and a pI of 9.5, was isolated from a library made from Norway spruce roots infected with the pathogenic oomycete *Pythium dimorphum* (Fossdal 1999). The *spi 2* transcript is present in healthy roots of Norway spruce, and the steady-state level increases transiently after infection with the pathogen *P. dimorphum*. Moreover, the level of SPI 2 protein as well as that of other highly basic peroxidase isozymes increases after the infection with *P. dimorphum* (Fossdal 1999).

To elucidate the role of *spi 2* in growth and lignification we have regenerated transgenic plants of Norway spruce that overexpress *spi 2*. The results presented here demonstrate that overexpression of *spi 2* increases total peroxidase activity and sensitivity to stress. Overexpression of *spi 2* also has a negative effect on height growth but no influence on the amount of Klason lignin.

Materials and methods

Plant materials

Embryogenic cell lines of Norway spruce [*Picea abies* (L.) Karst] were established from mature zygotic embryos (Egertsdotter and von Arnold 1993). Each cell line represents one genotype. Embryogenic cell lines representing the genotypes 95:88:22 and 95:61:21 were used in this study. The cell lines were grown as suspension cultures in proliferation medium (von Arnold 1987) supplemented with 9.0 µ*M* 2,4-D and 4.4 µ*M* BA. The cultures were subcultured weekly and incubated in darkness at 20°C.

Construction of *pUbi.Spi 2.bar*

To generate the plasmid for transforming Norway spruce, pAHC 25 (Christensen and Quail 1996) was cleaved by *Sac*I, and the 3′ overhang was removed by mung bean nuclease treatment. The linearised plasmid was restricted by *Sma*I to release the *uidA* gene from the plasmid. A 1,500-bp *Xho*I cDNA fragment (Fossdal 1999) carrying the *spi 2* gene was blunted by filling in the 5′ overhang and then ligated with the blunted *Sac*I/*Sma*I fragment from pAHC 25. This resulted in a transcriptional fusion between the maize *ubi-1* promoter and the *spi 2* gene. The resulting plasmid was denoted *pUbi.Spi 2.bar*.

Transformation of Norway spruce

Embryogenic cells of Norway spruce were transformed with *pUbi.Spi 2.bar* using a custom-made particle inflow gun as described in Clapham et al. (2000). Transformed cells were selected on proliferation medium containing 1 mg l^{-1} Basta, which corresponds to 0.2 mg l^{-1} phosphinothricin. Resistant colonies appeared 2–6 months after the bombardment and were transferred to proliferation medium supplemented with 450 mg l^{-1} glutamine.

Somatic embryos from the transformed sublines were initially allowed to mature according to Bozkhov and von Arnold (1998). Briefly, proliferating embryogenic cultures were suspended and exposed to a pre-maturation treatment in liquid medium without growth regulators for 1 week before being plated on filter papers placed on BMI-SI medium (Krogstrup 1986) containing 90 m*M* sucrose, 24 µ*M* ABA and 450 mg l^{-1} glutamine, and solidified with 3.5 g l^{-1} gelrite, for maturation. Mature embryos were partially desiccated under high humidity conditions for 21 days, and the partially desiccated embryos were transferred to Schenk-Hildebrandts (Sigma-Aldrich) medium for germination. The maturation protocol was adjusted for the transformed sublines by excluding culture in liquid medium and by shortening the time for partial desiccation to 10 days. The germination frequencies were scored after 1 month. An embryo was considered as germinated if it had developed cotyledons, hypocotyl and a radicle longer than 1 mm. Germinated somatic embryos were transferred to liquid medium (Ingestad 1979). The elongation of the roots (R) and the formation of epicotyls (E) was scored after 1 month on plants from five independent experiments. Due to the unequal distribution of plants in the experiments, the data were joined and the E/R ratio was taken. The E/R ratio denotes the ratio of the number of epicotyls and the number of elongated roots on the plants from each subline. Plants with a developed epicotyl and elongated roots were potted in mineral wool and grown under ex vitro conditions; they were fertilised twice a week with Wallcro nutrient solution. The plants were kept under a regime of 24-h light (220 µmol m^{-2} s^{-1}) and 20°C. Plant height was measured 5 months after potting.

Polymerase chain reaction analysis

Putatively transformed sublines were screened by polymerase chain reaction (PCR) using a forward primer (5′-GCT TTT TGT TCG CTT GGT TGT G-3′) annealing to the maize *ubi-1* promoter and a reverse primer (5′-TTA GGG CTG CTG GTC TTT TCC-3′) annealing to the *spi 2* coding sequence. The reactions contained 10 m*M* Tris-HCl pH 8.8, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1% Triton X-100 (Dynazymes reaction buffer), 0.2 µ*M* of each primer, 50 µ*M* each of dATP, dCTP, dGTP and dTTP, 0.5 U Dynazyme DNA polymerase and 100 ng of genomic DNA. The reaction mixtures were taken through 30 cycles of 94°C for 30 s; 56°C for 30 s; 72°C for 1 min. Positive sublines gave the expected single product of approximately 750 bp.

Total peroxidase activity

Total proteins were extracted from proliferating embryogenic cultures by homogenising the tissue in 100 m*M* Tris-HCl pH 7.5, 1.5 *M* NaCl, 13 m*M* β-mercaptoethanol and 2% polyvinylpyrrolidone. The homogenate was centrifuged for 10 min at 10,000 rpm and 4°C. The supernatant was then desalted to 50 m*M* Tris-HCl pH 7.5 on a Micro-BioSpin column (Bio-Rad), and the protein concentration in the extract determined using the Bradford reaction (Sigma-Aldrich). The peroxidase activity in the extract was determined in an assay solution with 100 m*M* citrate-phosphate buffer pH 3.8, 3.0 m*M* ABTS [2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)] and 3.0 m*M* H_2O_2 (Childs and Bardsley 1975). The increase in absorbance at 412 nm was followed for 70 s. The peroxidase activity was calculated as ΔA min^{-1} (mg protein)$^{-1}$.

RNA blot analysis

For the slot blot analysis, 30 µg of total RNA was blotted onto a Hybond-N^+ nylon membrane. For the RNA gel blot analysis, 15 µg of total RNA was separated by gel electrophoresis under denaturing conditions and blotted onto Hybond-N^+ nylon membranes. The membranes were probed with a 1.2-kb *Xho*I/*Eco*RI *spi 2* fragment labelled with [^{32}P]-dCTP using the oligolabelling-kit of Amersham-Pharmacia (Sweden). Hybridisation was performed overnight at 42°C in 5× SSC, 4× Denhardt's solution, 0.1% SDS, 40% formamide, 10% dextran sulphate and 100 µg ml^{-1} denatured salmon sperm DNA. The membranes were

washed in 0.2× SSC, 0.1% SDS, for 2×15 min at 37°C or at higher temperatures when necessary.

Histochemical analysis of peroxidase activity and lignin

Stems of top and side shoots were hand-sectioned and stained with guaiacol as follows to demonstrate peroxidase activity: fresh sections were treated with 1.5% guaiacol in water, followed by 0.3% H_2O_2 for 30 s and then washed thoroughly in water (Johansson and Stenlid 1985). Guaiacol gives a brown-red precipitation after a reaction with peroxidase. For the histochemical analysis of lignification, sections were stained with 2% (w/v) phloroglucinol in ethanol and mixed 2:1 with concentrated HCl. The sections were mounted in water and photographed.

For the estimations of cell-length in the regenerated material, side shoots from representative plants of sublines 2-4, 2-7, 2-8, 2-11 and 2-12 and from the control were fixed essentially as described by Mo and von Arnold (1991), embedded in Technovit (Hereaus) and cut into 10-µ*M* sections. The sections were stained with toluidine blue to accentuate the cell walls. The inner cortical cells were photographed at a magnification of 100×, and cell length was measured on the prints.

Determination of lignin content

Lignin content, including acid-soluble lignin, in the stem of 14-month-old plants from the control and sublines 2-4 and 2-12 was determined gravimetrically by the Klason technique (Schöning and Johansson 1965).

Results

Establishment of transgenic embryogenic sublines of Norway spruce overexpressing *spi 2*

Following bombardment of cell lines 95:88:22 and 95:61:21 with *pUbi.spi2.bar,*we were able to isolate 15 sublines of each cell line on selective medium. These sublines were then screened for the presence of the *ubi:spi2* construct by PCR on the nuclear DNA; a PCR product of the correct size, 750 bp, was amplified in 21 of them (data not shown). Thirteen proliferating transgenic sublines were also analysed for the expression of *spi 2* by RNA blot analysis. The expression of *spi 2* in non-transformed embryogenic callus was barely detectable by RNA blot analysis, while the levels of *spi 2* in transgenic cultures ranged up to three times the mRNA level found in the control (data not shown).

One week after subculture, total peroxidase activity in the proliferating transgenic sublines as well as in the control lines was measured spectrophotometrically, using ABTS as the substrate (Table 1). The total peroxidase activity in the untransformed control cultures varied between 200 and 235 ΔA min^{-1} (mg protein)$^{-1}$. Transgenic sublines with total peroxidase activities up to a threshold level of 1.5-fold the most active control (approximately 350 ΔA min^{-1} (mg protein)$^{-1}$) were classified as class-I sublines; those with a total peroxidase activity higher than 350 ΔA min^{-1} (mg protein)$^{-1}$ were classified as class-II sublines. The activity in each subline was measured in at least two independent experiments, and although variation was present between experiments the classification of the sublines remained constant.

Effects of increased total peroxidase activities on morphogenesis in vitro

Proliferating embryogenic cultures of Norway spruce consists of proembryogenic masses and somatic embryos (Filonova et al. 2000). The morphology of the proembryogenic masses and the somatic embryos was similar in both the transgenic sublines and control lines on solidified proliferation medium. Consequently, the transgenic sublines grew just as the controls on solidified medium.

Routinely, embryogenic cultures are transferred to liquid medium for proliferation. A high yield of well-developed mature somatic embryos is obtained by giving the proliferating cultures a pre-maturation treatment, which includes washing with liquid medium without plant growth regulators and culturing for 1 week in medium lacking plant growth regulators, before the maturation treatment. This standard protocol could not be applied to the *spi 2* transformed sublines. The transgenic sublines did not proliferate in liquid medium, and within 1 month they had turned brown and lost their embryogenic potential. Therefore, the *spi 2* transformed sublines were kept on solidified medium. This modified protocol enabled us

Table 1 Total peroxidase activity in proliferating cultures from control and transgenic *spi 2* sublines. Sublines 1-2 to 2-12 were derived from cell line 95:88:22 and sublines 3-2 to 4-10 from cell line 95:61:21. Total peroxidase activity in embryogenic cultures was measured using ABTS [2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)]. The value shown is a mean of at least two independent measurements, the variation between the measurements being normally about 10%. The sublines were classified as either class-I sublines, i.e. sublines with a total peroxidase activity lower than 350 ΔA min^{-1} (mg protein)$^{-1}$or as class-II sublines if they exhibited a total peroxidase activity higher than 350 ΔA min^{-1} (mg protein)$^{-1}$

Subline	ΔA_{412} min^{-1} mg protein^{-1}	Class	Subline	ΔA_{412} min^{-1} mg protein^{-1}	Class
95:88:22	207	I	95:61:21	234	I
1-2	129	I	3-2	4,374	II
2-4	8,375	II	3-3	188	I
2-5	594	II	3-4	310	I
2-7	593	II	3-5	2,093	II
2-8	394	II	4-1	420	II
2-11	1,161	II	4-10	623	II
2-12	324	I			

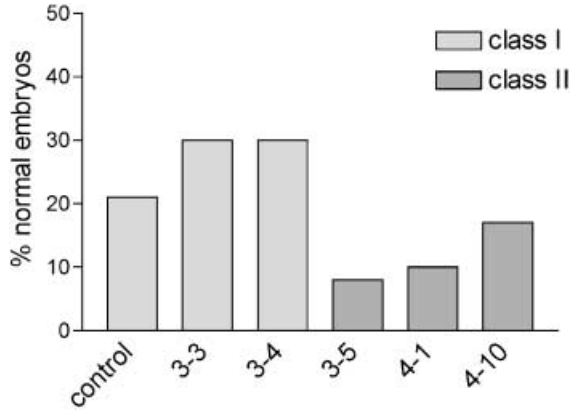

Fig. 1 Formation of normal mature somatic embryos in control and transgenic sublines. The classification of the control and the sublines is based on their total peroxidase activity as shown in Table 1. The percentage of normal mature somatic embryos is based on the classification of 300–600 maturing embryos after 4 weeks of growth on maturation medium

to generate mature, *spi 2* transformed somatic embryos. However, the yield of mature somatic embryos per gram of tissue in the control cell lines fell to 30 from about 300 using this modified protocol (data not shown).

Four weeks after the start of the maturation treatment the maturing embryos were classified into four groups: arrested nodules, normal mature embryos, precociously germinated embryos and overgrown nodules. The frequency of normal mature embryos was significantly (P=0.04) lower in the transgenic sublines classified as class-II sublines than in class-I sublines (Fig. 1). There were no differences in the kinetics between different groups of *spi 2* transformed sublines in their response to the maturation treatment, but the frequency of normal mature embryos was higher in sublines with a lower peroxidase activity.

Mature somatic embryos are routinely desiccated for 21 days under high humidity conditions (Bozkhov and von Arnold 1998), but this treatment was detrimental to the embryos after adjustment of the maturation protocol, the average germination frequency being only 29%. Shortening the desiccation period to 10 days increased the average germination frequency to 63% in both transgenic sublines and control cell lines.

Germinated embryos were transferred to liquid medium. After 1 month 70–90% of the plants had developed an actively growing root. There was no correlation between the total peroxidase activity in the proliferating cultures and root development. However, the E/R ratio in plants of *spi 2* transformed sublines was negatively correlated with the peroxidase activity in proliferating cultures (r^2=–0.6, P=0.04) (Table 2). The reduced E/R ratio was due to delayed development of the epicotyl. Within 6 months after transfer to liquid medium the epicotyls developed into normal shoots; this also occurred in plants regenerated from sublines with high peroxidase activities.

Table 2 Epicotyl (*E*) to root (*R*) ratios of germinated embryos regenerated from sublines classified as class-I and -II sublines. The classification of the sublines was made on the basis of total peroxidase activity in proliferating cultures (see Table 1). The E/R ratios in the class-I and -II transgenic sublines were scored after a 1-month culture in liquid medium. The data presented in the table is an average of three independent experiments. There is a weak but significant correlation between an increased total peroxidase activity in proliferating cultures and a decreased epicotyl-to-root elongation ratio (r^2=–0.6 P=0.04, using the Spearmans *r* test)

Subline	Class	E/R ratio
95:88:22	I	0.73
95:61:21	I	1.10
3-3	I	1.0
3-4	I	0.70
2-12	I	0.60
4-10	II	0.75
3-2	II	0.65
3-5	II	0.50
2-7	II	0.50
2-4	II	0.43
2-11	II	0.43
2-8	II	0.40

Table 3 Expression of *spi 2* in needles collected from control and transgenic plants. The classification of the sublines was made on the basis of total peroxidase activity in proliferating cultures (see Table 1). The relative mRNA levels of *spi 2* in needles collected from 6- to 9-month-old plants were determined by slot blot analysis. Total RNA (30 µg) was slot-blotted onto a membrane; the membrane was then hybridised with the *spi 2* probe, and the mRNA levels were quantified by a PhosphoImager. The mRNA levels in the transgenic plants were related to the sample corresponding to the median among the controls. The data are based on four to eight plants except for sublines 2-4 and 2-11

Subline	Class	Relative mRNA levels ±SE
95:88:22	I	1.0±0.1
2-4	II	4.5
2-5	II	3.6±0.6
2-7	II	3.4±0.5
2-8	II	5.0±1.0
2-11	II	4.0
2-12	I	2.3±0.2

Effects of overexpression of *spi 2* on plant growth and lignification ex vitro

About 40 transgenic plants were potted and grown ex vitro. The transgenic nature of the plants was verified by PCR, with primers amplifying a 750-bp *ubi/spi2* fragment (Fig. 2). The average expression level of *spi 2* in the needles varied among plants regenerated from different sublines (Table 3) and was correlated to the classification based on total peroxidase activity in proliferating cultures (P=0.04). Analysis of the total peroxidase activity in needles from two to three plants each from the control and the sublines showed that plants with an increased expression of *spi 2* also had an increased total peroxidase activity (data not shown).

The survival rate varied considerably between different sublines (Fig. 3). Higher expression levels of *spi 2* in

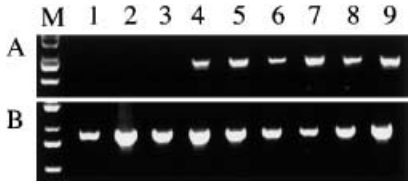

Fig. 2A, B Verification of the transgenic nature of regenerated plants. **A** The presence of the *pUbi.Spi 2.bar* construct in the regenerated plants was verified using specific *ubi/spi 2* primers to amplify a 750-bp fragment. **B** Amplification of *phy A* as a control that the DNA supports amplification. *M* Molecular size marker, *lanes 1–3* untransformed control plants, *lane 4* a plant regenerated from subline 2-5, *lanes 5, 6* plants regenerated from subline 2-8, *lanes 7, 8* plants from subline 2-11, *lane 9* a plant regenerated from subline 2-12

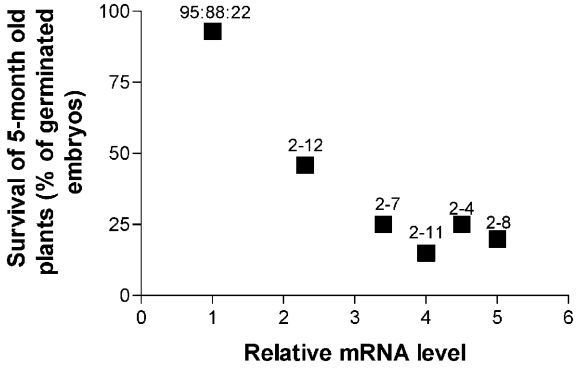

Fig. 3 Survival of germinated embryos after 5 months of growth ex vitro. Survival after 5 months of growth ex vitro is a measure of how well the germinated embryos from different sublines withstood the changes in growth conditions from germination to ex vitro growth. It is presented as the percentage of the germinated embryos that were successfully transferred ex vitro. The data presented in the graph is based on an average of 35 germinated embryos, except in sublines 2-4 and 2-12 where it is based on 4 and 13 germinated embryos, respectively

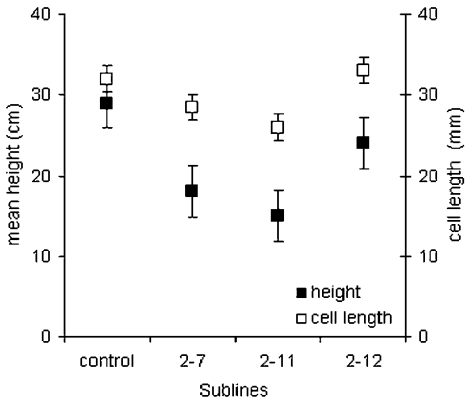

Fig. 4 Average height and cell length in *spi 2* transformed Norway spruce plants. The height of the plants was measured at 5 months after potting. Class-I sublines, i.e. subline 2-12 and the control, were significantly higher than the class-II sublines, i.e. sublines 2-7 and 2-11 (P=0.01, Spearmans correlation coefficient test). The length of the inner cortical cells of the side shoots from the transgenic sublines and the control was measured on microphotographs of sections. A minimum of 40 cells per subline was measured

Table 4 Assessment of lignification in *spi 2* transformed Norway spruce plants. Intensity of the phloroglucinol staining in the xylem of plants from five transgenic sublines was measured. Similar results were obtained in separate experiments carried out by independent persons. Klason lignin for representative material from sublines 2-4 and 2-12 as well as the control was analysed in duplicate; the value shown is a mean of the two measurements. The data shown is a mean of two independent measurements (*DW* dry weight; *n.a.* not analysed)

Subline	Intensity of phloroglucinol staining[a]	Klason lignin (% DW)
95:88:22	+++	26.0
2-4	+++++	25.5
2-7	+++	n.a.
2-8	+++	n.a.
2-11	++++	n.a.
2-12	++	23.9

[a] An increase in the number of '+' indicates an increase in the staining intensity

needles from the plants was correlated with lower survival rates (P=0.03). However, those plants still surviving 5 months after potting had a healthy appearance, with green needles and a growing apical shoot. At 5 months the average heights of plants regenerated from the control and subline 2-12, i.e. class-I lines, were significantly (P=0.01) higher than those of sublines 2-7 and 2-11 (Fig. 4).

In order to investigate if there was any correlation between height and cell length in the plants, side shoots were fixed and sectioned, and the cell length of the inner cortical cells was measured on microphotographs. The average cell length was shorter in plants regenerated from subline 2-7 and 2-11 than in plants regenerated from the control and subline 2-12 (Fig. 4). The average cell length of the inner cortical cells of subline 2-11 was significantly shorter (P=0.002) than that of the control.

Hand-cut sections of stems from both the controls and transgenic sublines 2-4, 2-7, 2-8 and 2-12 were stained with guaiacol to detect peroxidase activity. This treatment revealed a marked increase in peroxidase activity in sections from plants regenerated from sublines 2-4 (Fig. 5D), 2-7 and 2-8 compared with the control (Fig. 5C). There was a higher peroxidase activity both in the xylem and the cortex of the transgenic sublines, with it being most obvious in the cortex (Fig. 5E–H). Peroxidase activity in the cortex of plants regenerated from sublines originally classified as class-II sublines, i.e. sublines 2-4, 2-7 and 2-8, showed increased peroxidase activity in the cortex, while sections from plants regenerated from subline 2-12, a class-I subline, did not show stronger guaiacol staining than the controls. To investigate if the increased peroxidase activity led to increased lignification, we sectioned stems of plants regenerated from both the control and the sublines and treated these with acid phloroglucinol. The xylem of subline 2-4 stained more intensely with phloroglucinol than did the

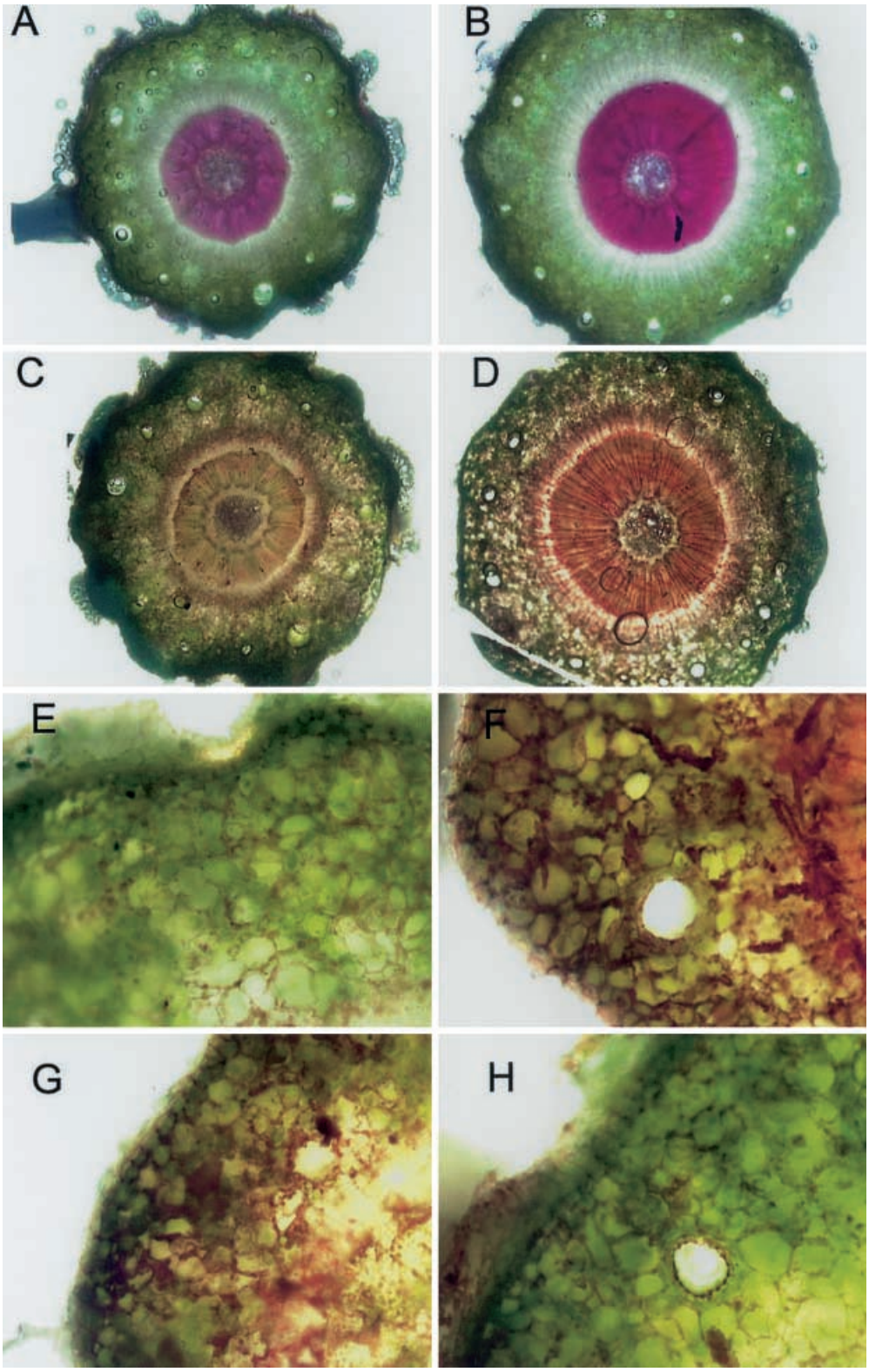

Fig. 5A–H Histochemical detection of lignin and peroxidase activity. Hand-cut sections of stems from control plants and plants regenerated from the transgenic sublines were stained for lignin using phloroglucinol-HCl and for peroxidase activity using guaiacol. **A, B** Phloroglucinol staining of the untransformed control (**A**) and subline 2-4 (**B**), **C, D** guaiacol staining of the untransformed control (**C**) and subline 2-4 (**D**). **E–H** Examples of increased peroxidase activity in the cortex of the transgenic plants: guaiacol staining in the cortex of the untransformed control (**E**) and transgenic plants regenerated from subline 2-4 (**F**), 2-8 (**G**) and 2-12 (**H**). The sections from the control and the class-I subline 2-12 show less guaiacol peroxidase activity than the class-II sublines 2-4 and 2-8

xylem of the control plants, giving a deeper red colour (Fig. 5A, B). No staining could be detected outside the xylem. Sections of plants from subline 2-11 showed a similar intensity of phloroglucinol staining of the xylem as did sections from 2-4. There were no obvious differences in the intensity of the phloroglucinol staining of the xylem in plants from subline 2-7, 2-8 and 2-12 compared to the control (Table 4). Interestingly, determination of the Klason lignin showed no clear differences between the control, 2-4 and 2-12 (Table 4).

Discussion

We have transformed embryogenic cultures of Norway spruce with an endogenous cDNA clone, *spi 2*, under the control of a strong promoter. The transgenic cultures showed increased total peroxidase activity. Plants that overexpressed *spi 2* were regenerated and transferred to ex vitro conditions. The regenerated plants showed an increased peroxidase activity in the stem and needles. Based on the overall sequence similarity with cationic peroxidases (Fossdal 1999) and the increased total peroxidase activity found in the transformed embryogenic cultures and regenerated plants overexpressing *spi 2*, we conclude that *spi 2* encodes a peroxidase. This is further supported by the fact that transgenic tobacco plants expressing *spi 2* also show an increased total peroxidase activity (Elfstrand et al. 2001).

Overexpression of *spi 2* in embryogenic cultures of Norway spruce made them more sensitive to changes in the growth regime than the control. This was seen as a degeneration of sublines in liquid culture and a need to adjust the maturation protocol at several steps. Reduced frequencies of normal mature embryos and increasing numbers of arrested as well as precociously germinated embryos were also found during maturation. The stress sensitivity is further illustrated by the reduced survival rate of germinated embryos throughout the acclimatisation process to ex vitro growth. The *ubi-1* promoter is a relatively strong promoter (Clapham et al. 2000) that is induced by abiotic stress (Takimoto et al. 1994). Therefore, it is likely that the peroxidase activity becomes too high in the overexpressing sublines and plants when they are exposed to stress.

The *spi 2* mRNA level was very low in the untransformed embryogenic cultures. This suggests that normally the *spi 2* gene is weakly expressed in proliferating embryogenic cultures. Fossdal (1999) did not detect any SPI 2 protein in Norway spruce seeds using Western analysis until the radicle had emerged, suggesting that *spi 2* is not expressed in the seeds either. Overexpression of *spi 2* during somatic embryogenesis reduced the frequency of normal mature embryos. This indicates that a high SPI 2 content has a negative effect on embryo development.

The plants derived from sublines with a high total peroxidase activity showed a normal root development but a reduced epicotyl development, resulting in decreased E/R ratios. This is in contrast to previous reports on tomato (El Mansouri et al. 1999) and tobacco (Lagrimini et al. 1997b) in which plants overexpressing peroxidases showed increased shoot/root ratios and a wilting phenotype, possibly due to hormonal imbalances in the overexpressing plants that resulted in an underdeveloped root system (El Mansouri et al. 1999). In Norway spruce *spi 2* has no inhibitory effect on root development, indicating that there is a direct negative effect on the epicotyl.

Plants overexpressing *spi 2* (levels three to five times that of the control) were significantly shorter than the control after 5 months of growth ex vitro. Similarly, Lagrimini et al. (1997a) reported reduced height growth in tobacco lines overproducing an anionic peroxidase compared to non-transformed plants under optimal growth conditions. They also observed that the tobacco lines overproducing the tobacco anionic peroxidase had thinner leaf lamina with smaller cells. The Norway spruce plants overexpressing *spi 2* showed more histochemical guaiacol peroxidase activity in the cortical cells. Furthermore, the inner cortical cells were shorter in side shoots from plants regenerated from class-II sublines 2-7 and 2-11 than those from the class-I lines and the control. Possibly, overexpression of *spi 2* in Norway spruce plants could result in shorter cells because of reduced cell-wall extensibility.

Compared with the controls, plants derived from sublines 2-4 and 2-11 showed more intense phloroglucinol-staining in the xylem, although no increase in the Klason lignin was found in subline 2-4. Phloroglucinol-HCl is known to react primarily with aldehydes. Tsai et al. (1998) showed that the more intense phloroglucinol staining seen in co-suppressed caffeic acid *O*-methyltransferase (COMT) transformed aspen plants was the result of an increased proportion of coniferaldehyde that had been incorporated into the lignin polymer. A deeper phloroglucinol staining was also observed in antisense cinnamyl alcohol dehydrogenase (CAD) transformed poplar (Baucher et al. 1996) and tobacco (Hibino et al. 1995) plants. In both these studies this deeper phloroglucinol staining was not accompanied by any changes in Klason lignin, indicating that overexpression of *spi 2* in Norway spruce could have altered the proportion of coniferaldehyde incorporated into the lignin polymer without changing the lignin content. A closer investigation of the lignin polymer composition in this material will be needed to determine if this is the case.

Acknowledgements We thank STFI for analysing the Klason lignin in the transgenic plants. We also thank Anette Silván and Aili Colbing for skilful technical assistance. This work was financed by the Swedish council for Agricultural and Forestry Research and by the Scandinavian Forest Research committee.

References

Amaya I, Botella MA, de la Calle M, Medina MI, Heredia A, Bressan RA, Hasegawa PM, Quesada MA, Valpuesta V (1999) Improved germination under osmotic stress of tobacco plants overexpressing a cell wall peroxidase. FEBS Lett 457:80–84

Asiegbu F, Daniel G, Johansson M (1993) Studies on the infection of Norway spruce roots by *Heterobasidion annosum*. Can J Bot 71:1552–1561

Baucher M, Chabbert B, Pilate G, Van Dooresslaere J, Tollier M-T, Petit-Conil M, Cornu D, Monties B, Van Montagu M, Inze D, Jouanin L, Boerjan W (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. Plant Physiol 112:1479–1490

Baumbusch LO, Eiblmeier M, Schnitzler J-P, Heller W, Sandermann HJ, Polle A (1998) Interactive effects of ozone and low UV-B radiation on antioxidants in spruce (*Picea abies*) and pine (*Pinus sylvestris*) needles. Physiol Plant 104:248–254

Bolwell GP, Wojtaszek P (1997) Mechanism for the generation of reactive oxygen species in plant defence-a broad perspective. Physiol Mol Plant Pathol 51:347–366

Bozkhov PV, von Arnold S (1998) Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. Physiol Plant 104:211–224

Bradley D, Kjellborn P, Lamb C (1992) Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell 70:21–30

Childs R, Bardsley W (1975) The steady-state kinetics of peroxidase with 2,2-Azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochem J 145:93–103

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Clapham D, Demel P, Elfstrand M, Koop H-U, Sabala I, von Arnold S (2000) Gene transfer by particle bombardment to embryogenic cultures of *Picea abies* and the production of transgenic plantlets. Scand J For Res 15:151–160

Egertsdotter U, von Arnold S (1993) Classification of embryogenic cell lines of *Picea abies* as regards protoplast isolation and culture. J Plant Physiol 141:222–229

El Mansouri I, Mercado JA, Santiago-Dómenech N, Pliego-Alfaro F, Valpuestra V, Quesada MA (1999) Biochemical and phenotypical characterization of transgenic tomato plants overexpressing a basic peroxidase. Physiol Plant 106:355–362

Elfstrand M, Sitbon F, Lapierre C, Bottin A, von Arnold S (2001) Altered lignin structure and resistance to pathogens in *spi 2*-expressing tobacco plants. Planta (in press)

Filonova LH, Bozhkov PV, von Arnold S (2000) Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. J Exp Bot 51:249–264

Fossdal CG (1999) Molecular and cytopathological host responses in root-infected Norway spruce [*Picea abies* (L.) Karst.] seedlings. PhD thesis, Norges Landbrukshogskole, Ås Norway

Hibino T, Takabe K, Kawazu T, Shibata D, Higuchi T (1995) Increase of cinnamaldehyde groups in lignin of transgenic tobacco plants carrying an antisense gene for cinnamyl alcohol dehydrogenase. Biosci Biotechnol Biochem 59:929–931

Hiraga S, Yamamoto K, Ito H, Sasaki K, Matsui H, Honma M, Nagamura Y, Sasaki T, Ohashi Y (2000) Diverse expression profiles of 21 rice peroxidase genes. FEBS Lett 471:245–250

Ingestad T (1979) Mineral nutrient requirements of *Pinus sylvestris* and *Picea abies* seedlings. Physiol Plant 45:137–148

Johansson M, Stenlid J (1985) Infection of roots of Norway spruce (*Picea abies*) by *Heterobasidion annosum.* 1. Initial reactions in sapwood by wounding and infection. Eur J For Pathol 15: 32–45

Kazan K, Goulter KG, Way HM, Manners JM (1998) Expression of a pathogenesis-related peroxidase of *Stylosanthes humilis* in transgenic tobacco and canola and its effect on disease development. Plant Sci 136:207–217

Kristensen BK, Brandt J, Bojsen K, Thordal-Christensen H, Kerby KB, Collinge DB, Mikkelsen JD, Rasmussen SK (1997) Expression of a defence-related intercellular barley peroxidase in transgenic tobacco. Plant Sci 122:173–182

Krogstrup P (1986) Embryo-like structures from cotyledons and ripe embryos of Norway spruce (*Picea abies*). Can J For Res 16:664–668

Lagrimini, LM, Bradford S, Rothstein S (1990) Peroxidase induced wilting in transgenic tobacco plants. Plant Cell 2:7–18

Lagrimini LM, Gingas V, Finger F, Rothstein S, Liu T-TY (1997a) Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. Plant Physiol 114:1187–1196

Lagrimini LM, Joly RJ, Dunlap JR, Liu T-TY (1997b) The consequence of peroxidase overexpression in transgenic plants on root growth and development. Plant Mol Biol 33:887–895

Mensen R, Hager A, Salzer P (1998) Elicitor-induced changes of wall-bound and secreted peroxidase activities in suspension-cultured spruce (Picea abies) cells are attenuated by auxins. Physiol Plant 102:539–546

Mo LH, von Arnold S (1991) Origin and development of embryogenic cultures from seedlings of Norway spruce (*Picea abies*). J Plant Physiol 138:223–230

Østergaard L, Pedersen AG, Jespersen HM, Brunak S, Welinder KG (1998) Computational analysis and annotations of the *Arabidopsis* peroxidase gene family. FEBS Lett 433:98–102

Polle A, Otter T, Seifert F (1994) Apoplastic peroxidases and lignification in needles of Norway spruce (*Picea abies* L.). Plant Physiol 106:53–60

Schöning A, Johansson G (1965) Sven Papperstidn 68:607

Smit F, Dubery IA (1997) Cell wall reinforcement in cotton hypocotyls in response to a *Verticillium dahliae* elicitor. Phytochemistry 44:811–815

Svalheim O, Robertsen B (1990) Induction of peroxidases in cucumber hypocotyls by wounding and fungal infection. Physiol Plant 78:261–267

Takimoto I, Christensen AH, Quail, PH, Uchimiya H, Toki S (1994) Non-systemic expression of a stress-responsive maize polyubiquitin gene (*Ubi-1*) in transgenic rice plants. Plant Mol Biol 26:1007–1012

Tsai C-J, Popko JL, Mielke MR, Hu W-J, Podila GK, Chiang VL (1998) Suppression of *O*-methyltransferase gene by homologous sense transgene in Quaking aspen causes red-brown wood phenotypes. Plant Physiol 117:101–112

von Arnold S (1987) Improved efficiency of somatic embryogenesis in mature embryos of *Picea abies* (L.) Karst. J Plant Physiol 128:233–244

Young SA, Gou A, Guikema JA, White FF, Leach JE (1995) Rice cationic peroxidase accumulates in xylem vessels during incompatible interactions with *Xanthomonas oryzae* pv. *oryzae*. Plant Physiol 107:1333–1341