

Structural and functional characteristics of two novel members of pathogenesis-related multigene family of class 10 from yellow lupine[☆]

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PR-10 proteins (pathogenesis-related), ubiquitous within the plant kingdom, are usually encoded by multigene families. To date we have identified 10 homologous *pr-10* genes in a yellow lupine cDNA library. Here, the structure and expression of two newly identified yellow lupine *pr-10* genes (*LlYpr10-2b* and *LlYpr10-2f*) are presented. Many potential regulatory sites were found in both gene promoters including common ones as well as those unique for each gene. However, promoter deletion analysis in transgenic tobacco plants revealed similar patterns of reporter gene (*gus*) expression. Shortened fragments of both gene promoters studied caused high GUS activity in leaves (along vascular bundles), stamen stigma, anthers and pollen grains. When conjugated with longer *LlYpr-10.2* promoter fragments, GUS was additionally present in petal edges. Only a long fragment of the *LlYpr10-2b* gene promoter caused GUS expression in the stem. In yellow lupine the *pr-10.2* genes are present in all studied organs, but their level of expression depends on the stage of development and is affected by wounding, oxidative stress and salicylic acid treatment. Silencing of the *Llpr-10.2b* gene in 4-week-old yellow lupine plants did not lead to any visible symptoms, which suggests that the function of the silenced gene is supplemented by its close homologues, still present in the studied plants.

Keywords: pathogenesis-related genes and proteins, yellow lupine, promoter analysis, salicylic acid, hydrogen peroxide, gene silencing

INTRODUCTION

Many eukaryotic proteins, including actins, hemoglobins, immunoglobulins, tubulins, interferons, histones or MAP kinases, are encoded by multigene families (Mishra *et al.*, 2006; Silverstein *et al.*, 2006; Schenk *et al.*, 2006). The coexistence of related genes in one organism presumably results from duplications and modifications of ancestral genes or whole genomes. Multigene families are particularly widespread in higher plants, which are often polyploid organisms possessing huge genomes (Schenk *et al.*, 2006).

An example of the numerous plant gene families is one encoding class 10 of pathogenesis-related proteins (PR-10). It often contains at least 10 homologous genes per species and, consequently, the same or a higher number of protein isoforms can be synthesized in plant cells (van Loom & van Strien, 1999; Liu & Ekramoddoullah, 2004; Beuning *et al.*, 2004; Schenk *et al.*, 2006). Despite their ubiquity within the plant kingdom, the function of PR-10 proteins remains unclear. The first *pr-10* gene was identified in parsley cell culture subjected to fungal elicitor treatment (Somssich *et al.*, 1986; 1988). During the last 30

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Abbreviations: auxRE, auxin-response element; Bet v 1, *Betula verrucosa* protein 1; Cdx, caudal type, homeobox transcription factor; Dof proteins, DNA-binding with One Finger proteins; dpi, days post infection/infiltration; GRE, glucocorticoid-response element; GUS, β -glucuronidase; HSF, heat-shock factor; Hyp 1, *Hypericum perforatum* protein 1; Llcsbp, *Lupinus luteus* cytokinin-specific binding protein; Llpr-10, *Lupinus luteus* pathogenesis-related class 10 gene/protein; MAP, mitogen-activated protein kinase; MtN13, *Medicago truncatula* nodulin gene; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance; WRKY, transcription factor family with WRKY domain.

years many reports on pathogenesis and stress-related expression of *pr-10* genes in different plant species have been published (Crowell *et al.*, 1992; Pinto & Ricardo, 1995; Pozueta-Romero *et al.*, 1995; Swoboda *et al.*, 1996; Walter *et al.*, 1996; Hoffmann-Sommergruber *et al.*, 1997; Wang *et al.*, 1999; McGee *et al.*, 2001; Liu & Ekramoddoullah, 2004; Beuning *et al.*, 2004; Finkler *et al.*, 2005). In addition, papers demonstrating constitutive expression of *pr-10* genes appeared, suggesting a more general biological role of PR-10 proteins in plant developmental program (Pozueta-Romero *et al.*, 1995; Vanek-Krebitz *et al.*, 1995; Balsamo *et al.*, 1995; Swoboda *et al.*, 1996; Huang *et al.*, 1997; Strömvik *et al.*, 1999; Sikorski *et al.*, 1999; Liu & Ekramoddoullah, 2003; Beuning *et al.*, 2004; Finkler *et al.*, 2005). Also, tissue-specific expression of *pr-10* genes, e.g. in roots (Sikorski *et al.*, 1999; Liu & Ekramoddoullah, 2003), flowers (Pozueta-Romero *et al.*, 1995), pods (Strömvik *et al.*, 1999), fruits (Vanek-Krebitz *et al.*, 1995), tapetum (Huang *et al.*, 1997) or pollen grains (Swoboda *et al.*, 1996) was documented. Many of the PR-10 class members were recognized as major food and pollen allergens. The best known pollen-associated allergens are PR-10 proteins encoded by birch genomes with Bet v 1 as a classic example (Schenk *et al.*, 2006). Some of the PR-10 proteins display weak ribonuclease activity (Moiseyev *et al.*, 1994; 1997; Swoboda *et al.*, 1996; Bufe *et al.*, 1996; Bantignies *et al.*, 2000; Zhou *et al.*, 2002; Wu *et al.*, 2003; Park *et al.*, 2004; Chadha *et al.*, 2006), but it is still difficult to estimate the contribution of this nucleolytic activity to their real function. PR-10 proteins also share homology with (*S*)-norcochlorine synthase (NCS), an enzyme implicated in biosynthesis of benzylisoquinoline alkaloids, but recombinant PR-10 polypeptides do not show a similar enzymatic activity (Liscombe *et al.*, 2005). Another enzymatic activity has been recently demonstrated for Hyp 1, a PR-10 protein extracted from St. John's wort (*Hypericum perforatum*), responsible for biosynthesis of hypericin from emodin (Bais *et al.*, 2003). The gene coding for Hyp 1 protein shows 45.1% identity to Bet v 1 class allergens.

In legumes, PR-10 proteins can be additionally considered in a symbiosis context. We discovered the first two yellow lupine (*Lupinus luteus*) PR-10 proteins (LIPR-10) in root extracts, as a result of differential expression analysis during symbiotic interaction with soil bacteria *Bradyrhizobium* sp. (*Lupinus*) (Sikorski, 1997; Sikorski *et al.*, 1999). Both proteins, consisting of 156 amino-acid residues, are highly expressed in root tissues except for mature root nodules (Sikorski *et al.*, 1999). This pattern of expression in roots is typical for all known legume PR-10 proteins except one — alfalfa MtN13 (*Medicago truncatula* nodulin gene), which is highly expressed exclusively in the nodule cortex (Gamas *et al.*, 1998).

Yellow lupine protein LIPR-10.1B is constitutively expressed in all aerial parts of plants whereas its close homologue, LIPR-10.1A, was detected only in stem and leaves of plants infected with the pathogen *Pseudomonas syringae* (Sikorski, unpublished).

Inspired with the idea of PR-10 proteins significance in the symbiotic interaction, we searched for a yellow lupine homologue of the nodule-specific MtN13. As a result of the screening of a yellow lupine cDNA library with MtN13 coding sequence as a probe, six new yellow lupine *pr-10* homologues were identified (Sikorski *et al.*, 2000; Handschuh *et al.*, 2004).

Summarizing, the yellow lupine *pr-10* gene family encompassed to date 10 homologues, including two genes encoding cytokinin-specific binding proteins (*csbp*), classified together with *pr-10* due to their similar tertiary structure (Pasternak *et al.*, 2006) (Supplementary Table 1). Three subclasses of PR-10 proteins (LIPR-10.1, LIPR-10.2 and LICSBP) were distinguished in yellow lupine, according to the level of predicted amino-acid sequence identity. The highest identity values at the nucleotide level were noticed within the subclass *Llpr-10.2*, where two pairs of very close homologues were identified: *Llpr-10.2a/Llpr-10.2d* and *Llpr-10.2b/Llpr-10.2c* (Supplementary Table 2).

Here, we present two new yellow lupine *pr-10* genes, which were identified in a yellow lupine genomic library and were classified as members of subclass LIPR-10.2: *LlYpr-10.2.b* and *LlYpr-10.2.f*. Their promoter activities were studied in transgenic tobacco. Expression of three *Llpr-10.2* genes (*LlYpr-10.2.a*, *LlYpr-10.2.b* and *LlYpr-10.2.e*) was also monitored in yellow lupine leaves during development, in wounded leaves and leaves infected with the pathogenic bacterium *P. syringae*. The expression profiles of five *Llpr-10* genes belonging to two *pr-10* subclasses were determined in roots and leaves of yellow lupine plants treated with hydrogen peroxide and salicylic acid. In addition, the effect of *Llpr-10.2b* gene silencing in yellow lupine plants was investigated.

MATERIALS AND METHODS

Screening of a yellow lupine genomic library. A genomic library of yellow lupine (made in λ -phage vector EMBL-3 by Clontech, USA) was screened according to the manufacturer's recommendations. Hybridization was performed overnight at 65°C using ³²P-labeled full coding sequence of *Llpr-10.2b* gene as a probe. The membranes were subsequently washed: first in 2 × SSC at room temperature, then in 1 × SSC at 60°C and in 0.1 × SSC at 60°C. After a 3-day-exposure of filters at -70°C, X-

ray films were developed and hybridizing colonies were collected.

Cloning of *LlYpr-10.2* genes. A 5 kb fragment of the *LlYpr-10.2.b* gene containing entire coding sequence, 5' UTR and promoter, was PCR-amplified and cloned into pDrive Cloning Vector (Promega). *LlYpr-10.2.f* gene, containing coding sequence and 1357 bp upstream from the start codon was cloned into pBS-SK plasmid. The prepared plasmids were used for DNA sequencing and construction of binary vectors for promoter deletion analysis.

In silico promoter analysis. Potential transcription factor-binding sites and regulatory motifs were searched for in the following promoter regions of the *LlYpr-10.2* genes: 1425 bases upstream from the start codon in the case of *LlYpr-10.2b* and 1353 bases upstream from the start codon in the case of *LlYpr-10.2f*, using web-available software: TRANSFAC (Heinemeyer *et al.*, 1998; <http://www.cbrc.jp/research/db/TFSEARCH.html>), TFSEARCH (Akiyama *et al.*, 1998; <http://www.rwcp.or.jp/papia/>) and TESS (Schug, 2003; <http://www.cbil.upenn.edu/cgi-bin/tess/tess>). Some additional regulatory motifs not available in the above softwares but present in other plant and *pr-10* gene promoters were also identified in the *Llpr-10.2* gene promoters studied here.

Promoter deletion analysis. Two promoter deletion fragments per gene were PCR amplified, digested and cloned to the binary vector pPR97 (kindly provided by Dr. Pascal Gamas from the Institut des Plantes Vegetales, Gif sur Yvette, France) upstream the coding sequence of β -glucuronidase (GUS). Then, the vectors were used for transformation of *Agrobacterium tumefaciens* with the three parental mating and electroporation methods. The bacteria carrying the plasmids were subsequently introduced to *Nicotiana tabacum*, cv. Wisconsin 38. Every transformation event gave 40–70 transgenic plants. Genomic DNA was isolated from over 130 plants and used as a template for PCR with universal primers, designed for the insert-flanking sequences of pPR97 plasmid. From the PCR-positive plants single organs were taken and incubated (24 h, 37°C) with GUS substrates (Jefferson *et al.*, 1987).

Transgenic plant regeneration. Leaves of *Nicotiana tabacum*, cv. Wisconsin 38, grown *in vitro*, were cut into small pieces (0.5–1 cm²) and incubated for 5 min in the YEB medium containing transformed *A. tumefaciens* cells (OD₆₀₀ 0.8–1). Then, the leaf segments were kept for 3 days in dark on MS permanent medium. Passaged to MS-T medium with antibiotics (kanamycin and carbenicillin), plants regenerated at 24°C, 16 h photoperiod. Single plants were cultivated on MS medium with selective antibiotics until the roots developed (Trinh *et al.*, 2001).

Plant genomic DNA isolation. The harvested plant tissues were immediately frozen in liquid ni-

trogen and ground using mortar and pestle. Gently shaken in buffer containing 1% CTAB, the plant powder was incubated for 1 h at 65°C. Afterwards, DNA was extracted with equal volume of chloroform/isoamyl alcohol (24:1, v/v) and precipitated overnight with isopropanol (0.7 volume) at –20°C. Following centrifugation (30 min, 13 200 rpm, 4°C) DNA pellet was dissolved in 0.5 × TE buffer.

Growth of yellow lupine plants. Yellow lupine plants were cultivated in a sterile growth chamber at 23°C with a 16 h day and 8 h night photoperiod. Symbiosis was induced by inoculation of 3-day-old seedlings with soil bacteria (*Bradyrhizobium* sp. *Lupinus*). Leaves of 4-week-old plants were mechanically wounded (using sterile glasspaper) or infiltrated (into bottom part of the leaf using syringe without needle) with the pathogenic bacterium *Pseudomonas syringae* pv. *pisi* grown in YDA medium at 30°C to OD₆₀₀ = 1.0 (according to Breda *et al.*, 1996). Control plants were infiltrated with 10 mM MgCl₂. Two other sets of 4-week-old plants were watered with 100 μ M salicylic acid (SA) solution and 10 mM hydrogen peroxide (H₂O₂) solution, respectively. Leaves were harvested directly before induction and 3, 6, 9, 24 and 48 h after treatment, frozen in liquid nitrogen and ground using mortar and pestle. Total RNA and proteins were extracted from the same tissue samples.

Gene silencing. The full coding sequence of the *Llpr-10.2b* gene was cloned into the binary expression vector pAWo59 in sense (**2b_{SE}**), antisense (**2b_{AS}**) or both orientations simultaneously, divided by a shortened intron of the chalcone synthase gene in order to produce a transcript adopting a long hairpin structure (**2b_{HP}**, Fig. 5A). Each construct was introduced into *Agrobacteria* and agroinfiltrated into 4-week-old yellow lupine plants. *A. tumefaciens* cells were transformed with the plasmids using electroporation and three parental mating methods. The transformed *Agrobacteria* were then grown in liquid YEB medium with kanamycin and rifampicin to OD₆₀₀ 0.5–0.8. Pelleted bacteria were suspended in 10 mM MgCl₂ and infiltrated with a syringe to leaf blades of 4-week-old yellow lupine plants in a sterile growth chamber. Leaves were collected 5, 11, 22, 33 and 57 days after infiltration and treated as after gene expression induction described above.

Plant RNA isolation and RT-PCR analysis. RNA was isolated with RNeasy Plant Mini Kit (Qiagen) and DNase I digested. RT-PCR reaction was performed with One-Step RT-PCR Kit (Qiagen) using specific primers complementary to the 3' and 5' untranslated regions of the *Llpr-10.1a*, *Llpr-10.1b*, *Llpr-10.2a*, *Llpr-10.2b* and *Llpr-10.2e* cDNA clones.

Western blot analysis. Ground tissues were shaken in 20 mM Tris/HCl buffer (pH 7.5)/5% glycerol/10 mM β -mercaptoethanol and kept on ice for 0.5

h. The extracts were then centrifuged (14 000 rpm, 30 min, 4°C), and the supernatants containing total soluble protein fraction were collected. The proteins (1 µg per well) were separated in 17% polyacrylamide/SDS gel in 1 × Laemmli buffer. After electrophoresis, the proteins were blotted to a PVDF membrane with a semi-dry method, using a Trans-Blot SD Semi Dry Transfer Cell (Biorad). The membranes were subsequently blocked 1 h in 1 × PBS/5% fat-free milk and incubated overnight with a polyclonal rabbit serum anti-LIPR-10.2Bs (Handschuh *et al.*, 2004; Figs. 3 and 4) diluted 1 : 3000 in 1 × PBS/5% fat-free milk/0.05% Tween 20 or with polyclonal rabbit serum anti-LIPR-10.1A (Sikorski *et al.*, 1999; Fig. 3) diluted 1 : 10 000. Next, the blots were incubated first with goat anti-rabbit biotinylated antibodies (Amersham), and then with streptavidin-CIP; thoroughly washed in 1 × PBS, 1 × PBS/Tween 20 and again 1 × PBS. The immune reaction was finally detected after short incubation with CIP substrates (BCIP and NBT, Sigma).

RESULTS

Identification of new *pr-10* genes in yellow lupine genome

In order to identify new genes encoding yellow lupine LIPR-10.2 proteins, we have screened a genomic library of yellow lupine with the *Llpr-10.2b* coding sequence as a probe. At high stringency conditions, we were able to find only two genomic sequences — one exactly overlapped the probe sequence (referred as *LlYpr-10.2b*), while the other was most similar but not identical with *Llpr-10.2e*. This gene was identified as a new *pr-10* lupine homologue and, consequently, named *LlYpr-10.2f*. The structure of *LlYpr-10* genes is schematically presented in Fig. 1. A TATA box sequence, TATAAATA, is localized in *LlYpr-10.2b* between 84 and 76 bp and in *LlYpr-10.2f* between 80 and 72 bp upstream of the ATG start codon. The conserved polyadenylation signal, AATAA, was identified in 3' UTRs. Both genes

contain a single intron of similar length (330 and 314 bp), placed in a conserved position — after the first base of the triplet encoding glycine 61 (Table 1). In contrast to the high degree of identity at the cDNA level (79%) and within the intron sequences (73%), the similarity within promoter sequences of *LlYpr-10.2b* and *LlYpr-10.2f* is significantly lower (49%).

Screening of the genomic library for other *LlYpr-10.2* gene sequences was not successful. Nevertheless, the *LlYpr-10.2a* intron sequence was identified as a result of PCR involving yellow lupine genomic DNA as a template and *Llpr-10.2a*-specific starters designed to be used in RT-PCR analysis. The obtained product was by about 200 bp longer than the corresponding RT-PCR product (not shown). Sequencing of the PCR product revealed the entire *Llpr-10.2a* coding sequence interrupted in the conserved position (as indicated above) by a 242 bp-long intron, sharing 54% and 55% identity with the intron sequences of *LlYpr-10.2b* and *LlYpr-10.2f* genes, respectively. All three introns have the same boundary sequences: TTGAGG/GTTAGTA...AG/GTGG. Table 1 summarizes basic information concerning all *pr-10* genes that were identified in yellow lupine genomic library, including two genes representing *Llpr-10.1* subclass described earlier (Sikorski *et al.*, 1998).

Screening for regulatory elements in yellow lupine *LlYpr-10.2* gene promoter

Analysis of the *LlYpr-10.2b* and *LlYpr-10.2f* promoter sequences *in silico* led to the identification of many potential regulatory elements (72 and 98, respectively) (Supplementary Table 3). More than half of them appear in both promoters, but with different frequency and orientation.

The most conservative regions of the *LlYpr-10.2b* and *LlYpr-10.2f* promoters are located within approx. 230 bp upstream of the start codon. Here, the basic signals determining transcription initiation are located. A conserved TATA box (TATAAATA), recognized by various transcription factors, is found

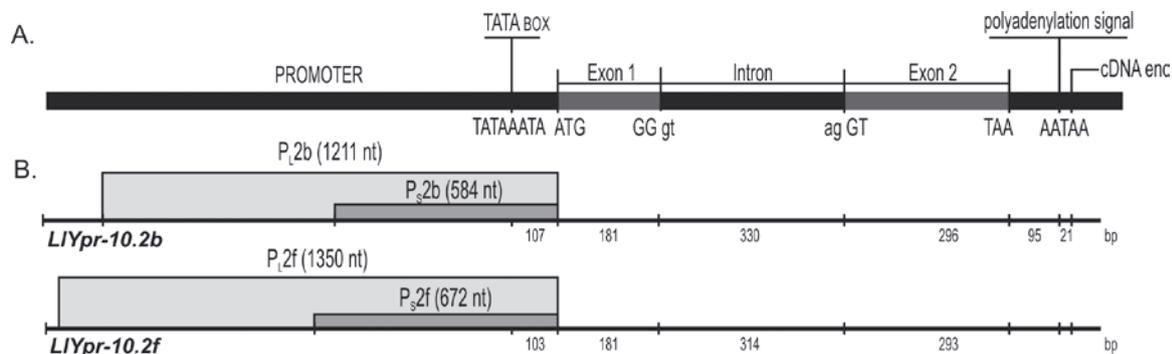


Figure 1. General scheme of the yellow lupine *LlYpr-10* gene structure (A) and two genes used in promoter deletion analysis (B).

Promoter fragments are indicated.

Table 1. Intron length and localization in *LlYpr-10* genes.

Introns are located after the first letter of the triplet coding for the 61st amino acid.

Gene name	Intron position	Intron length (bp)	GenBank Acc. No.
<i>LlYpr-10.1a</i>	61 (Asp)	457	AF002277
<i>LlYpr-10.1b</i>	61 (Gly)	797	AF002278
<i>LlYpr-10.2a</i>	61 (Gly)	242	AY729802
<i>LlYpr-10.2b</i>	61 (Gly)	330	AY377535
<i>LlYpr-10.2f</i>	61 (Gly)	314	AY303549

in the same position in both promoters, flanked by identical 5-nt-long sequences. The CAAT sequence frequent in both promoters probably plays a role of the CAAT box, implicated in transcription initiation, only in the *LlYpr-10.2b* promoter, where it is proximal to the TATA box. Within the most conservative regions of both promoters one site recognized by NIT2, activator of nitrogen-regulated genes, one gibberellin-response element and a few AT-com traits, specific for heat-shock protein coding genes were found. However, some significant differences appear in this region as well. For instance, only the *LlYpr-10.2f* gene possesses, in proximity of the TATA box, a motif similar to the GC box (GGCGG), present in many constitutively expressed genes. The same *LlYpr-10.2f* promoter fragment (236 bp upstream of the start codon) contains one PBF (prolamine binding factor) site, present in maize, wheat and barley gene promoters and recognized by a family of single zinc finger plant transcription factors, Dof proteins (DNA-binding with One Finger). One P motif, implicated in the flavonoid biosynthesis pathway, one E-box (hexanucleotide sequence CAAATG; binding site of many nuclear factors) and one AT-rich Ford consensus sequence, specific for late nodulin genes, are also located in this region of *LlYpr-10.2f* promoter. Within the corresponding region of *LlYpr-10.2b* promoter (232 bp upstream of the start codon) there is one motif recognized by Dof2 transcription factor, two TCA-like sequences, implicated in response to salicylic acid, two sites recognized by chicken CdxA homeobox gene (caudal type homeobox transcription factor, Cdx-1) and two heat-shock factor (HSF) sites described in *Drosophila*. More sites recognized by Dof proteins are located upstream of the most conservative regions and except for the common AAAG core, have different sequences in both *LlYpr-10.2* promoters. Additionally, the *LlYpr-10.2b* promoter contains three direct repeats of a characteristic sequence (TCATGNA) of unknown function.

Deletion analysis of *LlYpr-10.2* promoters

In order to complement the *in silico* analysis, *LlYpr-10.2* promoter deletion analysis was performed in transgenic tobacco. Four putative promoter fragments upstream of the start codon were amplified:

short **P₅2b** (584 bp) and long **P_L2b** (1211 bp) representing the *LlYpr-10.2b* gene, as well as short **P₅2f** (672 bp) and long **P_L2f** (1350 bp) representing *LlYpr-10.2f* (Fig. 1). Each fragment was fused to the coding sequence of β -glucuronidase gene and introduced into the model plant *Nicotiana tabacum*. Promoter activity was then analyzed in different plant organs. Generally, we observed similar, ubiquitous GUS expression in plants transformed with either of the four constructs (**P₅2b**, **P_L2b**, **P₅2f** and **P_L2f**) (Fig. 2). A definitely uniform, high expression pattern was noticed in stamens — limited to stigma, and leaves — associated with vascular bundles, which was clearly visible in light microscope pictures. Although the GUS expression pattern in anthers was not uniform, pollen grains clearly showed strong GUS activity in plants transformed with either of the four constructs. An evident discrepancy was found in stems where GUS was expressed exclusively in the case of the **P_L2b** construct, harboring the longer part of the *LlYpr-10.2b* gene promoter. Additionally, neither of the short promoter fragments (**P₅2b** and **P₅2f**) was sufficient to ensure β -glucuronidase expression in tobacco petals. The longer promoter fragments (**P_L2b** or **P_L2f**) caused GUS accumulation but limited to the edges of petals. The most diverse expression of the reporter gene was observed in the ovary, encompassing the whole organ in the case of the **P_L2f** construct, but concentrating in the bottom and the edges in the case of the **P_L2b** and **P₅2f** constructs. In the case of the **P₅2b** construct, containing the short *LlYpr-10.2b* promoter fragment, the activity of the reporter gene was undetectable in the ovary.

Llpr-10 gene expression pattern in leaves. Response to wounding, pathogen, salicylic acid and hydrogen peroxide

Independently of the promoter deletion analysis in transgenic tobacco we followed *Llpr-10* gene expression in the native system — yellow lupine plants, cultivated in a sterile growth chamber (Fig. 3). Results presented in Fig. 3A — semiquantitative RT-PCR — show the expression levels of three *Llpr-10.2* subclass members in yellow lupine leaves during development. In order to detect the *Llpr-10.2a* transcript, six times more template and

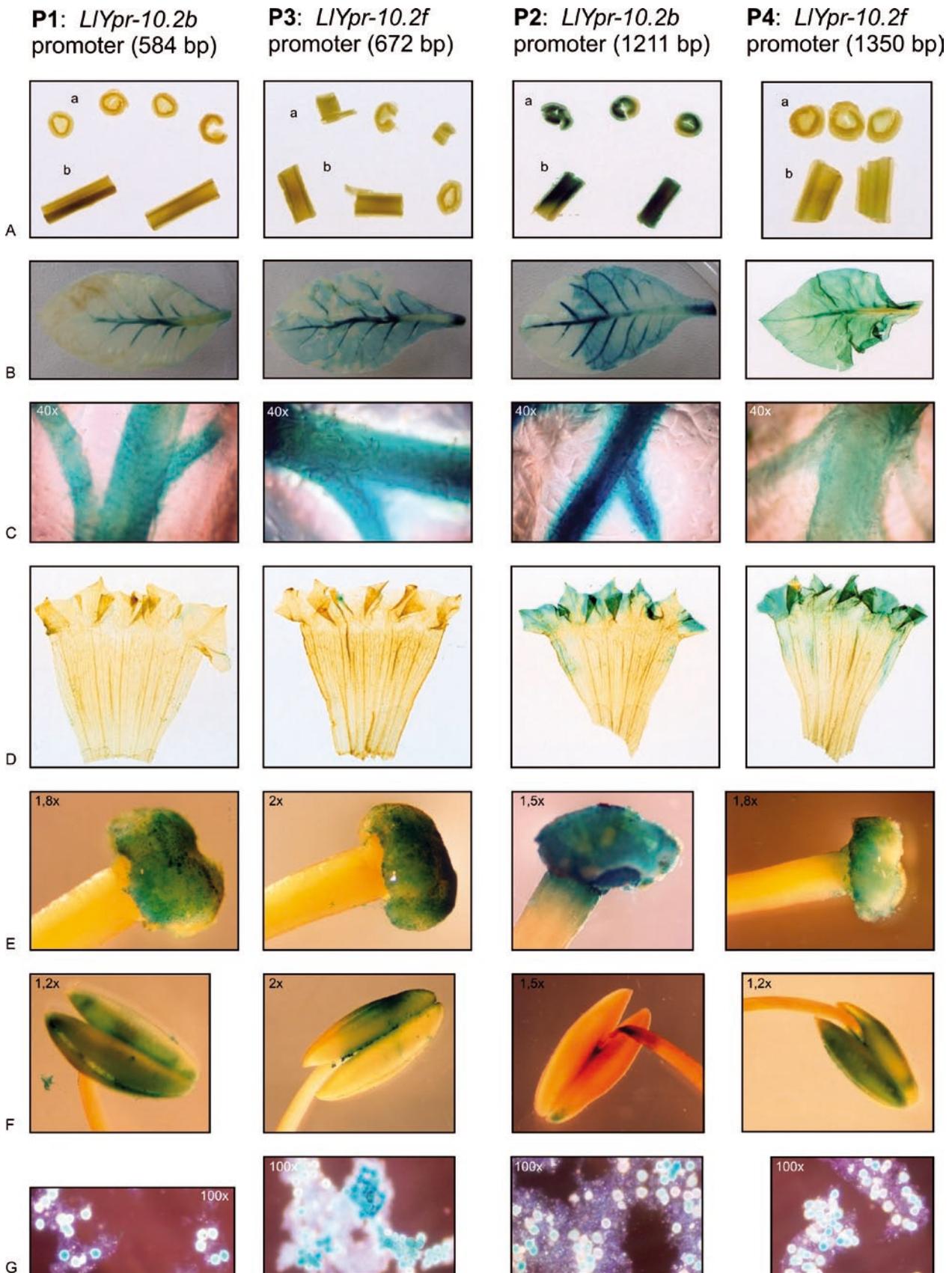


Figure 2. *In situ* histochemical localization of GUS activity in transgenic tobacco plants expressing *LIYpr-10:GUS* fusion under the control of different promoter regions of yellow lupine *pr-10* genes: *LIYpr-10.2b* and *LIYpr-10.2f*.

(A) stem: a. cross section, b. longitudinal section; (B) leaf; (C) magnified leaf (vascular bundles); (D) flower petals; (E) ovary; (F) stamen stigma; (G) anther; (H) pollen grains.

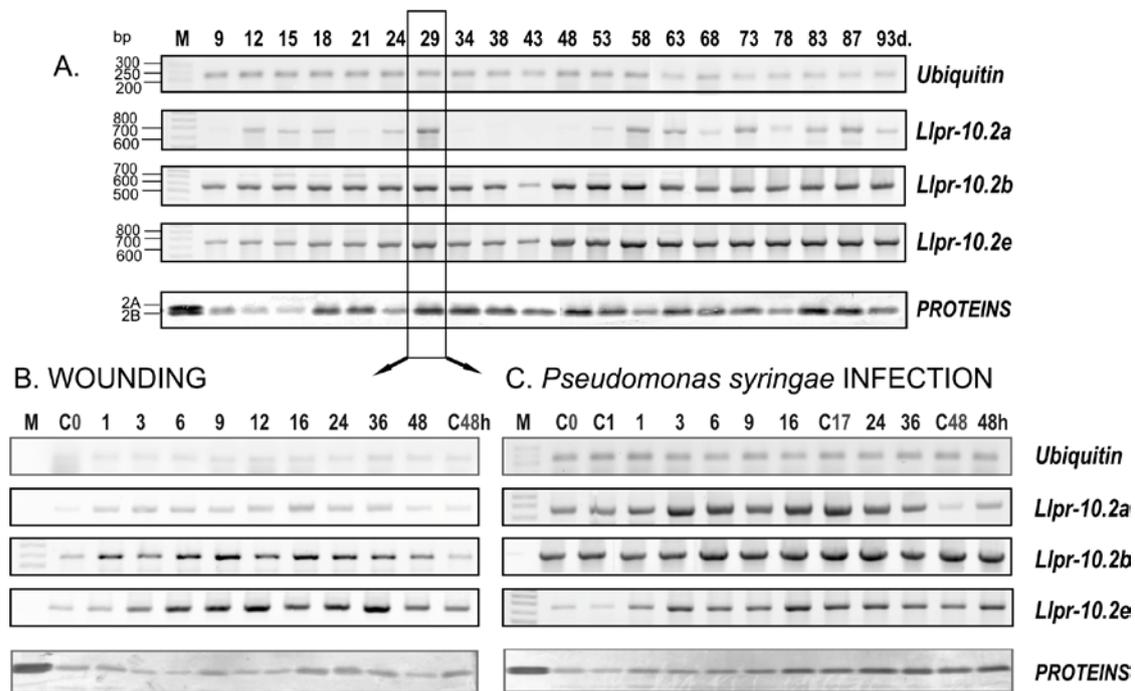


Figure 3. Expression pattern of genes representing *Llpr-10.2* subclass in yellow lupine leaves.

(A) leaves during development, (B) wounded leaves, (C) leaves infected with *Pseudomonas syringae*. Total RNA template concentration and PCR cycle number were as follows: with primers for ubiquitin 0.2 µg/35 cycles; for *Llpr-10.2a* 0.6 µg/40 cycles; for *Llpr-10.2b* and *Llpr-10.2e* 0.1 µg/30 cycles. RT-PCR product sizes were as follows: *Llpr-10.2b* – 583 bp; *Llpr-10.2e* – 666 bp; ubiquitin – 230 bp. Protein accumulation was analyzed using Western blot performed after SDS/PAGE of 1 µg total protein extract per well and antibodies anti-LIPR-10.2 subclass. **M** – molecular weight markers; **C0**, **C1**, **C17**, **C48** – leaves of control plants 0, 1, 17 and 48 h after treatment, respectively; **B** – wounded; **C** – infiltrated with 10 mM MgCl₂.

more PCR cycles were needed than in the case of the *Llpr-10.2b* and *Llpr-10.2e* transcripts. This difference in the expression level confirms our earlier, preliminary results (Hands Schuh *et al.*, 2004). In addition, there were time points where the *Llpr-10.2a* transcript was undetectable (9, 21 and 34–48-day-old plants). In contrast, *Llpr-10.2b* and *Llpr-10.2e* genes reveal similar, constitutive expression profiles. The only difference is that *Llpr-10.2b* gene expression is more uniform, while *Llpr-10.2e* transcript undergoes slight changes during plant development. In young leaves the amount of *Llpr-10.2e* transcript is clearly smaller than in ageing ones. The same tendency can be observed for the level of protein subclass LIPR-10.2.

Taking into account the fact that in 29-day-old plants all *Llpr-10.2* genes studied were expressed, we decided to check their response to wounding and selected exogenous elicitors in 4-week-old yellow lupine plants. First, leaves of one group of plants were mechanically wounded (Fig. 3B) while the second group was infiltrated with pathogenic bacteria *Pseudomonas syringae* pv. *pisi* (Fig. 3C). Comparing to ubiquitin, expression of all *pr-10* genes studied was elevated in both plant groups: *Llpr-10.2a* between 3–36 h, *Llpr-10.2b* and *Llpr-10.2e* between 1–48 h, which was reflected also in a gradual protein increase

(Fig. 3B and C). However, as shown in panel C, control leaves (C17), infiltrated with buffer only instead of the pathogenic bacteria suspension, reveal the same level of increase independently of the studied gene. This evidently proves that the observed elevated expression of *Llpr-10.2* genes is a consequence of mechanical wounding, not pathogen infection *per se*. Thus, the following experiment was based on another approach – two elicitors – hydrogen peroxide, involved in the oxidative stress (Ślesak *et al.*, 2007) and salicylic acid, one of the crucial signaling molecule in plant defense response (Shah, 2003), were supplied as water solutions into the soil, not directly into leaves. Then, expression of *Llpr-10* genes was determined in roots as well as in leaves within 48 h after treatment (Fig. 4). The effect of hydrogen peroxide was definitely weaker than that caused by salicylic acid. Only in roots treated with hydrogen peroxide solution, the expression of two genes slightly increased – *Llpr-10.1a*, representing yellow lupine subclass *Llpr-10.1*, and *Llpr-10.2a*, representing subclass *Llpr-10.2*. Quite the opposite, in the same tissues the expression level of *Llpr-10.2b* and *Llpr-10.2e* gradually diminished, while the *Llpr-10.1b* gene in roots and all the studied genes in leaves seemed not to be sensitive to hydrogen peroxide. Salicylic acid (SA) caused changes in the expression level all

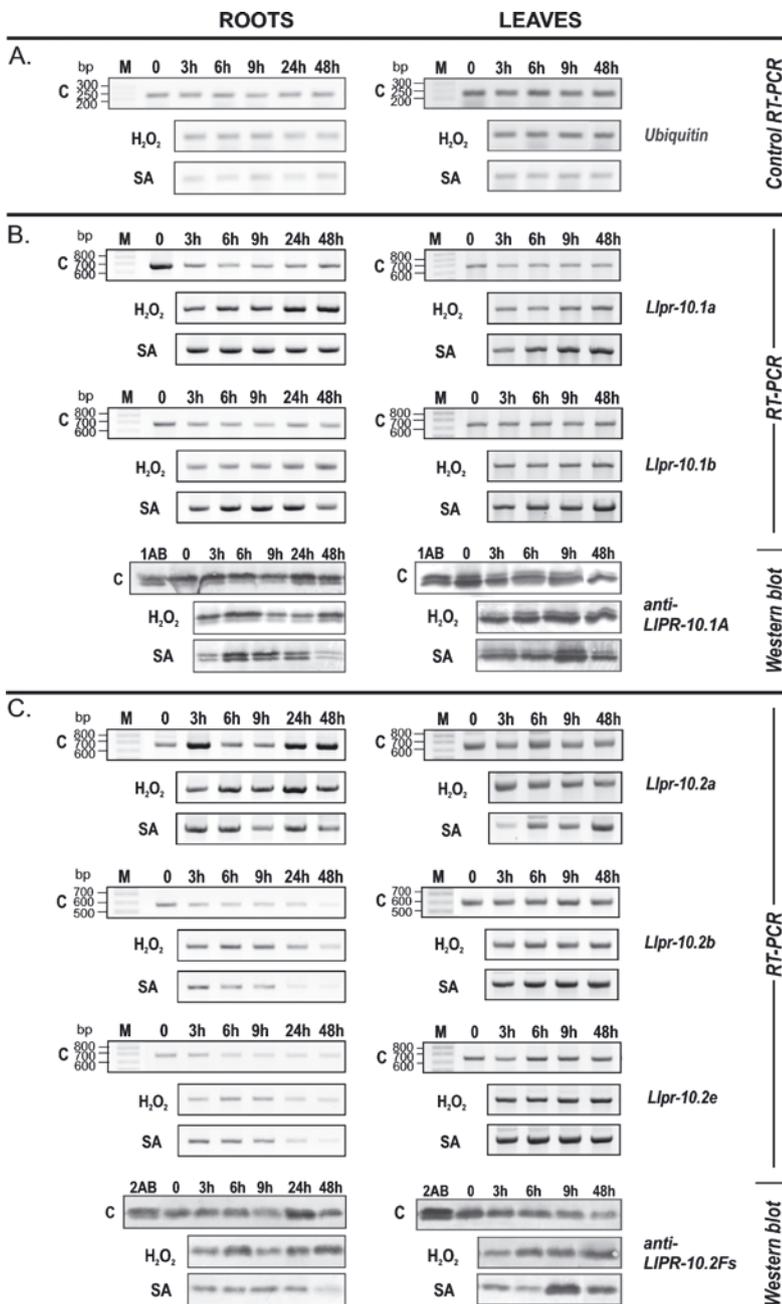


Figure 4. Expression of selected *Llpr-10* genes in yellow lupine plants in response to elicitors: hydrogen peroxide (H_2O_2) and salicylic acid (SA).

(A) Ubiquitin control; (B) subclass *Llpr-10.1*; (C) subclass *Llpr-10.2*. Total RNA template concentration and PCR cycle number were as follows: for ubiquitin 0.6 μ g/35 cycles; for *Llpr-10.2a* 0.6 μ g/40 cycles; for *Llpr-10.1a*, *Llpr-10.1b*, *Llpr-10.2b* and *Llpr-10.2e* – 0.1 μ g/30 cycles. Western blot was performed separately with specific polyclonal antibodies against subclass LIPR-10.1 and LIPR-10.2.

Llpr-10 genes, generally increasing it in leaves. Especially both *Llpr-10.1* subclass members and one gene representing subclass *Llpr-10.2*, *Llpr-10.2a*, gradually increased up to 48 h after treatment. *Llpr-10.2b* and *Llpr-10.2e* genes revealed a permanently enhanced expression level in leaves of plants watered with SA solution, comparing to control plant leaves. Interestingly, the same genes were clearly decreased in roots of the SA-treated plants. This antagonistic effect of salicylic acid was also visible at the LIPR-10 protein level – evidently decreasing in roots 48 h after treatment and increasing in leaves 9 h after treatment. Both Western blots, with antibodies specific for LIPR-10.1 subclass as well as for LIPR-10.2 subclass, reflected this bias.

Llpr-10.2b gene silencing

Another approach applied for a functional analysis of yellow lupine *pr-10* genes was gene silencing (Fire *et al.*, 1998; Tijsterman *et al.*, 2002; Szweykowska-Kulińska *et al.*, 2003; Meister & Tuschl, 2004; Baulcombe, 2005). RNA interference (RNAi) was used to switch off the *Llpr-10.2b* gene expression in yellow lupine plants. As any effective method of the gene silencing in legumes was elaborated up to date, we decided to prepare three versions of silencing vector differing in the orientation of the target gene: sense (**2b_{SE}**), antisense (**2b_{AS}**) and a hair pin (**2b_{HP}**, Fig. 5A). Plant leaves (infiltrated ones as well as those located above and below the

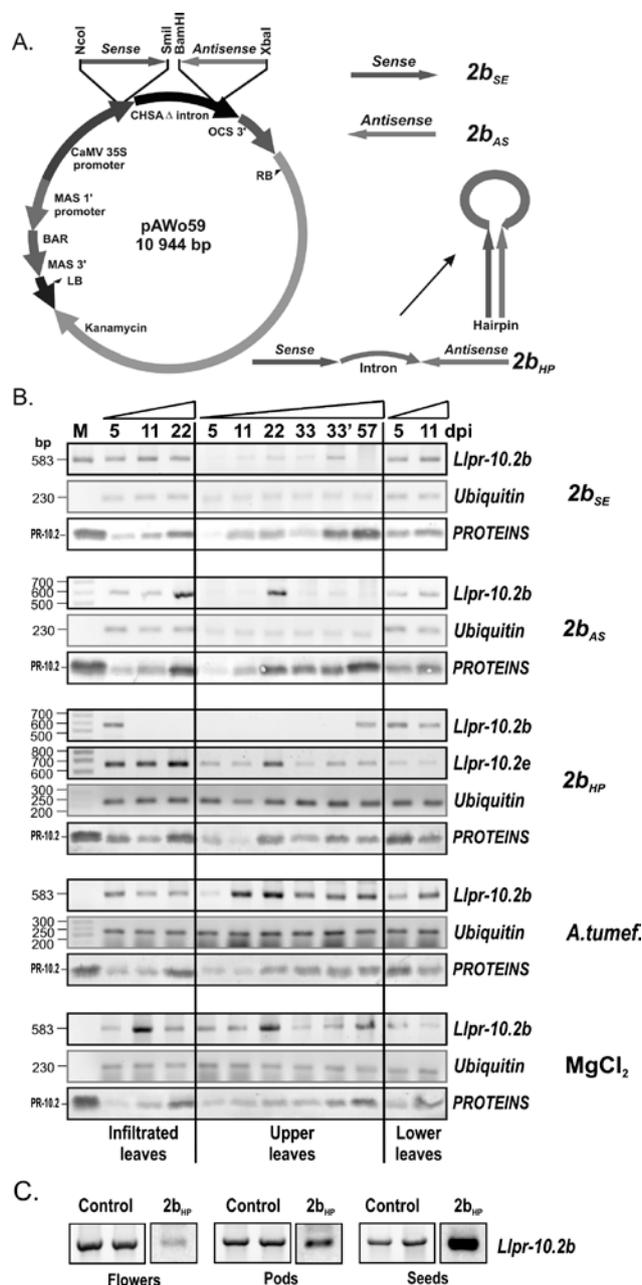


Figure 5. *Llpr-10.2b* gene silencing in yellow lupine.

(A) Structure of the gene silencing vector and three *Llpr-10.2b* gene silencing constructs: sense ($2b_{SE}$), antisense ($2b_{AS}$) and hairpin ($2b_{HP}$). (B) Gene and protein expression pattern in yellow lupine leaves infiltrated with *A. tumefaciens* transformed with prepared silencing vectors. (C) Flowers, pods and seeds of plants used in silencing assay. As a control, plants infiltrated with wild type *Agrobacterium* or with 10 mM $MgCl_2$ were applied. The effect of gene silencing was followed using RT-PCR with primers specific to *Llpr-10.2b* gene. For comparison ubiquitin expression level was monitored as a constitutively expressed gene. In the case of effective silencing of the *Llpr-10.2b* gene (hairpin construct; $2b_{HP}$) RT-PCR was also performed with primers specific for homologous gene *Llpr-10.2e*. PCR and Western parameters were as described in Fig. 3. dpi – days post infiltration; 33 and 33' – younger and older leaves from the same plants 33 dpi, respectively.

infiltration site) were separately collected 5, 11, 22, 33 and 57 days post infiltration (dpi). Target gene expression was analysed using semi-quantitative RT-PCR method, supplemented by Western blot analysis with anti-LIPR-10.2B polyclonal antibodies as described earlier (Handschuh *et al.*, 2004). RT-PCR with starters specific for lupine ubiquitin gene was applied as a control.

The sense construct ($2b_{SE}$) very slightly suppressed expression of the *Llpr-10.2b* gene in young leaves above the infiltration sites (Fig. 5B). Better results were obtained with the antisense construct ($2b_{AS}$) – for all upper leaves the level of *Llpr-10.2b* expression was clearly decreased, except for leaves collected 22 days after infiltration. Here, the *Llpr-10.2b* gene was even overexpressed, comparing to the other samples and the ubiquitin control, which

was observed in all infiltrated leaves. Only the hairpin construct ($2b_{HP}$) completely silenced the *Llpr-10.2b* gene in leaves (Fig. 5B). Here, the RT-PCR product specific for the *Llpr-10.2b* gene was detected in infiltrated leaves only 5 days after infiltration whereas the upper leaves were free from *Llpr-10.2b* gene transcript until the 57th day after infiltration. Eleven days after infiltration *Llpr-10.2b* mRNA was detected only in leaves below the infiltration sites.

Surprisingly, the suppression events were not confirmed by the results of Western blot analysis. In all cases, the PR-10.2 protein level represents an upward tendency correlated with the age of the plants (Fig. 5B). This bias is, however, less visible in plants infiltrated with the hairpin construct.

In the control plants infiltrated with wild type *Agrobacterium* or with infiltration buffer only, tran-

siently increased *Llpr-10.2* expression was noticed 11 or/and 22 days after induction. No phenotype differences were observed between any groups of plants used for this experiment.

In order to confirm the specificity of silencing, RT-PCR with primers specific for the *Llpr-10.2e* gene, a close homologue of *Llpr-10.2b*, was performed. As shown in Fig. 5B the *Llpr-10.2e* gene remains expressed in the plants with silenced *Llpr-10.2b* gene, despite the high level of identity of both genes (90.7%; Table 2). Nonetheless, the *Llpr-10.2e* expression seems to be slightly suppressed in the leaves above and below the infiltration site, comparing to infiltrated leaves.

DISCUSSION

In earlier reports we showed that yellow lupine *pr-10* genes form a multigene family and despite a high protein sequence identity, they are differentially expressed in plant tissues (Sikorski *et al.*, 1999; 2000; Handschuh *et al.*, 2004). LIPR-10.1B, member of subclass LIPR-10.1, was constitutively expressed in the whole plant whereas its homologue, LIPR-10.1A, only in root and stem, being induced in leaves by pathogenic bacteria *Pseudomonas syringae* (Sikorski, unpublished). Expression of the LIPR-10.2 subclass members was detected in all yellow lupine organs, but at different levels. Preliminary results showed that one of the *Llpr-10.2* genes (*Llpr-10.2a*) was induced in leaves by salicylic acid (Handschuh *et al.*, 2004). It was proved that all yellow lupine *pr-10* genes are suppressed in nodulated roots (Sikorski *et al.*, 2000).

The two new *pr-10.2* genes presented here were identified in a yellow lupine genomic library. Their general structure is very similar to that of the previously discovered *LlYpr-10.1* genes. Also the gene expression level of the new *Llpr-10.2* subclass members resembles that of the *Llpr-10.1* subclass, which can be concluded from the fact that identical RT-PCR parameters could be applied in order to detect the transcripts corresponding to the *Llpr-10.1a*, *Llpr-10.1b*, *Llpr-10.2b* and *Llpr-10.2e* genes (Fig. 4B and C). The only exception is the *Llpr-10.2a* gene that is detectable by RT-PCR only when six times more of total RNA template is used and ten more PCR cycles follow reverse transcription. The expression of this gene, as it was expected, was increased in leaves of yellow lupine plants treated with salicylic acid (Fig. 4C). However, elevated levels of expression in leaves in response to salicylate were also observed for the other *Llpr-10* genes studied. It corresponds with the fact that SA-response elements, such as TCA-like sequences described in tobacco, are present in all known yellow lupine *pr-10* gene pro-

moters, including the two presented in this report. *pr-10* genes from other plant species also respond to salicylate and hydrogen peroxide, however, the kinetics of response often differs (Jwa *et al.*, 2001; Rakwal *et al.*, 2001). The antagonistic effect of SA on *Llpr-10.2* gene expression in yellow lupine roots and leaves suggests that complex, organ-dependent regulation pathways are stimulated in plants by this elicitor. Endogenous SA, a key mediator of systemic acquired resistance (SAR), increases in plant tissues following pathogen infection and stimulates the expression of many genes engaged in defense response (Kunkel & Brooks, 2002; Shah, 2003). Exogenously applied salicylate generates a similar effect. Salicylate-induced enhanced expression of *Llpr-10* genes in yellow lupine leaves supports the hypothesis relating PR-10 proteins to pathogenesis.

Apart from the SA-response elements other regulatory motifs implicated in plant defense mechanisms were identified in the two *Llpr-10.2* gene promoters studied. The most abundant are different variants of Dof motifs, recognized by plant-specific transcription factors regulating expression of a variety of signal-responsive genes, including those responsive to pathogens (Yanagisawa & Schmidt, 1999; Yanagisawa, 2002). A single AC-rich H-box, present in defense-related genes, was found in both *LlYpr-10.2* promoters. Additionally, in the *LlYpr-10.2f* promoter another element, W-box, occurs in two copies. Vast families of plant transcription factors that bind to the W-boxes, WRKY proteins, regulate many pathogenesis- and senescence-related genes (Eulgem *et al.*, 1999; 2000; Turck *et al.*, 2004). W-box presence in *LlYpr-10.2f* promoter seems to be one of the key arguments supporting this gene's classification as pathogenesis-related. Although functional W-boxes are often gathered in clusters and act synergistically, even a single W-box is sufficient for WRKY-dependent regulation of transcription (Turck *et al.*, 2004). In *LlYpr-10.2f* two W-boxes are separated from each other, so they rather act as two single, independent elements. The lack of a W-box in the *LlYpr-10.2b* promoter is a significant feature that distinguishes the two homologous gene promoters presented in this report.

It must be stressed here that some other important defense-related elements are absent in the studied *LlYpr-10.2b* and *LlYpr-10.2f* gene promoters. These motifs include G-box (CCACGTGG), responsible for reaction to biotic stress, GCC-box (AGCCGCC), ERE (ethylene-responsive element) and PR-box (GGCGGC), specific for some promoters of genes implicated in systemic acquired resistance (Singh *et al.*, 2002). In fact, in wounded leaves we observed an elevated level of the studied *Llpr-10.2* gene transcripts (Fig. 3B). However, our results revealed that the same reason (wounding) caused

the increase of *Llpr-10.2* genes expression observed after pathogen infiltration, not the infection *per se*. That agrees with our preliminary Northern blot results (Sikorski *et al.*, 2000) showing high level of transcripts belonging to the *Llpr-10.2* subclass 1 h after *Pseudomonas syringae* infection, in contrast to the later response of *Llpr-10.1a*, a pathogen-inducible member of subclass *Llpr-10.1*. The kinetics of the response of *Llpr-10.2* genes also suggested reaction to mechanical stress rather than to pathogen infection. It is possible that despite the high homology within the *pr-10* gene family particular homologues can play slightly different roles or be recruited after different kinds of stress and pathogen.

Besides the pathogenesis-related aspect discussed above, it must be underlined that PR-10 proteins are postulated to participate in general plant developmental program (Pozueta-Romero *et al.*, 1995; Vanek-Krebitz *et al.*, 1995; Balsamo *et al.*, 1995; Swoboda *et al.*, 1996; Huang *et al.*, 1997; Strömvik *et al.*, 1999; Sikorski *et al.*, 1999; Liu & Ekramoddoullah, 2003; Beuning *et al.*, 2004; Finkler *et al.*, 2005). Both *pr-10* gene promoters studied here contain hormone-response element (HRE) specific for genes regulated by gibberellins (TAACAA). Additionally, the majority of transcription factors that potentially bind to the sequences identified in the *Llpr-10.2* gene promoters and mediate stress-response can also participate in developmental processes, revealing time- and organ-dependent activities and mediating tissue-specific expression. For instance, Dof proteins mediate not only defense but also light and phytohormone response, seed development and germination (Yanagisawa & Schmidt, 1999; Yanagisawa, 2002). The expression analysis results presented here evidently prove that *pr-10.2* genes are generally sensitive to any kind of environmental or developmental changes. The elicitor-independent increase of PR-10 protein amount in yellow lupine leaves with ageing (Figs. 3 and 5) reaches the highest level in senescent leaves, which is typical for many *pr-10* proteins (Crowell *et al.*, 1992; Puhlinger *et al.*, 2000). *Arabidopsis* genomic studies of 402 transcription factors revealed that the expression of stress-related genes is often correlated with senescence (Yanagisawa, 2002).

The analysis of the two *pr-10.2* promoter sequences revealed many common sites as well as substantial variations that can determine their expression pattern in plants. Within the *LlYpr-10.2b* gene promoter also an auxin-response element (auxRE) was identified. This motif consists of two 6-nucleotide parts (TGTCTC and AATAAG) separated by several nucleotides (here 12 nt). Only one of these parts is present in the *LlYpr-10.2f* gene promoter, which is probably not sufficient for interaction with auxin-dependent transcription factors. Instead, the *LlYpr-10.2f* promoter has one glucocorticoid-response

element (GRE), absent in the *LlYpr-10.2b* gene promoter. These differences can determine the diverse mode of expression of the yellow lupine *pr-10.2* genes, as a consequence of regulation by different plant hormones. Although no substantial differences in expression were observed between the *Llpr-10.2b* and *Llpr-10.2f* genes in yellow lupine or in transgenic tobacco during plant development, a distinct expression pattern after stimulation by factors not assayed in this study cannot be excluded. The influence of plant hormones on expression of *Llpr-10.2* genes needs further experimental verification. The impact of jasmonate, ethylene, abscisic acid, kinetin and zeatin is under investigation.

Llpr-10.2b gene silencing, although successful, did not help to explain the function of PR-10 proteins, either. We succeeded in silencing *Llpr-10.2b* gene in leaves of mature yellow lupine plants using *Agrobacterium tumefaciens* as a carrier. The *Llpr-10.2b* gene silencing was effective only when the hairpin construct was introduced into leaves, in compliance with literature data reporting better efficiency of gene silencing triggered by double-stranded RNA (Chen *et al.*, 2002; Colditz *et al.*, 2007). However, it was difficult to conclude the function of *pr-10* genes as the *Llpr-10.2b* gene absence did not cause any visible symptoms. The most probable explanation bases on the fact that the studied gene belongs to a multigene family. Presumably we observed a situation when one absent gene was substituted for another family member. The fact that silencing of the target gene was not accompanied by efficient silencing of its close homologue, *Llpr-10.2e*, sharing 90% identity within the coding sequence and representing a similar constitutive expression pattern, seems to support this hypothesis. Also Western blot analysis shows an invariable protein accumulation level, despite *Llpr-10.2b* transcript absence. Western analysis reported here represents the whole LIPR-10.2 subclass expression, comprising at least six different proteins. Therefore, subtle differences in the levels of individual proteins cannot be noticed. Additionally, protein accumulation does not always reflect the fluctuations observed at the transcript level. Apparently, silencing of the whole *pr-10* family would be more informative. Alternatively, another plant model could be used, with fewer *pr-10* family members than in yellow lupine. It is also possible that *pr-10* gene silencing is not harmful to a mature plant and that it should be performed at an early stage of plant development. Moreover, gene silencing conjugated with elicitor-induction experiments can be helpful. Such an approach was recently successfully applied by Colditz *et al.* (2006) who silenced PR10-1 protein in *Medicago truncatula*. Despite the knockdown *Medicago* transgenic line revealing almost complete silencing of five additional homologues of the target

gene, the authors did not observe any morphological differences in the studied root cultures. Changes appeared only when the silenced line was submitted to infection with the oomycete pathogen *Aphanomyces euteiches*, the major parasite of legumes. Here, the lack of PR10-like proteins was accompanied by overexpression of other, non-homologous, PR defense proteins and in consequence the suppression of pathogen infection development, contrary to the expected result. Undoubtedly, to confirm the contribution of the yellow lupine *pr-10.2* genes presented here to the plant defense reactions, further functional analysis is necessary, including studies of gene expression response to other pathogens and stress-related factors.

Summarizing, the presented results showed that yellow lupine *pr-10* genes are precisely regulated and can be differentially expressed despite high sequence homology. They are implicated not only in plant defense mechanisms, but constitute a substantial part of plant developmental program. However, temporal silencing of one *pr-10* family member is neutral to the mature yellow lupine plants while the other homologues are still present. The exact biological role of PR-10 proteins still needs to be determined.

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