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Nonspecific Lipid-Transfer Protein Genes Expression in Grape (*Vitis* sp.) Cells in Response to Fungal Elicitor Treatments

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Nonspecific lipid transfer proteins (nsLTPs) are small, basic cystein-rich proteins believed to be involved in plant defense mechanisms. Three cDNAs coding nsLTPs from grape (*Vitis vinifera* sp.) were cloned by reverse-transcriptase-polymerase chain reaction (RT-PCR) and PCR. The expression of nsLTP genes was investigated in 41B-rootstock grape cell suspension, in response to various defense-related signal molecules. Ergosterol (a fungi-specific sterol) and a proteinaceous elicitor purified from *Botrytis cinerea* strongly and rapidly induced the accumulation of nsLTP mRNAs. Jasmonic acid, cholesterol, and sitosterol also promoted nsLTPs mRNA accumulation, although to a lesser extent, whereas salicylic acid had no effect. High performance liquid chromatography analysis indicated that the amounts of three LTP isoforms (previously named P1, P2, and P4) were increased by ergosterol. None of the four isoforms displayed any significant antifungal properties, with the exception of the P4 isoform, which reduced *Botrytis* mycelium growth in vitro, but only in calcium-free medium. The results are discussed in the context of plant–pathogen interactions.

Additional keywords: Botrytis cinerea, ergosterol, gene expression, plant defense.

Plant nonspecific lipid-transfer proteins (nsLTPs) are small (91 to 95 residues), basic polypeptides that were first characterized in potato in the mid-1970s (Kader 1975). They share several structural features, the most striking being eight strictly conserved cystein residues engaged in four disulfide bridges (Kader 1996; Yamada 1992). Despite efforts of numerous research groups, the role of these proteins is still a matter of debate. Based on experimental results, several hypothesis have been suggested. First, the in vitro lipid transfer capacity shown by Kader (1975) suggested that these proteins were involved in the intracellular traffic of phospholipids. However, the extracellular location of the protein and the fact that all the sequences known to date exhibit a secretion signal in the N-terminal part of the peptide seem to rule out such a role for nsLTPs. These proteins are largely secreted during somatic embryogenesis of grape and carrot cell (Coutos-Thévenot et al. 1992a, 1993; Fleming et al. 1992) and might be involved in plant development mechanisms. For these reasons, and based on the binding properties of fatty acid derivatives, Sterk and associates (1991) and Meijer and associates (1993) suggested that nsLTPs may be involved in the transport of cutin monomers during cuticle layer formation. However, this hypothesis has not yet been validated. More recently, several results suggested a role for nsLTPs in plant defense mechanisms. Indeed, different 10-kDa basic proteins sharing homology with plant nsLTPs are induced during plant microorganism interactions (Blilou et al. 2000). Moreover, some of them are active against several pathogens with various degrees of specificity (Cammue et al. 1995; Carvalho et al. 2001; Kristensen et al. 2000; Molina et al. 1993). Expression of a barley nsLTP in transgenic tobacco and *Arabidopsis* spp. enhances tolerance to bacterial pathogens (Molina and Garcia-Olmedo 1997). However, the antimicrobial activity of the nsLTP does not seem to be related to their lipid transfer capacity (Cammue et al. 1995; Regente and De la Canal 2000).

Most of the nsLTPs described in literature were extracted from seed (Douady et al. 1985; Guerbette et al. 1999; Regente and De la Canal 2000), herbaceous plants (Kader et al. 1984; Kristensen et al. 2000; Molina et al. 1993), or beer (Jégou et al. 2000) by harsh treatments followed by chromatographic purification under drastic conditions. The 41B embryogenic grape cell suspensions, in which 30% of the extracellular proteins are nsLTPs when an auxin-free medium is used, provide an interesting alternative to obtain nsLTPs under much milder conditions (Coutos-Thévenot et al. 1992a, 1993). In this model system, the nsLTPs are purified to homogeneity by cation exchange chromatography followed by hydrophobic interaction chromatography. This allows the purification and characterization of four isoforms (named P1, P2, P3, and P4) of auxin down-regulated nsLTPs characterized by their amino acid sequence, maize and carrot antibody detection, and in vitro lipid transfer activity. Grape suspension cells are convenient for establishing structure–function relationships in the nsLTPs family and for studying the expression of nsLTP in response to various inducers. The nsLTPs are encoded by a multigenic family (Arondel et al. 2000; Kader 1997); therefore, different isoforms may correspond to different functions. It is, therefore, relevant to find out whether some isoforms are involved in grape defense reactions. The present article describes the cloning of several cDNAs encoding grape nsLTPs, and the accumulation of the corresponding mRNAs in the 41B cells after treatment with signal molecules known to induce defense reactions in plants.

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RESULTS

Cloning grape LTP cDNAs.

To obtain nsLTP cDNAs, two strategies were developed which allowed the isolation of three full-length clones. First, alignment of 47 LTPs sequences at the amino acid level led to the identification of two conserved regions, which were used to design degenerate primers (forward primer: YTI GGI TAY YTI AAR AAY GG; reverse primer: RTT IAC ICC RCA IKY IBH NGG). Using this pair of primers, reverse transcriptase-polymerase chain reaction (RT-PCR) run on mRNA extracted from auxin-depleted suspension cell cultures generated a 193-bp fragment. This fragment was subcloned in the pGEM-T easy vector and sequenced. Database interrogation showed that the sequence obtained was highly homologous to the central part of LTPs cDNAs. The 5′ and 3′ missing ends of the 41B rootstock LTP cDNA were obtained by rapid amplification of cDNA ends (RACE)-PCR. The full-length cDNA was amplified by PCR with primers, including the start and the stop codons. The deduced amino acid sequence exhibited typical signatures of plant nsLTPs, including the strictly conserved cysteine residues, at positions 31, 41, 57, 58, 78, 80, 104, and 118 (Fig. 1). In addition, amino acids 28 to 65 were identical to the 37 N-terminal residues of the P1 (accession # P80275) isoform characterized previously (Coutos-Thévenot et al. 1993).

Second, a primer was defined at the 5′ end of the 193-bp fragment, and PCR was performed on a λZapII grape (cv. Pinot noir) cDNA library (Fillion et al. 1999) using the phage-resident T7 primer sequence. A 299-bp fragment containing the

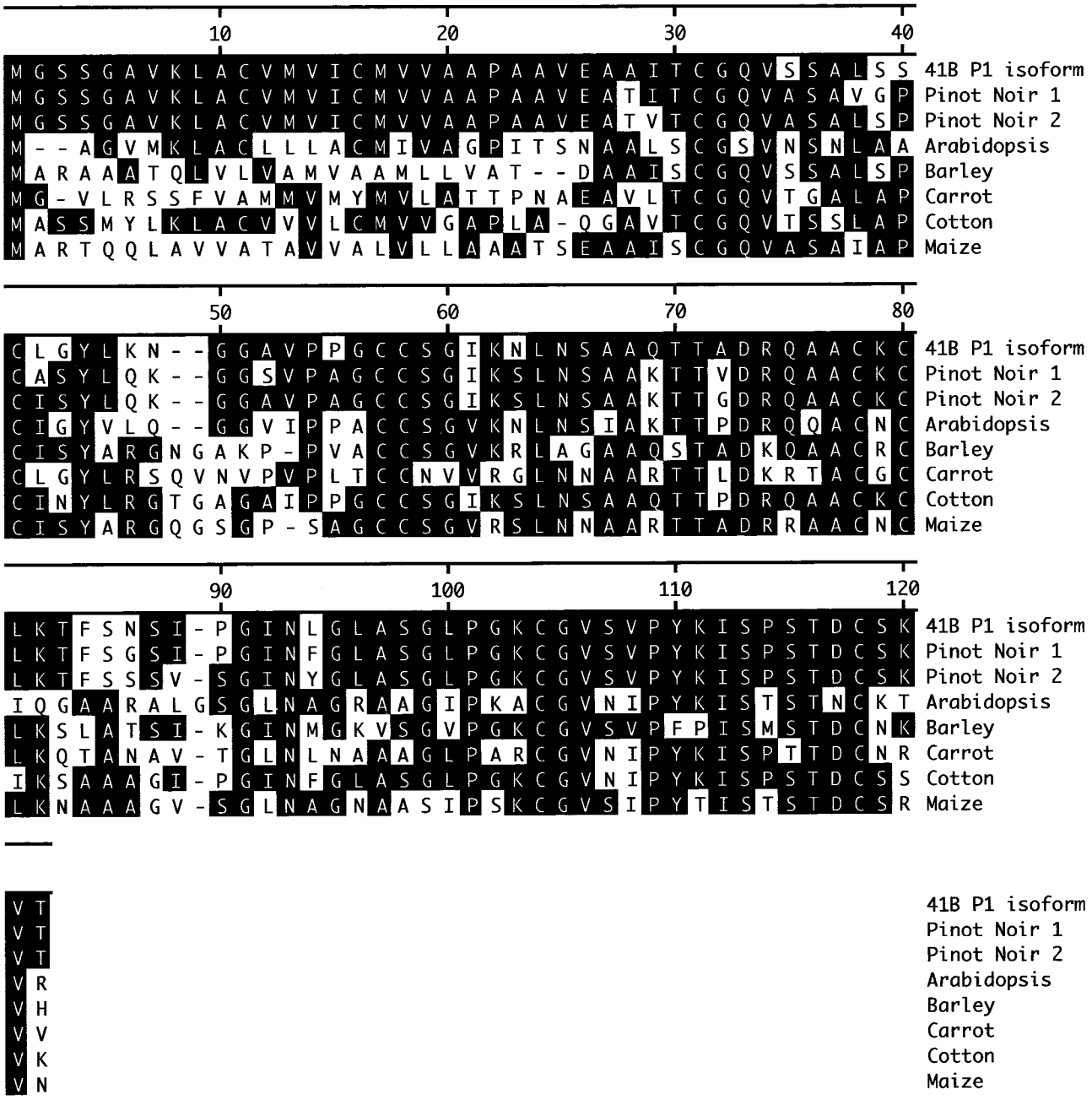

Fig. 1. Sequence alignment of different nonspecific lipid transfer proteins (nsLTPs). Grapevine sequences of P1 isoform cloned from 41B rootstock were compared with nsLTPs from Pinot Noir, *Arabidopsis* spp., barley, carrot, cotton, and maize. Black-boxed residues indicate conserved amino acids. Dashes indicate gaps. Alignments were performed with the clustal V program of the DNA Star package. Grapevine sequences have deposited in the GenBank database (accession numbers: 41B, AF465408; Pinot noir 1, AF467945; Pinot noir 2, AF467946).

stop codon was obtained. A reverse primer including the 3′ end of the open reading frame was used in conjunction with the T3 primer to amplify the full-length cDNA. After subcloning and sequencing, it appeared that two different Pinot noir LTPs cDNA were amplified. One sequence matched with the 41B P4 isoform (accession #P80274) previously described (Coutos-Thevenot et al. 1993), and the other one was more closely related to P1 (Fig. 1).

Induction of nsLTP gene expression in 41B cell suspension cultures.

Potential inducers of LTPs expression in grape are unknown. In this context, the effects of several compounds related to defense mechanisms on LTP expression were investigated. Northern blot analysis (Fig. 2A, upper panel) were performed with RNA extracted from cells treated by jasmonic acid (JA), salicylic acid (SA), or different sterols, including ergosterol, which is fungus specific and is an active elicitor of defense reactions (Granado et al. 1995). All of these chemicals were dissolved in ethanol; therefore, an identical volume of ethanol was added in the control experiment. Using the entire 41B LTP cDNA as a probe, the strongest hybridization signals were detected in ergosterol treatments. A faint signal also appeared in the control treated cells, indicating that expression was not induced by ethanol. SA treatment did not induce a significant and reproducible increase of LTP transcripts compared to the ethanol control. However, the amount of LTP transcripts was slightly but consistently increased by JA, sitosterol, and cholesterol.

To confirm induction of LTPs expression by fungal elicitors, 41B grape cells were treated by a water-dialyzed culture filtrate of *Botrytis cinerea*, a well-known grape pathogen. This treatment strongly increased the amount of LTP transcripts compared with the water control (Fig. 2B, upper panel). The active molecule in this filtrate was further purified by exclusion chromatography and identified as a protein fraction. Final purification, biochemical characterization, and identification of the active protein are under way (B. Poinssot and A. Pugin, *unpublished data*).

The most potent inducers were chosen to characterize more precisely the kinetics of expression (Fig. 3). LTPs mRNA levels started to increase 5 h after the addition of either ergosterol or the purified proteinaceous elicitor of *Botrytis* spp., and continued accumulating throughout the 30-h duration of the ex-

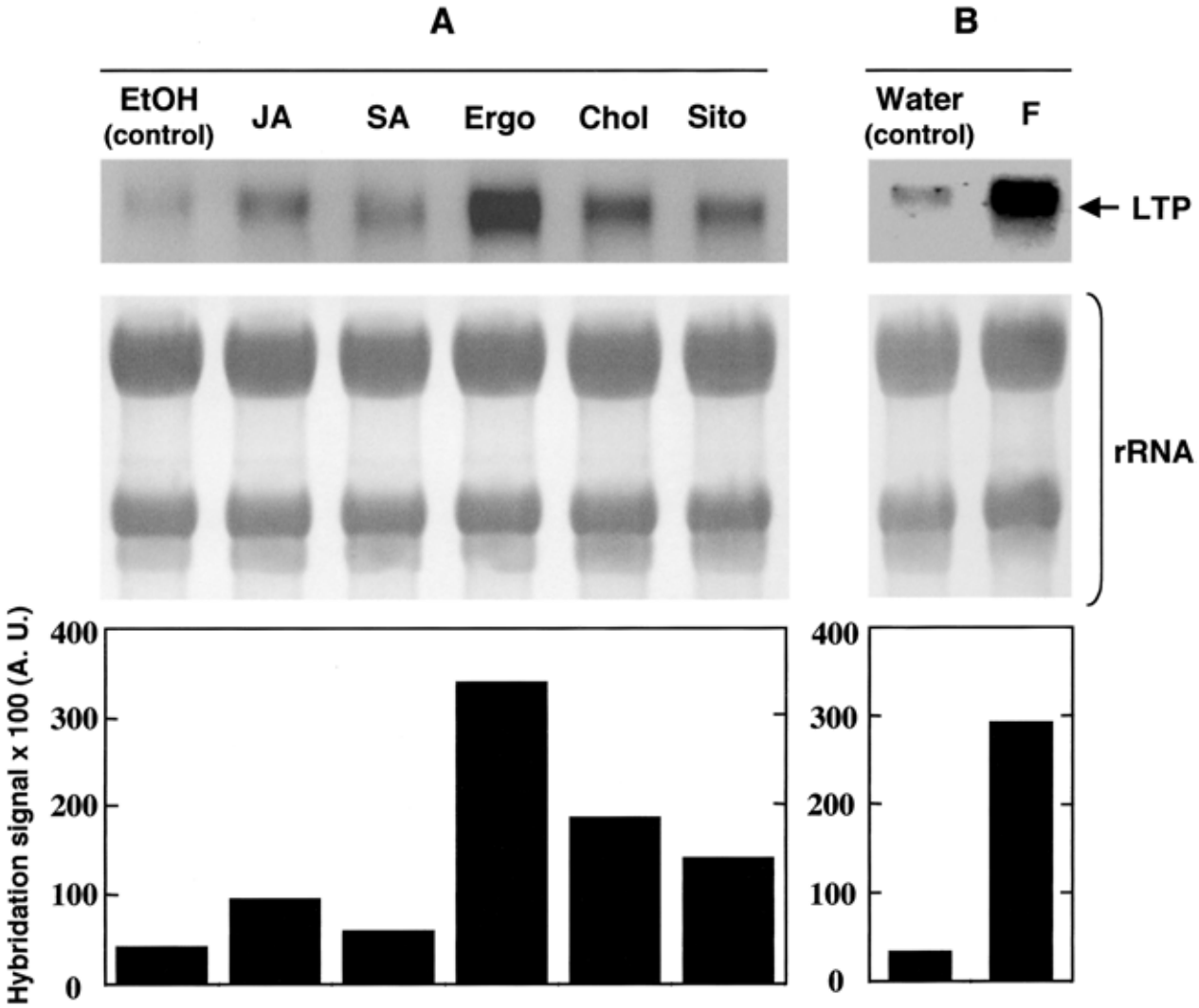

Fig. 2. Northern blot analysis of nonspecific lipid transfer proteins (nsLTPs) expression in 41B cell suspension cultures. RNAs were isolated from cells cultured in presence of 5 µM of NOA (β-naphtoxyacetic acid). **A,** JA: jasmonic acid (5 µM); SA: salicylic acid (5 µM); Ergo: ergosterol (5 µM); Chol: cholesterol (5 µM); Sito: sitosterol (5 µM), dissolved in ethanol. A control was performed using the same amount of ethanol (EtOH lane). **B,** F: water-dialyzed *Botrytis cinerea* culture filtrate. A control was performed using the same amount of water (W lane). Upper panels: hybridization with full-length 41B-LTP cDNA probe; middle panels: methylene blue staining of rRNAs as a control for equal lane loading; lower panel: hybridization signal quantification.

periment (Fig. 3). This visual estimation was confirmed by Phosphor-Imager quantification of the Northern blot membrane signals. The quantification was achieved by subtracting from the values obtained for the elicitor-treated cells, for each time point, the value obtained in the untreated control kinetics. At 30 h after induction, a 150-fold and 190-fold increase in hybridization signal was observed for ergosterol and *Botrytis* spp. elicitor treatment, respectively, compared with control cells. This reflects a strong and linear induction of mRNA accumulation with time in both cases, albeit a little more delayed in *Botrytis* spp. elicitor-treated cells.

Isoform expression specificity.

LTPs are encoded by multigenic families (Arondel et al. 2000; Coutos-Thévenot et al. 1993); therefore, there was a high probability that the probe used for Northern blot would not discriminate between different isoforms. The 3′ regions of the grape LTPs cDNAs are identical (Fig. 1), making it very difficult to design specific probes for each isoform. Furthermore, the transcript level is not always strictly related to the protein amount, due to translational control. To circumvent these limitations, cation-exchange high performance liquid chromatography (HPLC) analysis was used to investigate the diversity of isoform expression (Fig. 4). In the control, grape cells secreted only the P3 isoform, and lacked P2 and P1. However, a small amount of P4 was detected (just above the detection threshold). Such an expression pattern, particularly the positive response of the P3 isoform in control conditions, is new, because all results in the literature indicate that LTP genes are negatively regulated by auxin in suspension cells (Coutos-Thévenot et al. 1993; Song et al. 1998). The expression pattern in ergosterol-treated cells was different. P1, P2, and P4 isoforms were induced by ergosterol, whereas P3 was barely detectable. P2 was the major protein secreted by ergosterol-elicited cells. This pattern was qualitatively similar to the one already published for auxin-starved cells (Coutos-Thévenot et al. 1993), although there were differences in the relative level of each isoform expression. The proteins detected in each of the four HPLC fractions (P1, 2, 3, and 4) were assessed as nsLTP by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Western blot analysis. Each fraction displayed a single, 9-kDa band which cross-reacted with a polyclonal antibody

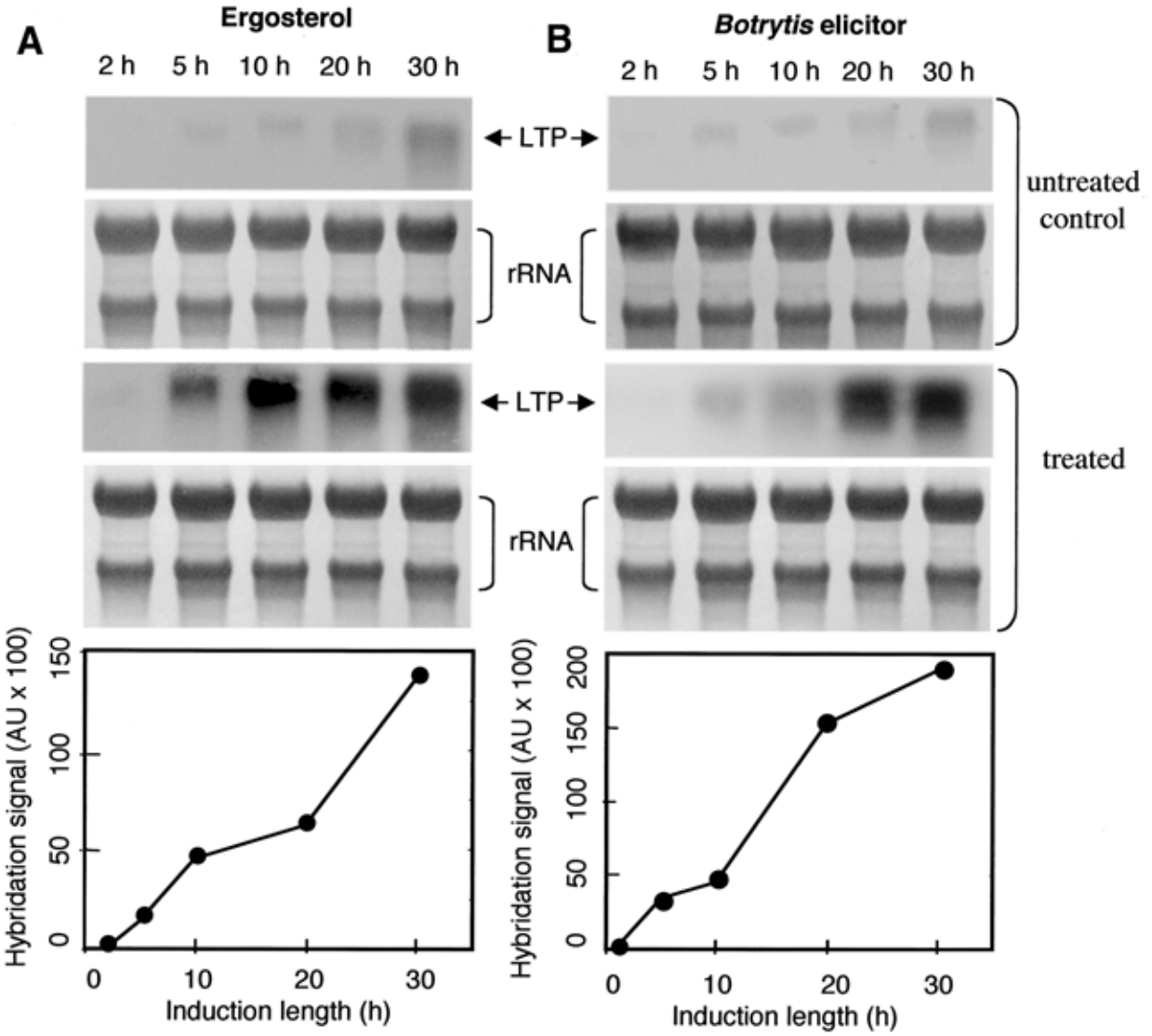

Fig. 3. Time-course analysis of nonspecific lipid transfer protein (nsLTP) mRNA accumulation in ergosterol or *Botrytis* spp. elicitor-treated cells. Cells were treated with **A,** 5 µM of ergosterol or **B,** 0,085 µg ml^{-1} of the proteinaceous *Botrytis* spp. elicitor, in the presence of 5 µM of NOA. Upper panels show hybridization signals and methylene blue staining of rRNAs, for both treated and untreated (control) kinetics. Lower panels show quantification of the hybridization signals with the Phosphor Imager. Hybridization signals are normalized by subtracting to each time point the hybridization value obtained at time zero.

raised against a Maize nsLTP (data not shown). Antibody was a generous gift of J.-C. Kader (Université Pierre et Marie Curie, Paris).

Fungitoxicity of LTPs.

B. cinerea is the major fungal pathogen for grape. With regard to the numerous results involving LTPs as antifungal proteins (Cammue et al. 1995; Carvalho et al. 2001; Kristensen et al. 2000; Molina et al. 1993; Wijaya et al. 2000), the activity of the different isoforms was checked on *B. cinerea* conidia germination and mycelium growth. Compared with an untreated control, addition of different LTP concentrations affected neither germination of conidia in 100 mM glucose solution supplemented with 1 mM $CaCl_2$ (Fig. 5A) nor mycelium growth. Moreover, the morphology of hyphae was not altered (Fig. 5C). LTPs are small, highly basic proteins (isoelectric point around 10); therefore, two control treatments were done. Bovine serum albumin (BSA) was used as a negative control, and histones, which present the same physicochemical properties as LTPs (i.e., a highly basic isoelectric point) were used as a positive control. The same results were obtained with these two treatments: neither BSA nor histones affected conidia germination and mycelium growth (Fig. 5B).

When the same assay was performed in the absence of calcium, BSA did not affect germination of conidia. P1, P2, and P3 (F1 fraction) isoforms had no effect for concentrations up to 30 μg ml^{-1} and induced a slight decrease (10%) of conidia germination for higher concentrations. The germination rate was markedly decreased in the presence of P4 isoform (approximately 50% less germination at the highest concentration) and totally blocked with histones at concentration as low as 15 μg ml^{-1} (Fig. 5D). In addition, mycelium growth was markedly reduced by the P4 isoform of LTP (Fig. 5F) and no growth was observed with histones (Fig. 5E).

DISCUSSION

The nsLTPs were originally described as proteins that have the ability to transfer lipid between membranes in vitro. This suggested a role for these proteins in intracellular lipid trafficking. The extensively described extracellular localization of the LTP (Coutos-Thévenot et al. 1992a, 1993; Kader 1997; Sterk et al. 1991; Thoma et al. 1993, 1994) has ruled out such a function in vivo. This raises the question of the in vivo role of LTPs.

Based on results published on onion (Cammue et al. 1995), sunflower (Regente and De la Canal 2000), cowpea (Carvalho et al. 2001), and sugar beet (Kristensen et al. 2000), a role was proposed for LTPs in plant defense mechanisms. In particular, Cammue and associates (1995) tested the inhibition of fungal growth by an LTP-like protein, (Ace-AMP1) that does not exhibit any lipid transfer activity, and three true nsLTPs (Rs-nsLTP, Ta-nsLTP, and Zm-nsLTP). Twelve different fungi species, including *B. cinerea,* were tested. Ace-AMP1 was shown

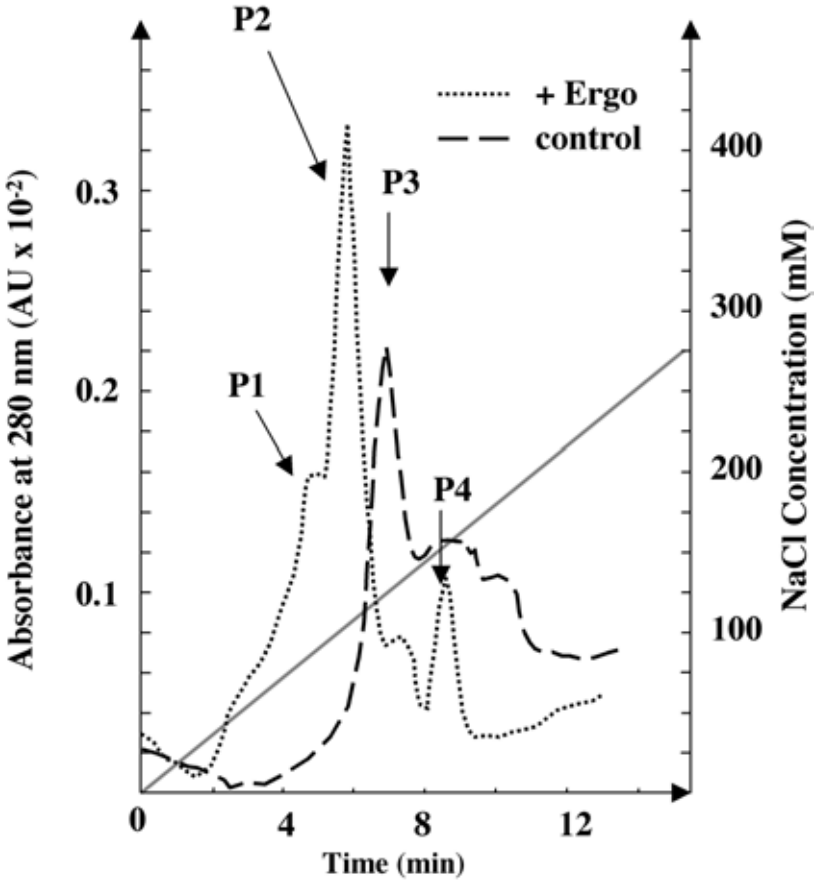

Fig. 4. Nonspecific lipid transfer protein (nsLTP) isoforms expression analysis in 41B cells. Isoform expression patterns were analyzed by ion-exchange high-pressure chromatography (HPLC) in various conditions. Cell (packed cell volume, 1 ml) was inoculated in 250 ml of glycerol-maltose medium supplemented with 5 μM NOA. Extracellular proteins were extracted as described after treatment (10 days) by ergosterol (5 μM) dissolved in ethanol (+ Ergo), and compared with the protein secreted in the control supplemented with an equivalent amount of ethanol (Control). After a 200-fold concentration, the total extract was loaded onto the column. Proteins were eluted by a linear gradient of NaCl as shown on the chromatogram. Elution was monitored by following absorbance at 280 nm. Proteins are named P1 to P4 according to their elution order from the column, as previously described (Coutos-Thévenot et al. 1993).

to be the most inhibitory. In addition, it was quite clear that the antifungal activity of Rs-nsLTP, Ta-nsLTP, and Zm-nsLTP strongly depended on the ionic strength of the assay medium. Indeed, addition of 1 mM Ca^{2+} (SMF^{+} assay medium) almost abolished the antifungal effect. Even for the ns-LTP-like Ace-APM1 protein, the presence of 1 mM calcium markedly decreased the 50% inhibitory concentration (IC_{50}) 2- to 10-fold. This led to the classification of LTPs in the pathogenesis-related proteins family (PR-14) (Van Loon and Van Strien 1999). The effects of calcium and ionic strength on LTP antifungal activity are surprising. Recent results indicate that some nsLTP-like proteins (CaMBP-10) may have calmodulin-binding properties (Liu et al. 2001). The experiments with the histone controls performed here (Fig. 5E) provide new insight. Histones, which are not antifungal protein but share with LTPs their small size and their high isoelectric point, are strongly antifungic (Fig. 5). This suggests that the effects of histones and grape LTPs on fungal growth is rather nonspecific and due to their physicochemical properties, which may affect, for example, the integrity of the fungus plasma membrane. A comparison of the isoelectric points of 10 LTPs from different species (including the grape P1) with 10 histones (based on sequences extracted from GenBank and calculation by the "PI/MW" software from Swiss-Prot) was made. This analysis gave values ranging from 9.02 to 9.5 and from 10.3 to 11.4 for nsLTP and histones, respectively. The most basic proteins (histones) were the most potent for fungal growth inhibition. None of the four LTP isoforms produced by 41B cells has any effect on *B. cinerea* growth in the presence of calcium at a concentration very close to the physiological conditions (i.e., in the calcium-supplemented medium). It is well known that high calcium concentrations are found in the apoplast, where the fungus resides (Bush 1995). This supports the idea that grape nsLTPs probably are not antifungal protein in vivo.

Previous studies at the protein level showed that, in grape suspension cells, nsLTPs are induced by a somatic embryogenesis-related signal: auxin starvation (Coutos-Thévenot et al. 1993; Sterk et al. 1991). The present data, obtained by Northern blot and HPLC analysis (Figs. 2, 3, and 4), indicate that nsLTPs also are induced by defense-related signals such as ergosterol, a proteinaceous elicitor secreted by *B. cinerea* and, to a lesser extent, JA. The fact that this increase in protein level is accompanied by an increase of LTP transcripts suggests a transcriptional control. HPLC analysis further distinguished between different isoforms, and showed that the P2 isoform is the major LTP produced in response to the ergosterol treatment. This differs from the induction of LTPs by auxin starvation, in which the most-produced isoform is P1 (Coutos-Thévenot et al. 1993). This means that, although LTPs are expressed in both situations (somatic embryogenesis and defense response), the isoform expression patterns are different, suggesting that the response of LTP genes to auxin and elicitor signals is different. The kinetics of transcript accumulation indicate that the response of LTP genes to elicitor treatment occurs quite fast, the mRNA level starting to increase 5 h after the treatment by ergosterol or by the elicitor from *Botrytis* spp. This kind of time course also has been observed for other PR proteins in grape, such as chitinase, β-1,3-glucanases, or class 10 pathogenesis-related (PR) proteins (Derckel et al. 1998; Robert et al. 2001). All these results concur with the conclusion that at least some grape LTP isoforms (i.e., P2 and P4) can be described as defense-related proteins, and may play a role in plant defense mechanisms, even if this is not a direct role as antibiotic compounds. These results are in agreement with the classification of plant LTPs in the PR proteins by Van Loon and Van Strien (1999).

However, the precise function of LTPs in plant defense mechanisms is still unclear. An important point is the function, within the context of plant defense response, of proteins excreted in the extracellular compartment and able to bind hydrophobic (Meijer et al. 1993) and amphiphilic molecules (Pato et al. 2001). Another class of cystein-rich proteins, named elicitins, which are secreted by oomycete fungi such as *Phytophtora cryptogea*, also are able to bind lipid molecules and various sterols, including the fungal-specific ergosterol (Mikès et al. 1998; Vauthrin et al. 1999). Moreover, the interaction of the elicitin–sterol complex with a plant plasma-membrane receptor triggers the elicitation of defense mechanisms in tobacco (Osman et al. 2001). No plant elicitin homolog is known so far. Nevertheless, even though little homology can be found between the primary sequences of nsLTPs and elicitins, their three-dimensional structures present significant analogies (Blein et al. 2002). A similar signaling role for an LTP–ergosterol complex during plant–fungus interaction constitutes an appealing hypothesis, but the existence of such a complex has never been shown. Indeed, according to literature, the rigid backbone of sterol molecules seems to prevent their binding to nsLTPs (Blein et al. 2002; Pato et al. 2001). The other potential ligands that may play a role in the LTP signaling pathway still need to identified.

Recently, new and decisive results emerged in the literature. It has been demonstrated that nsLTPs bind to specific sites on plasma membrane vesicles from tobacco. Moreover, nsLTP and elicitins appear to compete for the same binding sites (Buhot et al. 2001). It is, therefore, tempting to think that receptors for nsLTPs exist at the plant cell surface, and that these are identical to the elicitins' own binding sites. In addition, a T-DNA tagged *Arabidopsis* LTP mutant (*dir 1-1*) recently was shown to be affected in the systemic acquired resistance (SAR) signaling pathway (Maldonado et al. 2002). According to these authors, the *DIR 1* gene product could be the SAR mobile signal or may transport the SAR signal to the phloem for export to distant tissue. Future work will focus on this line of research.

MATERIAL AND METHODS

Elicitation of grape cell suspension cultures.

Grape cells (embryogenic line derived from the 41B rootstock *Vitis vinifera* × *V. berlandieri* hybrid, supplied by Moët-et-Chandon, Epernay, France) were established in the presence of 5 μM NOA (β-naphtoxyacetic acid) at 21°C in the dark on glycerol-maltose medium (pH 5.8) (Coutos-Thevenot et al. 1992b) and subcultured weekly under orbital shaking (100 rpm). Specific elicitation treatments are indicated in the figure legends. JA, SA, and sterols were purchased from Sigma and were at the highest purity available. All chemicals were dissolved in ethanol.

RNA extraction and Northern blot analysis.

Total RNAs were extracted from 18-day-old cell cultures using the Perfect RNA Eukaryotic mini kit (Eppendorf, Barkhausenweg, Germany) according to the manufacturer's instructions. Total RNA (20 μg) was mixed with loading buffer (32.7% formamide, 1.21 M formaldehyde) and loaded onto a 1.2% agarose gel made with MEN buffer (20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA) and supplemented with 2.25 M formaldehyde. The gels were run for 5 h at 50 V in MEN buffer, and capillary blotted onto a Hybond N nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) with 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNAs were cross linked to the membrane by heating for 2 h at 80°C. At this stage, the membrane was stained with methylene blue to check for equal RNA loading on the gel. Hybridization was performed overnight at 65°C in a buffer containing 0.25 M sodium phosphate (pH 7.2), 1%

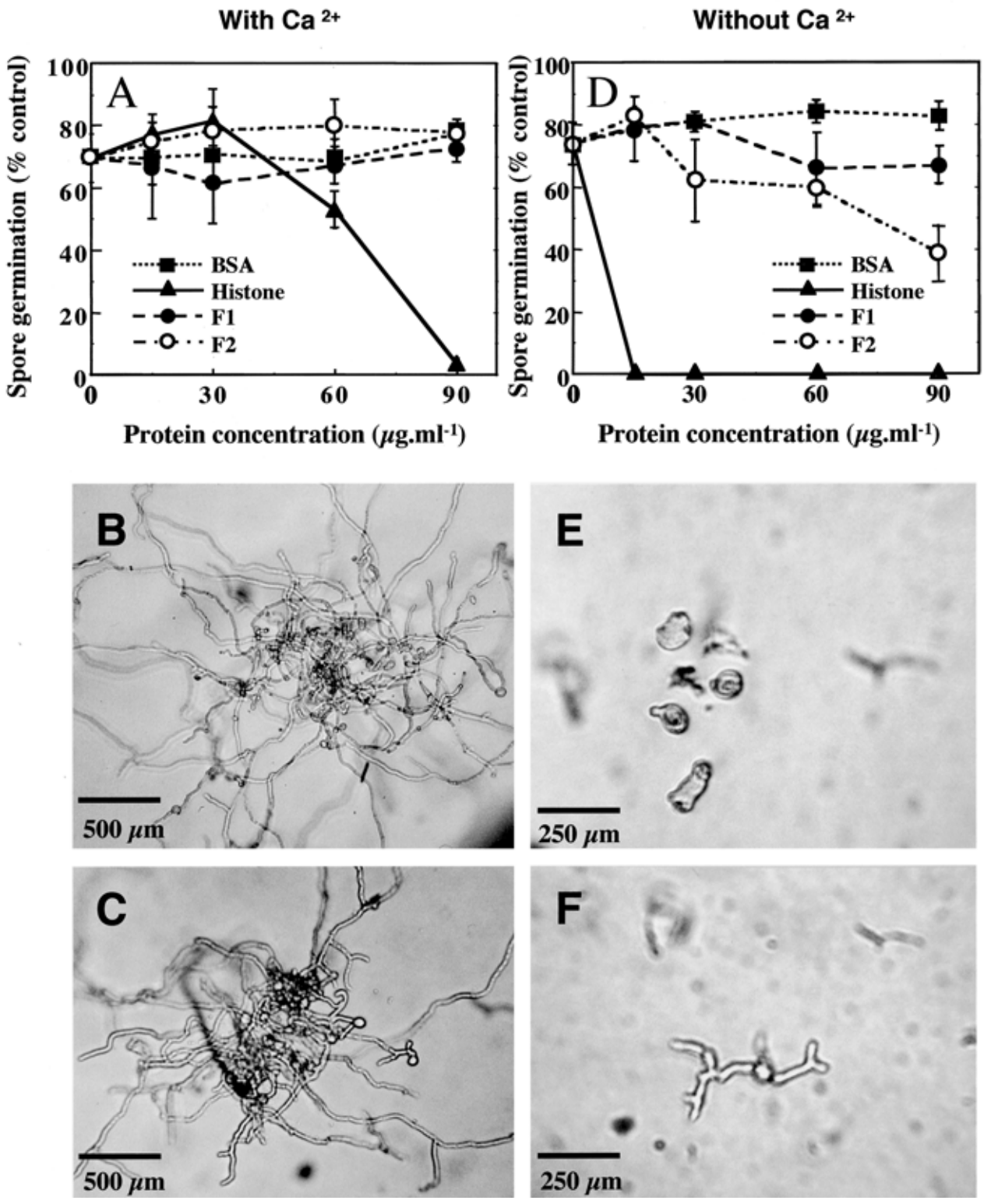

Fig. 5. Antifungal activity trials of grapevine nonspecific lipid transfer proteins (nsLTPs) compared with bovine serum albumin (BSA) and histone controls. Conidia germination and mycelium growth were monitored 96 h after inoculation for four concentrations of protein (15, 30, 60, and 60 μg ml^{-1}). The number of germinated conidia were counted in two independent experiments with three replicates. The percentage of germination was calculated as the number of germinated conidia compared with the total number of conidia in each well. The test was performed in 100 mM glucose, in the presence of 1 mM $CaCl_2$ (with Ca^{2+}) or in absence of calcium (without Ca^{2+}). Each well was pictured after 96 h to visualize mycelium growth. Conidia were considered germinated when a mycelium filament was emitted. **A,** Spore germination in the presence of Ca^{2+}; **B** and **C,** pictures of germinated conidia in the presence of Ca^{2+} and BSA or F1 LTP fraction (mix of P1, P2, and P3) at 30 μg ml^{-1}, respectively. Magnification ×100. **D,** Spore germination in the absence of Ca^{2+}. **E** and **F,** Pictures of conidia in the absence of Ca^{2+} and histones or F2 LTP fraction (pure P4) at 30 μg ml^{-1}, respectively. Magnification ×200.

BSA, 7% SDS, and 1 mM EDTA. Radiolabeled DNA probes were synthesized by random priming, using the ready-to-go DNA labeling beads kit (Amersham Pharmacia Biotech) and [^{32}P]-dCTP (Amersham Pharmacia Biotech). After hybridization, the membranes were washed at 65°C twice in 2× SSC/0.1% SDS, twice in 0.5× SSC/0.1% SDS, and once in 0.2× SSC/0.1% SDS. Membranes finally were exposed to hyperfilms MP (Amersham Pharmacia Biotech) at –80°C for autoradiography. Alternatively, membranes were exposed for 24 h to phosphoscreens for quantification with a Storm Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RT-PCR and RACE-PCR

The mRNA were purified from total RNA (prepared as described above) using oligo-dT cellulose type 7 spin columns (Amersham Pharmacia Biotech). First-strand and double-strand cDNA synthesis was performed using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, U.S.A.), according to the manufacturer's instructions.

PCR using the degenerated primers (sequences described above) were performed onto a PCR Express thermocycler (Hybaid Instruments, Holbrook, NY, U.S.A.) according to the following temperature program: 95°C, 5 min (initial denaturation step); then 95°C, 30 s; 50°C, 45 s (primer annealing), and 72°C, 2 min (primer extension) for a total of 30 cycles.

RACE-PCR was performed with the Marathon cDNA amplification kit (Clontech), using the following gene-specific non-degenerated primers: 5′RACE primer 5′-TTGCAAGGCCGAGATTGATG and 3′RACE primer 5′-GGATTAAGAATCTCAACAGCG). Thermal cycling was set as recommended by the manufacturer.

Lipid transfer protein purification.

Purification was performed using an HPLC system (Spectra Physics SP 8800 linked to a variable wavelength detector SP 700 and a Chromjet integrator) in a two-step method described previously (Coutos-Thévenot et al. 1993). Briefly, an 18-day-old culture medium was concentrated 200-fold using an Amicon ultrafiltration system (Amicon WR Grace and Co., Beverly, MA, U.S.A.) equipped with a YM3 membrane (cut off, 3 kDa). The concentrate was loaded onto a cation exchange column (Selfpack POROS 20CM, 8.5 by 0.8 cm; Applied Biosystems, Foster City, CA, U.S.A.) previously equilibrated with 25 mM sodium phosphate buffer (pH 6.5). The bound proteins were eluted by a linear NaCl gradient (0 to 275 mM in 15 min). The collected fractions were further purified on a hydrophobic interaction column (Applied Biosystem Selfpack POROS HP2, 13.5 by 0.8 cm) equilibrated with a buffer containing 50 mM sodium phosphate and 1.8 M ammonium sulfate (pH 7.0). Bound proteins were eluted by a linear ammonium sulfate gradient (1.8 M to 0 M in 20 min). Protein elution was monitored at 280 nm.

Preparation of the elicitor fraction from *B. cinerea.*

B. cinerea (*Botryotinia fucheliana* p.f.) strain T4 (kind gift of Y. Brygoo, INRA Versailles) was multiplied in a 9.5-cm petri dish with 15 ml of half-strength V8 medium supplemented with KH_2PO_4 at 5 g liter^{-1} and bacto agar (pH 6.0) at 30 g liter^{-1} and subcultured every month. For elicitor production, Roux flasks containing 250 ml of Nitsch-Nitsch medium, pH 6.0 (Nitsch and Nitsch 1969) were inoculated with 2,000 conidia of the T4 strain and cultured for 14 days at 25°C with a photoperiod of 16 h light, 8 h dark. The crude elicitor preparation obtained from 4 liters of culture filtrate was first concentrated by dialysis (10,000-molecular weight [MW] cutoff) against polyethylene glycol (15,000 to 20,000 MW). The concentrate then was sterilized in a 0.2-µm filter (Sterivex, Millipore, Bedford, MA, U.S.A.). The 15-ml concentrated fraction was diluted to 50 ml with anion equilibration buffer (20 mM ethanolamine, pH 10.8) and loaded onto the column (Hi-trap Mono Q, 5 ml, Amersham Pharmacia Biotech) through a superloop injector using an AKTA purifier system. The column was washed with 10 ml of the equilibration buffer and the adsorbed proteins were eluted with a linear NaCl gradient (0 to 700 mM in equilibration buffer). The flow rate was 5 ml min^{-1} with a total volume of 150 ml. The purification of the elicitor was monitored by measuring the ability of the fractions to induce H_2O_2 production in grape cell suspensions. In all, 20 µl of each fraction was applied on 1-ml aliquots of grape cell suspension and H_2O_2 production was determined 20 min after treatment. Active fractions were pooled and concentrated by ultrafiltration (Macrosep 10 K, Pall, Zaventem, Belgium). Part of the concentrated fraction (0.5 ml) was further purified by size exclusion chromatography on a Superdex 75 HR/6/30 column (Amersham Pharmacia Biotech). The elution buffer (0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, 0.15 M NaCl, pH 7.0) was eluted at 0.5 ml min^{-1}. Active fractions were dialyzed against water and pooled (500 µl) before use.

Antifungal assays.

The *Botrytis cinerea* strain was obtained from B. Dubos (INRA, Bordeaux France) and subcultured each month on malt-agar culture medium (malt extract at 15 g liter^{-1}, bacto agar at 20 g liter^{-1}) in the dark at 21°C. Conidia were obtained by fungus inoculation on V8 (commercial Campbell's vegetable juice) culture medium diluted to half and supplemented with bacto agar at 20 g liter^{-1} under light at 21°C. Antifungal activity of LTPs was tested both on germination and mycelium growth in 24-well culture boxes (Nunc, Roskilde, Denmark). Conidia (50 per well) were inoculated in 1 ml of liquid 100 mM glucose solution, supplemented or not with 1 mM $CaCl_2$, at four protein concentrations (15, 30, 60, and 90 µg ml^{-1}). Controls were made using water, BSA, or histones (Sigma, Saint Quentin, France), the latter having an isoelectric point close to that of basic nsLTPs. Conidia in germination were counted under an inverted microscope at different times, and mycelium growth was estimated by the length of the filaments.

Sequence analysis.

For each cDNA, products from two independent PCR reactions were sequenced (Eurogentec, Ivoz-Ramet, Belgium) in both directions. Sequence alignments were performed by the Clustal method, using the Megalign program of the DNA Star package.

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