Journal of Biochemistry and Molecular Biology, Vol. 36, No. 5, September 2003, pp. 433-441

JOURNAL OF Biochemistry and Molecular Biology

Characterization of a Salicylic Acid- and Pathogen-induced Lipase-like Gene in Chinese Cabbage

Kyung-Ah Lee and Tae-Ju Cho*

Division of Life Sciences, College of Natural Sciences, Chungbuk National University, Cheongju 360-763, Korea

Received 10 February 2003, Accepted 11 March 2003

A cDNA clone for a salicylic acid-induced gene in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) was isolated and characterized. The cabbage gene, designated *Br-sil*1 (for *Brassica rapa* salicylate-induced lipase-like 1 gene), encodes a putative lipase that has the family II lipase motif GDSxxDxG around the active site serine. A database search showed that plant genomes have a large number of genes that contain the family II lipase motif. The lipase-like proteins include a myrosinase-associated protein, an anther-specific proline-rich protein APG, a pollen coat protein EXL, and an early nodule-specific protein. The *Br-sil*1 gene is strongly induced by salicylic acid and a non-host pathogen, *Pseudomonas syringae* pv. *tomato*, that elicits a hypersensitive response in Chinese cabbage. Treatment of the cabbage leaves with BTH, methyl jasmonate, or ethephon showed that the *Br-sil*1 gene expression is induced by BTH, but not by methyl jasmonate or ethylene. This indicates that the cabbage gene is activated *via* a salicylic acid-dependent signaling pathway. An examination of the tissue-specific expression revealed that the induction of the *Br-sil*1 gene expression by BTH occurs in leaves and stems, but not in roots and flowers. Without the BTH treatment, however, the *Br-sil*1 gene is not expressed in any of the tissues that were examined.

Keywords: *Brassica rapa*, Chinese cabbage, Defense-related, Lipase II, GDSL motif

*To whom correspondence should be addressed.
Tel: 82-43-261-2309; Fax: 82-43-267-2306
E-mail: tjcho@cbucc.chungbuk.ac.kr

The nucleotide sequence data that is reported in this paper was submitted to the GenBank nucleotide sequence database and assigned the accession number AY101366.

Introduction

Like animals, plants also have defense mechanisms against various pathogens and pests, although the underlying mechanisms differ from vertebrate immune responses. Salicylic acid is a messenger molecule in the activation pathway of one of the disease-resistance responses that are known as systemic acquired resistance (SAR). SAR is induced locally by pathogen or pest attack and spreads systemically, resulting in protection of the whole plant. SAR is also broad-spectrum and long-lasting in effect (Ryals *et al.*, 1996). Defense responses are triggered by the perception of invading pathogens by specific receptors that are encoded by disease resistance genes (Bent *et al.*, 1996; Dangl and Jones, 2001). This recognition in turn activates a complex array of signaling pathways in plant cells. Many of the signaling components, such as EDS1, NDR1, and NPR1, have been studied by mutant analysis (Delaney, 1997; Feys and Parker, 2000). Biochemical studies have shown that active oxygen species and nitric oxide are important signaling molecules in the activation of disease resistance (McDowell and Dangl, 2000; Nuerenberger and Scheel, 2001). These studies have also shown that MAP kinase cascades play a critical role in plant defense responses, as in animals.

A picture of defense mechanisms in plants is emerging. However, there is still a lot to be elucidated in order to clarify the uncertainties and ambiguities. Moreover, studies on defense mechanisms have focused on a limited number of model plants, such as *Arabidopsis thaliana*, tomato, and tobacco. Although the information that was obtained from these model plants has advanced our understanding of defense responses in plants as a whole, certain plants may have unique resistance mechanisms. The genus *Brassica* includes many important vegetable crops, such as broccoli, cabbage, Chinese cabbage, cauliflower, mustard, rape, kale, and turnip. Although these *Brassica* species have served as good model plants to study self-incompatibility (Takasaki *et al.*, 2000), there have been few studies on the defense mechanisms in *Brassica*. So far, only a few defense-related genes in *Brassica*

have been reported. These include the pathogenesis-related protein 1a (Hanfrey *et al.*, 1996), β-1,3-glucanase (Newman *et al.*, 1994; Newman *et al.*, 1995), myrosinase (Thangstad *et al.*, 1993), and myrosinase-associated protein (Taipalensuu *et al.*, 1996; 1997). Progress on identifying the defense mechanisms in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), an important vegetable crop in Asia, has also been very slow, and only a few defense-related genes in Chinese cabbage have been reported.

Previously, we performed a subtractive hybridization experiment to isolate salicylic acid-induced genes in *Brassica napus* as the first step toward understanding the defense mechanisms in *Brassica* plants (Chu and Cho, 1996). Of the isolated genes, a gene encoding a putative lipase exhibited strong induction by salicylic acid. Here, we report that the salicylic acid-induced lipase-like gene is also present in Chinese cabbage, and that it is a member of lipase II superfamily. We also show that the gene expression is pathogen-inducible and tissue-specific.

Materials and Methods

Plant materials and chemical treatments *Brassica rapa* subsp. *pekinensis* (cultivar Norang) seedlings were grown on potting compost after germination. Unless stated otherwise, the experiments were performed with cabbage seedlings at the seven- or eight-leaf stages. For the salicylic acid treatment, fully developed and healthy leaves from the plants were cut into 1 × 1 cm pieces and floated onto a 20 mM MOPS buffer (pH 7.5) that contained either 5 mM or no salicylic acid (Sigma Chemical Co., St. Louis, USA) in a 10 cm or 15 cm petri dish. The leaf samples were incubated at 25°C under continuous fluorescent light. After one to three days of treatment, the leaf squares were harvested, weighed, and frozen immediately in liquid nitrogen. Treatment of cabbage leaves with a 0.3 mM BTH solution was performed using methods that are similar to those that are used for salicylic acid treatment. Methyl jasmonate (1 mM in 0.1% [v/v] ethanol), ethephon (1 mM), and 0.1% ethanol were sprayed on the leaves of intact plants. BTH (5% active ingredient in wettable powder) was a kind gift from Novartis, Korea. Methyl jasmonate (Me-JA) and ethephon were purchased from Aldrich Chemical Co. (Milwaukee, USA) or Sigma Chemical Co. (St. Louis, USA). For the analysis of the tissue-specific expression, the seeds were vernalized three weeks at 4°C after sowing. Each germinated seed was then transferred to a vinyl pot (9 cm in diameter) and grown until flowering. The aerial parts of the plants were treated with 0.3 mM BTH by spraying, and the roots were treated with BTH by pouring 200 ml of a 0.3 mM BTH solution into each pot. The control plants were similarly treated with sterile water.

Pathogen treatment *Pseudomonas syringae* pv. *tomato* (*Pst*) 259 was a kind gift from Dr. Cha at the Phytopathogenic Bacteriology Laboratory at Chungbuk National University, Korea. The *Pseudomonas* bacteria for inoculation were freshly grown on a NA agar medium (5 g of peptone, 3 g of beef extract, 2 g of yeast extract, and 15 g of Bacto-agar per liter) for one or two days at 30°C. The bacteria were collected by gently scraping the colonies from the agar medium with a sterile glass rod after adding 2 ml of sterile water per 85-mm plate. After centrifugation at 5,000 × *g*, the bacterial pellet was resuspended in sterile water and adjusted to OD_{600} = 0.1. The cabbage leaves were inoculated with the bacterial suspension by syringe infiltration. *Pst*-treated cabbage plants were then transferred to a growth chamber and incubated at 25°C under continuous light. Visible hypersensitive response (HR) usually developed 24 to 36 h after inoculation. The control plants were similarly treated with sterile water.

RNA extraction and Northern analysis Total RNA was prepared from frozen plant materials using the "hot phenol" method of De Vries *et al.* (1988). For the Northern analysis, 10 μg of total RNA was separated on a 1.0% formaldehyde-agarose gel and blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) using the standard capillary transfer method. After UV-crosslinking at 125 mJ, the blots were hybridized using the DNA probe that was labeled with digoxigenin (DIG). Chemiluminescent detection of the hybridized probe was carried out as described by Kim *et al.* (2002). The probe DNA was prepared by PCR amplification of the insert DNA of the cDNA clones. The DNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPD) was obtained using a Chinese cabbage GAPD cDNA clone (GenBank accession no. AF536826). The DNA probes for the cabbage CYP79 (GenBank accession no. AF528173) and chitinase genes (GenBank accession no. AF528178) were prepared using the cDNA clones (CPE32 and CPL24-1, respectively) that were isolated in our laboratory (Ryang *et al.*, 2002). The DIG-labeling, hybridization, and chemiluminescent immunodetection were performed using kits from Roche Molecular Biochemicals (Mannheim, Germany).

cDNA library construction and screening A Chinese cabbage cDNA library was constructed using mRNA from the cabbage leaves that were treated with salicylic acid. The total RNA, which was prepared from the leaf samples that were treated with salicylic acid for 24 h and the total RNA from the leaves that were treated for 72 h, were mixed at a 3 : 1 ratio, and mRNA was purified from the total RNA mixture using biotin-labeled oligo (dT) and streptavidin-conjugated paramagnetic particles (Promega, Madison, USA). The cDNA library was constructed using Lambda ZapII cDNA cloning and Gigapack *in vitro* packaging kits from Stratagene (La Jolla, USA).

Aliquots of the library were grown on ten LB agar plates at a density of 2.5×10^4 pfu per plate, and the phages were eluted with a SM buffer. The phage suspension from each plate was then examined by PCR for the presence of the lipase-like clone. Fight μl of the PCR mixture contained 5 μl of denatured phage suspension, 2.5 units of *Taq* DNA polymerase, 20 pmol of each primer in a PCR buffer (10 mM Tris (pH 8.9), 50 mM KCl, 1.5 mM $MgCl_2$). One of the PCR primers was a lipase-like gene-specific primer: 5' CGCTTGATTACCGATTTCATC 3'. The other primer was of the vector sequence near the cloning site: 5' CGCCAGGGT TTTCCCAGTCACGAC 3'. The conditions of thermal cycling for PCR were as follows: 94°C for 3 min, and 25-30 cycles of 94°C for 1 min, 55°C for 0.5 min, 72°C for 1.5 min with a final extension at 72°C for 10 min.

The phages in the sub-library fraction that yielded positive DNA bands were then diluted and grown on 24 plates at the density of 2,000 pfu per plate. Eluted phages from the plates were again examined by PCR for the presence of the lipase-like clone. Approximately 80% of the phage samples produced a PCR product of 1.1-1.2 kb, an expected size for the lipase-like cDNA insert. To confirm whether the DNA contained the lipase-like gene sequence, a DNA blot hybridization was performed using a partial rapeseed lipase-like gene as a probe. Preparation of the alkaline phosphatase-conjugated DNA probe and chemiluminescent detection were carried out as described by Min *et al*. (2001), using a AlkPhos Direct labeling and detection kit from Amersham Pharmacia Biotech (Buckinghamshire, UK). The DNA blot analysis showed that the 1.1-1.2 kb DNAs strongly hybridized to the probes. The phage sample that contained the largest insert was then screened by *in situ* plaque hybridization. Subcloning of the cDNA insert was performed by an *in vivo* excision of the pBluescript SK phagemid DNA with ExAssist helper phage, as described in Stratagene's protocol.

DNA sequence analysis and database search The nucleotide sequence of the cDNA insert was determined with an Applied Biosystems automatic sequencer at the Korea Basic Science Institute (Daejeon, Korea). Conventional M13-forward and M13-reverse primers were initially used to determine the sequence. Based on the sequence information, three oligonucleotide primers for each strand of the cDNA insert were synthesized and used for a further sequence analysis. The gene-specific sequencing primers were spaced at about 300-bp intervals. The sequence analysis showed the presence of an internal *Xho*I site. Since the cDNA insert has another *Xho*I site at the 3' end, *Xho*I digestion yielded a 0.7 kb DNA fragment. The *Xho*I fragment was subcloned into a M13 vector, and the deletion construct without the *Xho*I fragment was also cloned. These subclones were used to confirm the sequence around the internal *Xho*I site. The database search and computation were performed at the NCBI (National Center for Biotechnology Information) using the BLAST network service. Motif searches were made using the PROSITE database at http://expasy.hcuge.ch/sprot/prosite.html. A signal peptide prediction was performed using TargetP (Emanuelsson *et al.*, 2000) and http://www.cbs.dtu.dk/services/TargetP/. A multiple sequence alignment was done with the CLUSTAL W software (Thompson *et al.*, 1994) at http://www.ebi.ac.uk. The TreeView software (Page, 1996) was run to display a phylogenetic tree using the tree files that were generated by CLUSTAL W.

Results and Discussion

Isolation of a Chinese cabbage lipase-like protein gene To obtain a cDNA clone for the salicylate-induced lipase-like gene in Chinese cabbage, a cDNA library was constructed and screened by PCR and *in situ* plaque hybridization, as described in Materials and Methods. The cDNA clone contains a 1,113-bp ORF with 5' and 3' noncoding sequences of 10-bp and 138-bp, respectively (Fig. 1). The ORF encodes a 371 amino acid protein (GenBank accession no. AY101366) that contains the motif [FT]G[ND]SxxDxG[NG]x(10,20)PYG that is found in the family II lipases (Brick *et al.*, 1995). One of the characteristic features of the family II lipases is that they have a GxSxxxxG motif around the active site serine residue rather than the GxSxG that is found in classical lipases. The other feature is that the serine-containing motif is much closer to the amino terminus of most of these proteins (Upton and Buckley, 1995). These features, common to the family II lipases, are also found in the cabbage lipase-like protein. Active-site aspartate and histidine amino acids, two other residues of catalytic triads of lipases, can also be identified in the cabbage lipase-like protein (Fig. 1). The Chinese cabbage gene was designated *Br-sil*1 (for *Brassica rapa* salicylate-induced lipase-like 1 gene).

Scanning the PROSITE database revealed six possible protein kinase C phosphorylation sites (amino acids 3, 58, 75, 141, 148, and 340 in Fig. 1), three possible casein kinase II phosphorylation sites (amino acids 45, 205, and 282), and a leucine zipper region (amino acids 262-283). The significance of these motifs, however, is unclear. Running the TargetP program (Emanuelsson *et al.*, 2000), a prediction program for subcellular localization of proteins, identified a signal peptide. The difference between the highest and second-highest output score was 0.953. Thus, the prediction reliability class (RC) was 1. Considering that 99% of the sequences with RC = 1 were correctly predicted by the TargetP program, it seems highly probable that the cabbage lipase-like protein contains a signal peptide. As in typical signal peptides, the signal peptide in the cabbage protein has two N-terminal positively-charged residues, a stretch of hydrophobic residues in the central region and a following region of polar residues. The result of the prediction of cleavage site using TPlen (Nielsen *et al.*, 1997) indicated that the most likely cleavage site was between positions 22 and 23.

Database searches show the presence of a lipase II superfamily in plant genomes A BLAST analysis of the cabbage gene showed that the most similar gene was the *Arabidopsis* gene At1g53990 that encodes a lipase-like protein (GenBank accession no. NP_175801); the *Arabidopsis* protein exhibits 84% similarity and 75% identity with the Chinese cabbage lipase-like protein. In addition, three other *Arabidopsis* proteins (At1g53940, At3g14225, At5g40990) have scores that are better than BLASTP $E < 10^{-140}$. Like the cabbage lipase-like protein, all of these four *Arabidopsis* proteins have the FGDSxxDxGx(10,20)PYG motif and the putative signal peptide. The physiological functions of these proteins have not yet been reported. Besides these four *Arabidopsis* proteins, many other proteins were found to be similar to the cabbage protein. Most of these proteins were of unknown functions and have less than a 50% similarity (30% identity) with the cabbage protein. Among the proteins, functionally-characterized proteins include the *Arabidopsis* lipase Arab-1 (Brick *et al.*, 1995), *Brassica napus* myrosinase-associated proteins (Taipalensuu *et al.*, 1997), anther-specific

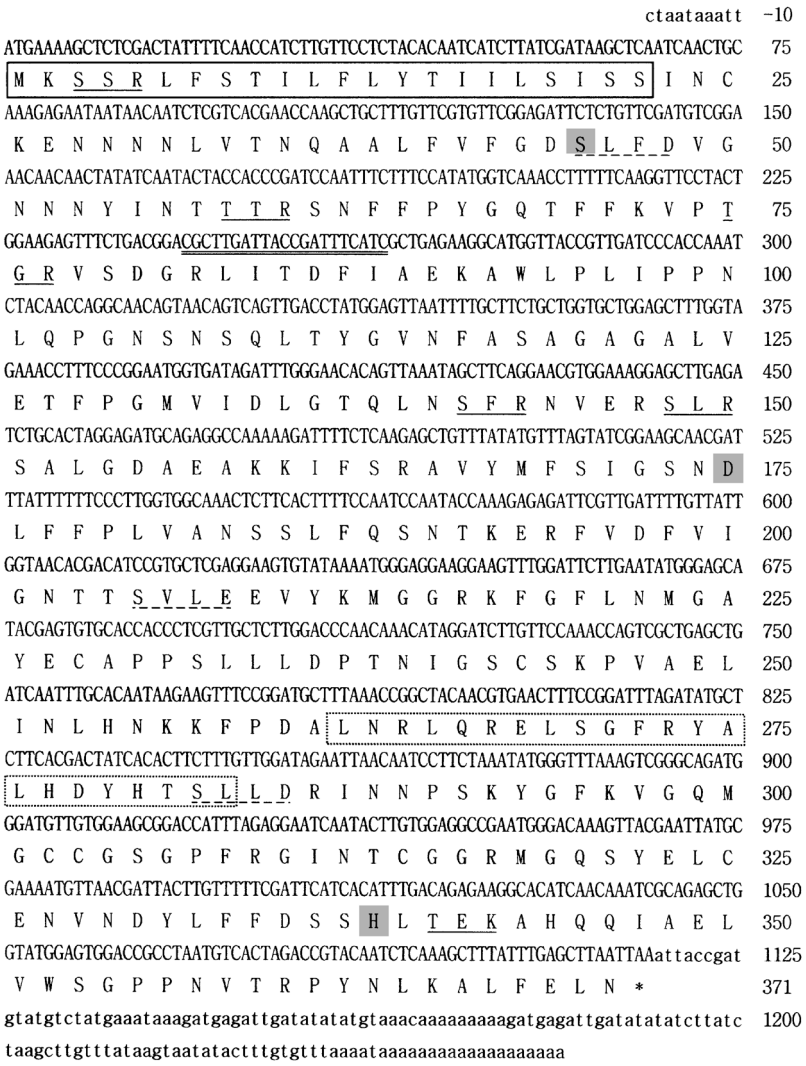

Fig. 1. Nucleotide and deduced amino acid sequences of a lipase-like gene in Chinese cabbage. The 5' and 3' noncoding cDNA sequences are represented by lowercase letters. The 21-bp oligonucleotide primer that was used for cDNA screening is shown by a double-solid underline. Three predicted lipase catalytic residues (S45, D175, and H338) are shaded, and the putative signal peptide is indicated by a solid-line box. Putative protein kinase C phosphorylation site (single solid underline), putative casein kinase II phosphorylation site (broken single underline), and putative leucine zipper (broken-line boxed region) are also shown.

proline-rich proteins APG in *Arabidopsis* and CEX in *B. napus* (Roberts *et al.*, 1993), extracellular lipase EXLs in *Arabidopsis* pollen coat (Mayfield *et al.*, 2001), *Medicago* nodule-specific protein (Dickstein *et al.*, 1993), *Medicago* pollen-specific coil protein PO22 (Wu *et al.*, 1996), and *Digitalis* lanatoside O-acetylesterase (Kandzia *et al.*, 1998). Among the bacterial enzymes that have been functionally characterized, lipases from *Aeromonas hydrophila* and *Vibrio parahaemolyticus* share homology with the cabbage protein, although the % similarity is much lower. The *Aeromonas* enzyme, a lipase/acyltransferase, is one of the best-characterized bacterial family II lipases; its active site was carefully studied by site-directed mutagenesis (Hilton and Buckley, 1991). The *Vibrio* enzyme is a phospholipase with hemolytic activity (Shinoda *et al.*, 1991).

All of these proteins have the family II lipase motif (Fig. 2A). The amino acid sequence around the active site serine is particularly well conserved, and all of the plant proteins conform to the consensus sequence motif [FT]G[ND] SxxDxG[NG]x(10,20)PYG that was described by Brick *et al.* (1995). The bacterial lipases lack the PYG motif that is conserved in plant proteins. The multiple alignments with CLUSTAL W revealed that all of these proteins contained two invariant glycine residues at the distance of 14 to 30-aa residues from the active site serine. As shown in Fig. 2A, these proteins also display consensus sequences at the active site aspartate and histidine residues. All of the proteins have glycine residues at the −3 position from the active site aspartate. This invariant glycine residue is also present in other family II bacterial lipases, described by Upton and

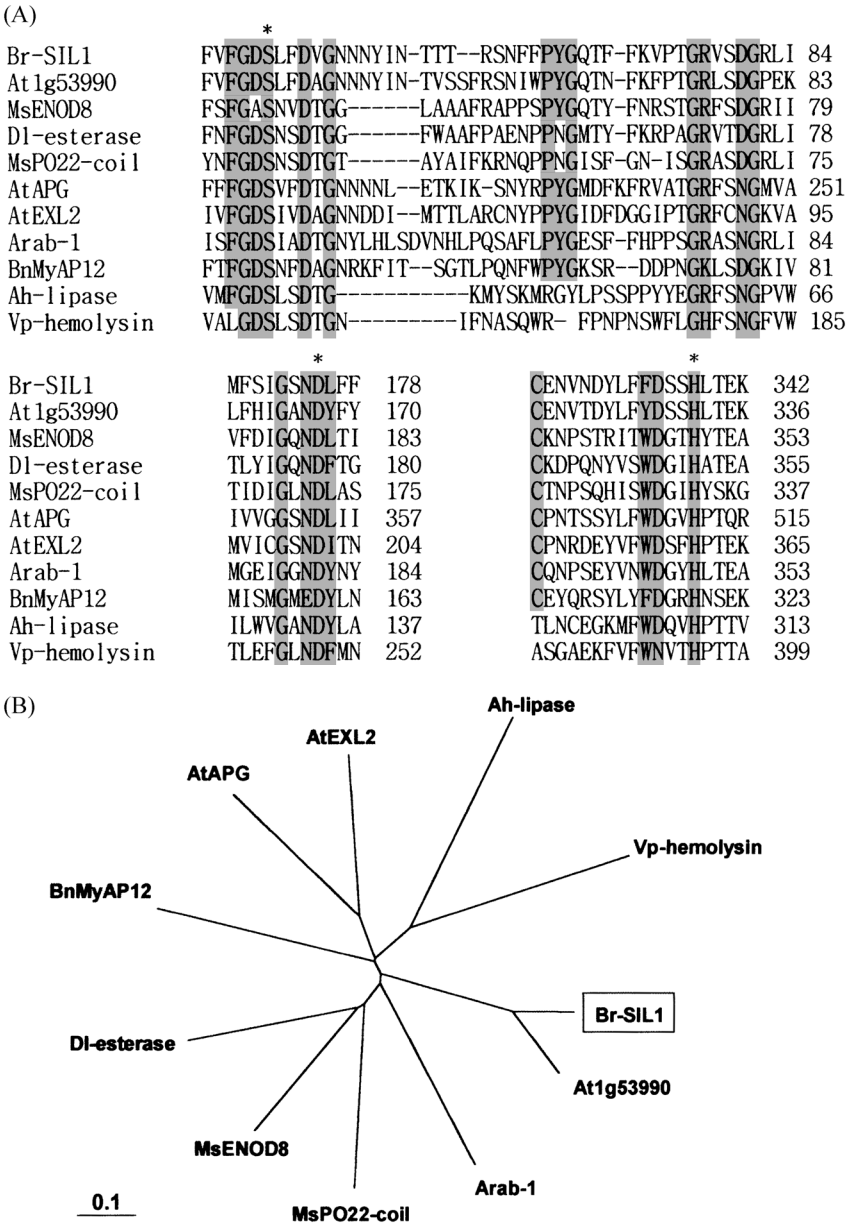

Fig. 2. Amino acid sequence comparison of the predicted cabbage lipase-like Br-SIL1 with other proteins with lipase motif. (A) Multiple sequence alignment by CLUSTAL W. The putative lipase catalytic triad consisting of a serine, histidine, and aspartate is indicated by asterisks. Conserved amino acids or amino acids with similar properties are shaded. (B) Cladogram showing the relationship between Br-SIL1 and other proteins with lipase motif. The phylogenetic tree was constructed by TreeView software using the tree file data that was obtained from a CLUSTAL W analysis. Arab-1, lipase from *Arabidopsis thaliana* (Accession no. U38916); At1g53990, putative lipase from *Arabidopsis* (Accession no. NP_175801); AtAPG, anther-specific proline-rich protein from *Arabidopsis* (Accession no. CAA42925); AtEXL2, extracellular lipase in *Arabidopsis* pollen coat (Accession no. AY028610); BnMYAP12, myrosinase-associated protein 12 from *Brassica napus* (Accession no. T08099); MsPO22-coil, pollen coil protein from *Medicago sativa* (Accession no. AAA83209); Dl-esterase, lanatoside 15-O-acetylesterase in *Digitalis lanata* (Accession no. CAA09694); MsENOD8, early nodule-specific protein in *Medicago sativa* (Accession no. AAB41547); Ah-lipase, lipase/acyltransferase in *Aeromonas hydrophila* (Accession no. P10480); Vp-hemolysin, phospholipase in *Vibrio parahaemolyticus* (Accession no. Q99289).

Buckley (1995). The sequence around the active site histidine is less well conserved, although there is a tendency for hydrophobic and bulky amino acid to be present at the –4 position, aspartate at the –3 position, and a small amino acid residue at the –2 position from the active site histidine. The cysteine residue around the active site histidine is also conserved among the proteins of plant origin. The proteins that were similar to Br-SIL1 were found to show no strong

homology to each other. As shown in Fig. 2B, a phylogenetic tree that was constructed from the CLUSTAL W data shows the distant relationships between these proteins. Overall, this implies the presence of diverse groups of lipase II motif proteins in plant genomes.

A BLAST search with the NCBI database or TAIR database (http//www.Arabidopsis.org) revealed that there are more than 90 *Arabidopsis* proteins that showed the BLASTP score $E < 10^{-20}$. Most of these proteins, between 350-aa and 400-aa in size, share at least a 25% identity (40% similarity) with the cabbage Br-SIL1 protein over 300-aa length. Thus, there is obviously a large lipase II superfamily in *Arabidopsis*. Most of these proteins conform to the lipase II amino acid signature. The consensus sequence around the active site serine in these proteins is FGDSxxDxGx(10,20)PYGx(7,10)G [RK]xx[DN]G[RK]. Amino acid sequences around the other two active site residues aspartate and histidine are also well conserved. The consensus sequences around these active site residues are GxND[FYLI] and [WFY]DxxH, respectively. A CLUSTAL W analysis of these proteins shows that these proteins are very diverse. This implies that these lipase-like proteins carry out various functions in plants. Available data at the TIGR (http://tigrblast.tigr.org) whole rice genome annotation database also revealed that there might be as many lipase II motif protein genes in rice genome as in *Arabidopsis*. Therefore, the presence of a vast number of the GDSxxDxG motif proteins might be a common feature of plant genomes.

As described earlier, the family II lipases that contain the GDSxxDxG motif are also found in bacteria. These bacterial lipases have a variety of different lipase activities, including arylesterase, acyltransferase, and thioesterase (Upton and Buckley, 1995). This also suggests that the lipase II motif proteins in *Arabidopsis* carry out many different lipase functions, although lipase activity has only been demonstrated with the Arab-1 among the *Arabidopsis* proteins (Brick *et al*., 1995). Not surprisingly, plants have far more different kinds of lipid-modifying enzymes than animals, considering that plants have a variety of lipid secondary products, such as terpenes. An example that demonstrates this activity would be the lanatoside O-acetylesterase that was previously mentioned. The enzyme catalyzes the deacetylation of the lanatoside, cardenolide steroids found in the genus *Digitalis* (Kandzia, *et al.*, 1998). However, it should be pointed out here that there are some proteins that display a similarity to the cabbage Br-SIL1, but lack the active site serine. An example is a carrot protein iEP4, which is induced by elicitors (Bertnetti and Ugalde, 1996). The iEP4 protein, which shows a >25% identity with the cabbage protein, has an alanine residue instead of a serine at the active site, although the sequence around the active site matches well with the consensus sequence. In some of the lipase II family proteins, there are also truncated versions of a lipase-like protein, where either the N-terminal part that contains the active site serine or the C-terminal part that contains the active site histidine is missing. Apparently, therefore, some members in this lipase II superfamily do not function as lipases.

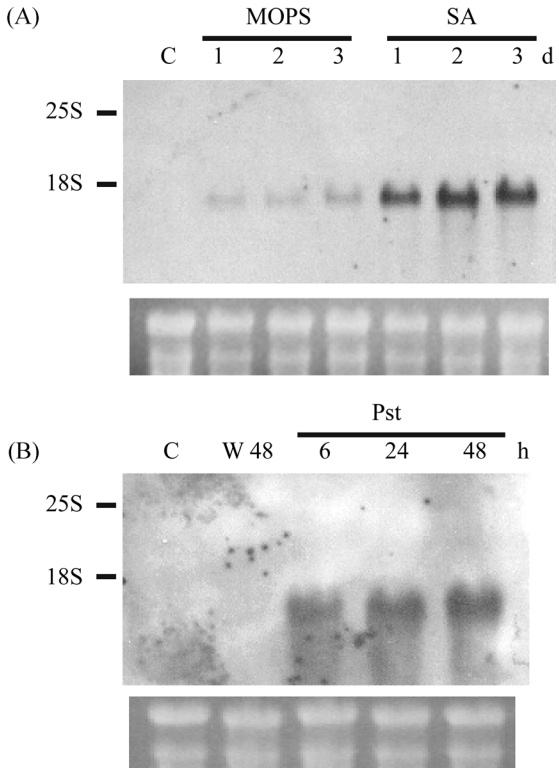

Fig. 3. Induction of *Br-sil*1 gene expression by salicylic acid and pathogen. (A) Chinese cabbage leaves were cut into 1 × 1 cm squares and floated onto a 20 mM MOPS buffer (pH 7.5) containing 5 mM salicylic acid. The control samples were treated with the MOPS buffer without salicylic acid. C, control leaf sample with no treatment; MOPS, 1 × 1 cm leaf squares treated with MOPS buffer; SA, leaf squares treated salicylic acid. 1, 2, and 3 represent the duration of the treatment in days. (B) Chinese cabbage leaves were infiltrated with either sterile water (W) or *Pseudomonas syringae* pv. *tomato* (Pst). C and W48 represent the control leaf sample with no treatment and the leaf sample that was harvested 48 h after infiltration with sterile water, respectively. After the indicated time, the leaf samples were collected and analyzed by Northern blot hybridization. Next, 10 μg total RNA from each sample was size-fractionated on a 1% formaldehyde agarose gel. The agarose gel that was stained with ethidium bromide is represented in the lower panel. The fractionated RNA was blotted onto a nylon membrane and hybridized with a DIG-labeled *Br-sil*1 DNA probe. Chemiluminescent detection was done using alkaline phosphatase-conjugated anti-DIG antibody and CSPD, according to the protocol provided by Roche Molecular Biochemicals.

Expression patterns of the cabbage lipase-like gene To examine whether the expression of the Chinese cabbage *Br-sil*1 gene is induced by salicylic acid, as in *Brassica napus*, the Chinese cabbage leaf was cut into 1 × 1 cm pieces and treated with a MOPS buffer that contained 5 mM salicylic acid. As shown in Fig. 3A, the salicylic acid treatment strongly

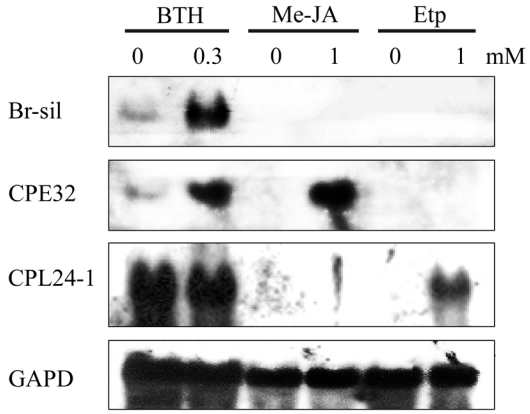

Fig. 4. Induction by BTH, methyl jasmonate, and ethylene. The leaves were treated with BTH by methods that are similar to those used for salicylic acid treatment. One mM methyl jasmonate (MeJA) in 0.1% ethanol, 1 mM ethephon solution, or 0.1% ethanol and sterile water (control) was sprayed onto cabbage leaves. After 24 h, the leaf samples were collected and analyzed by Northern blot hybridization using DIG-labeled probes (Fig. 3). The transcript levels of the cabbage CYP79B1 (CPE32) and chitinase (CPL24-1) genes were assayed as positive controls for induction by MeJA and ethylene, respectively. As a loading control, the samples were also hybridized with the cDNA specific for the cabbage glyceraldehyde-3-phosphate dehydrogenase (GAPD).

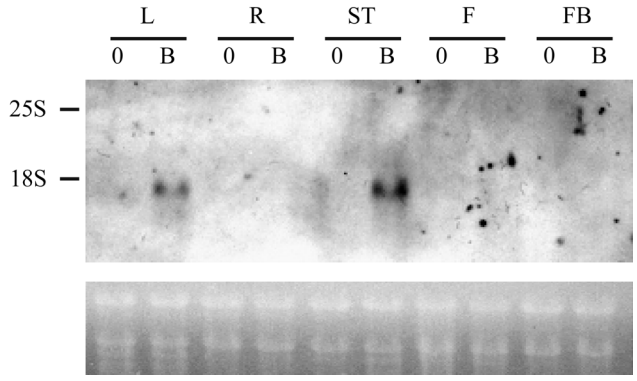

Fig. 5. Tissue-specific expression of the *Br-sil*1 gene. Chinese cabbage plants were induced to bolt by vernalizing the seeds. The cabbage plants bearing flowers were treated with BTH (B) by spraying (aerial parts) and soaking (roots) the plants with a 0.3 mM BTH solution. The control plants were similarly treated with sterile water. The leaf (L), root (R), stem (ST), flower (F), and flower bud (FB) tissues were collected from the plants and analyzed by Northern blot hybridization using a DIG-labeled *Br-sil*1 DNA probe (Fig. 3).

induced the expression of the cabbage lipase-like gene. The mock treatment without salicylic acid weakly induced the gene. The *Br-sil*1 mRNA was not detected in the untreated leaf sample.

We were then interested in whether the gene could be induced by the stimuli that accompany the hypersensitive response (HR), since HR is often a prerequisite for induction of disease resistance in plants. We used *Pseudomonas syringae* pv. *tomato* (*Pst*) to induce HR in Chinese cabbage. *Pst* causes bacterial speck disease in tomatoes and *Arabidopsis* (Bashan *et al.*, 1981; Whalen *et al.*, 1991). However, Chinese cabbage is not a host to this pathogen. In the *Pst*-infiltrated cabbage leaves, visible necrosis was evident 24-36 h after inoculation. As shown in Figure 3B, a Northern analysis showed that the gene was strongly induced in cabbage leaves that were infiltrated with *Pst*. The gene was not induced in the control leaves that were infiltrated with water.

SAR can also be induced by chemicals other than salicylic acid. One of the most potent SAR-inducing chemicals is benzothiadiazole (Goerlach *et al.*, 1996). To see if the *Br-sil*1 gene is also induced by benzothiadiazole (BTH), 1×1 cm squares of cabbage leaves were treated with a MOPS buffer that contained 0.3 mM BTH, and analyzed by Northern blot hybridization. The result showed that the cabbage *Br-sil*1 gene was also strongly induced by BTH (Fig. 4). This confirms the result that was obtained with salicylic acid, since BTH is known to be a salicylic acid-mimicking chemical. We then examined the effect of other activators of plant defense responses, since it was reported that systemic resistance is also mediated by jasmonate or ethylene (Piterse and van Loon, 1999). To examine how the cabbage gene responds to ethylene or jasmonates, the cabbage leaves were treated with methyl jasmononate (Me-JA) or with ethephon, an ethylene-releasing compound. Since ethylene and jasmonate act as wound signals in defensive responses to insect attacks (O'Donnell *et al.*, 1996; McConn *et al.*, 1997), these chemicals were sprayed onto intact cabbage leaves in order to protect them from physical injury. As shown in Fig. 4, the *Br-sil*1 gene was not induced, either by ethylene or by Me-JA.

To verify that appropriate induction conditions were used, we included as positive controls the expression of the Me-JA-inducible Chinese cabbage gene CPE32 and the ethylene-inducible gene CPL24-1, which were isolated in our laboratory as *Pst*-induced genes (Ryang *et al*., 2002). The CPE32 gene encodes a putative CYP79B1 that catalyzes the conversion of amino acids to aldoxime precursors for glucosinolate biosynthesis (Bak *et al.*, 1998). The cabbage cytochrome P450 gene was induced by both BTH and Me-JA (Fig. 4). The CPL24-1 that was used as a positive control for the induction by ethylene encodes a chitinase. Figure 4 clearly shows that the expression of the Chinese chitinase gene is induced by ethylene. The chitinase gene is also strongly expressed in MOPS-treated leaf samples, as well as in BTH-treated samples. This indicates that wounding also induces the expression of the chitinase gene, since the BTH treatment and the mock control involved physical injury. These results clearly show that the Br-*sil*1 gene is induced by salicylic acid or BTH, but not by Me-JA or ethylene.

We then the examined the tissue specificity of the gene expression. To this end, cabbage plants were induced to bolt

by vernalizing the seeds. After flowering, the whole plants were treated with BTH by spraying (aerial parts) and soaking (roots) with a 0.3 mM BTH solution. A Northern analysis of the total RNAs from various tissues showed that the gene was barely expressed in the tissues that were examined, unless treated with BTH (Fig. 5). The result also showed that the induction by BTH occurred only in the leaf and stem tissues, but not in the root and floral tissues.

EDS1 and PAD4 represent examples of the lipases that play important roles in the plant defense system. Both EDS1 and PAD4 play pivotal roles in the salicylic acid-dependent defense signaling pathways, operating upstream of pathogen-induced salicylic acid accumulation (Falk *et al.*, 1999; Jirage *et al.*, 1999). These two proteins displayed a similarity to eukaryotic lipases, although hydrolytic activities have not been clearly demonstrated. Like the cabbage *Br-sil*1 gene, the *eds*1 and *pad*4 genes are induced by salicylic acid and pathogens. Unlike the cabbage lipase-like protein, however, both the EDS1 and PAD4 proteins display a classical lipase motif GxSxG around the active site serine. Another difference could be the subcellular location. The EDS1 and PAD4 proteins are predicted to be cytosolic, as expected from their intracellular signaling functions. In contrast, it is predicted that the cabbage Br-SIL1 protein is targeted to the secretory pathway. This implies that the cabbage lipase-like protein could perform a function that is different from the intracellular signaling functions.

In conclusion, we isolated and characterized a Chinese cabbage lipase-like *Br-sil*1 gene whose expression is strongly induced by salicylic acid and non-host pathogen *Pseudomonas syringae* pv. *tomato*. These findings suggest a defensive role of the *Br-sil*1 gene. A BLAST analysis showed the presence of a superfamily of lipase II motif proteins in plant genomes. The functions of these proteins are mostly unknown. Therefore, what the lipase II superfamily members do in plants must still be elucidated.

Acknowledgments This work was supported by a grant from the Korea Research Foundation (KRF-99-041-D00356).

References

Bak, S., Nielsen, H. L. and Halkier, B. A. (1998) The presence of CYP79 homologues in glucosinolate-producing plants shows evolutionary conservation of the enzymes in the conversion of amino acid to aldoxime in the biosynthesis of cyanogenic glucosides and glucosinolates. *Plant Mol. Biol.* **38**, 725-734.

Bashan, Y., Sharon, E., Okon, Y. and Henis, Y. (1981) Scanning electron and light microscopy of infection and symptom development in tomato leaves infected with *Pseudomonas tomato*. *Physiol. Plant Pathol.* **19**, 139-144.

Bent, A. F. (1996) Plant disease resistance genes: Function meets structure. *Plant Cell* **8**, 1757-1771.

Bertinetti, C. and Ugalde, R. A. (1996) Studies on the response of carrot cells to a *Sclerotinia sclerotiorum* elicitor: induction of the expression of an extracellular glycoprotein mRNA. *Mol. Plant Microbe Interact.* **9**, 658-663.

Brick, D. J., Brumlik, M. J., Buckley, J. T., Cao, J. -X., Davies, P. C., Misra, S., Tranbarger, T. J. and Upton, C. (1995) A new family of lipolytic plant enzymes with members in rice, arabidopsis and maize. *FEBS Lett.* **377**, 475-480.

Chu, H. -S. and Cho, T. -J. (1996) Isolation of salicylic acid-induced genes in *Brassica napus* by subtractive hybridization. *Mol. Cells* **6**, 766-772.

Dangl, J. L. and Jones, J. D. (2001) Plant pathogens and integrated defense responses to infection. *Nature* **411**, 826-833.

De Vries, S., Hoge, H. and Bisseling, T. (1988) Isolation of total and polysomal RNA from plant tissues; in *Plant Molecular Biology*, Gelvin, S. B. and Schilperoot, R. A. (eds), pp. B6/1-5, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Delaney, T. P. (1997) Genetic dissection of acquired resistance to disease. *Plant Physiol.* **113**, 5-12.

Dickstein, R., Prusty, R., Peng, T., Ngo, W. and Smith, M. E. (1993) ENOD8, a novel early nodule-specific gene, is expressed in empty alfalfa nodules. *Mol. Plant Microbe Interact.* **6**, 715-721.

Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **300**, 1005-1016.

Falk, A., Feys, B. J., Frost, L. N., Jones, J. D. G. and Daniels, M. J. (1999) EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. USA* **96**, 3292-3297.

Feys, B. and Parker, J. E. (2000) Interplay of signaling pathways in plant disease resistance. *Trends Genet.* **16**, 449-455.

Goerlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.-H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. and Ryals, J. (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* **8**, 629-643.

Hanfrey, C., Fife, M. and Buchanan-Wollaston, V. (1996) Leaf senescence in *Brassica napus*: Expression of genes encoding pathogenesis-related proteins. *Plant Mol. Biol.* **30**, 597-609.

Hilton, S. and Buckley, J. T. (1991) Studies on the reaction mechanism of a microbial lipase/acyltransferase using chemical modification and site-directed mutagenesis. *J. Biol. Chem.* **266**, 997-1000.

Jirage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E. and Ausubel, F. M. (1999) *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. USA* **96**, 13583-13588.

Kandzia, R., Grimm, R., Eckerskorn, C., Lindemann, P. and Luckner, M. (1998) Purification and characterization of lanatoside 15-O-acetylesterase from *Digitalis lanata* Ehrh. *Planta* **204**, 383-389.

Kim, M., Lim, C.-J. and Kim, D. (2002) Transcription of *Schizosaccharomyces pombe* thioltransferase-1 in response to stress conditions. *J. Biochem. Mol. Biol.* **35**, 409-413.

Mayfield, J. A., Fiebig, A., Jhonstone, S. E. and Preuss, D. (2001) Gene families from the *Arabidopsis thaliana* pollen coat proteome. *Science* **292**, 2482-2485.

McConn, M., Creelman, R. A., Bell, E., Mullet, J. E. and Browse, J. (1997) Jasmonate is essential for insect defense in

Arabidopsis. Proc. Natl Acad. Sci. USA **94**, 5473-5477.

McDowell, J. M. and Dangl, J. L. (2000) Signal transduction in the plant immune response. *Trends Biochem. Sci.* **25**, 79-82.

Min, H. -J., Park, S. -S. and Cho, T. -J. (2001) A simple and efficient subtractive cloning method. *J. Biochem. Mol. Biol*. **34**, 59-65.

Newman, M. A., Conrads-Strauch, J., Scofield, G., Daniels, M. J. and Dow, J. M. (1994) Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. *Mol. Plant Microbe Interact.* **7**, 553-563.

Newman, M. A., Daniels, M. J. and Dow, J. M. (1995) Lipopolysaccharide from *Xanthomonas campestris* induces defense-related gene expression in *Brassica campestris*. *Mol. Plant Microbe Interact*. **8**, 778-780.

Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**, 1-6.

Nuernberger, T. and Scheel, D. (2001) Signal transmission in the plant immune response. *Trends Plant Sci.* **6**, 372-379.

O'Donnell, P. J., Calvert, C., Atzorn, R., Wastemack, C., Leyser, H. M. O. and Bowles, D. J. (1996) Ethylene as a signal mediating the wound response of tomato plants. *Science* **274**, 1914-1917.

Page, R. D. (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**, 357-358.

Pieterse, C. M. J. and van Loon, L. C. (1999) Salicylic acid-independent plant defense pathways. *Trends Plant Sci.* **4**, 52-58.

Roberts, M. R., Foster, G. D., Blundell, R. P., Robinson, S. W., Kumar, A., Draper, J. and Scott, R. (1993) Gametophytic and sporophytic expression of an anther-specific *Arabidopsis thaliana* gene. *Plant J.* **3**, 111-120.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y. and Hunt, M. D. (1996) Systemic acquired resistance. *Plant Cell* **8**, 1809-1819.

Ryang, S. -H., Chung, S. -Y., Lee, S. -H., Cha, J. -S., Kim, H. Y. and Cho, T. -J. (2002) Isolation of pathogen-induced Chinese cabbage genes by subtractive hybridization employing selective adaptor ligation. *Biochem. Biophys. Res. Commun.* **299**, 352-359.

Shinoda, S., Matsuoka, H., Tsuchie, T., Miyoshi, S., Yamamoto, S., Taniguchi, H. and Mizuguchi, Y. (1991) Purification and characterization of a lecithin-dependent haemolysin from *Escherichia coli* transformed by a *Vibrio parahaemolyticus* gene. *J. Gen. Microbiol*. **137**, 2705-2711.

Taipalenssu, J., Falk, A. and Rask, L. (1996) A wound- and methyl jasmonate-inducible transcript coding for a myrosinase-associated protein with similarities to an early nodulin. *Plant Physiol.* **110**, 483-491.

Taipalensuu, J., Andersson, E., Eriksson, S. and Rask, L. (1997) Regulation of the wound-induced myrosinase-associated protein transcript in *Brassica napus* plants. *Eur. J. Biochem.* **247**, 963-971.

Takasaki, T., Hatakeyama, K., Suzuki, G., Watanabe, M., Isogai, A. and Hinata, K. (2000) The S receptor kinase determines self-incompatibility in *Brassica* stigma. *Nature* **403**, 913-916.

Thangstad, O. P., Winge, P., Husebye, H. and Bones, A. (1993) The myrosinase (thioglucoside glucohydrolase) gene family in *Brassicaceae*. *Plant Mol. Biol.* **23**, 511-524.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Upton, C. and Buckley, J. T. (1995) A new family of lipolytic enzymes? *Trends Biochem. Sci.* **20**, 178-179.

Whalen, M. C., Innes, R. W., Bent, A. F. and Staskawicz, B. J. (1991) Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* **3**, 49-59.

Wu, Y., Qui, X., Du, S. and Erickson, L. (1996) Cloning and characterization of PO22, a pollen-expressed gene in alfalfa. *Plant Mol. Biol.* **32**, 1205-1207.