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ESTs reveal a multigene family for plant defensins in *Arabidopsis thaliana*

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Abstract Plant defensins, formerly named γ-thionins, are a group of small, cysteine-rich, basic, and antimicrobial plant proteins. Random sequencing of expressed sequence tags (ESTs) in *Arabidopsis thaliana* has revealed several different plant defensin genes in this plant species which can be grouped into two subfamilies. We have used one EST of each subfamily to study the expression of the corresponding genes in *A. thaliana*. *Pdf2.3* is constitutively expressed in seedlings, rosettes, flowers, and siliques and is not inducible in seedlings either by methyl jasmonate, salicylate, ethephon, and silver nitrate or by several different phytopathogenic fungi. The expression of a second gene, *Pdf1.2*, is in untreated plants only detectable in rosettes. In seedlings, it is inducible by methyl jasmonate, silver nitrate, and different phytopathogenic fungi, notably *Fusarium oxysporum f.* sp. *matthiolae*. The regulation of *Pdf1.2* resembles that of the pathogen-inducible thionin gene *Thi2.1*.

Key words: Plant defence; Cysteine-rich protein; Antimicrobial

1. Introduction

Plants have evolved different mechanisms to cope with the constant threat by phytopathogenic microorganisms. These include preformed physical barriers and antimicrobial compounds [1] as well as the induction of defence compounds after infection by pathogens. Well known examples of the latter group comprise phytoalexins [2] and PR proteins [3]. During recent years, it has become evident that, in addition to the above-mentioned defence mechanisms, several families of small, cysteine-rich, basic polypeptides play a role in the defence of plants against microorganisms. Vertebrates also contain a variety of antimicrobial polypeptides in epithelial tissues and macrophages [4,5] in addition to their immunoglobulin system. The synthesis of antimicrobial polypeptides in the fat body is also part of the immune response of insects [4].

In plants, the antimicrobial and toxic activities of thionins, basic, cysteine-rich polypeptides of 5 kDa molecular mass, have been known for some time (for a review, see [6,7]). In several plant species the induction of thionin genes following pathogen attack has been documented [8–11] and it has been shown that the expression of a hordothionin in transgenic tobacco leads to an enhanced resistance against a phytopathogenic bacterium [12]. In 1990, Colilla et al. [13] and Mendez et al. [14] reported the isolation of new, so-called γ-purothionins and γ-hordothionins from the endosperm of wheat and barley, respectively. As it turned out, these proteins are not homologous to the classical thionins but are members of another group of small, basic, and cysteine-rich plant proteins [7], homologs of which have since then been found in a large number of other plant species. These include sorghum [15], pea [16], tobacco [17], potato [18,19], petunia [20], sugar beet [21], and several members of the family Brassicaceae [22–24].

Since their three-dimensional structure is similar to that of pathogen-inducible insect defensins, the name plant defensins has been proposed [24] and will be used here. Plant defensins have in vitro antimicrobial activities against different fungi [19,21,22]. Expression of the radish plant defensin AFP2 in tobacco resulted in a reduction of the average lesion area after inoculation with *Alternaria longipes* [24]. In some cases it has been shown that plant defensins are induced after pathogen attack [16,24]. Taken together, these results strongly suggest a role for plant defensins in the resistance of plants against pathogens, especially fungi.

In the work reported here, we made use of ESTs generated for *Arabidopsis thaliana* [25,26] to study the plant defensin gene family of *A. thaliana*. Searching the *A. thaliana* databases revealed several ESTs for plant defensins. Clones supplied by the Arabidopsis Stockcenter (ABRC, OH, USA) were taken as hybridisation probes to study the expression of plant defensin genes in *A. thaliana*.

2. Materials and methods

2.1. Sequencing

The EST clone 80D5T7 (*Pdf2.3*) was sequenced using the dideoxynucleotide chain termination method [27]. Sequences were analysed with the GCG programs [28].

2.2. Growth and treatment of plants

The *A. thaliana* ecotype Col-2 was used. For seed production, isolation of genomic DNA, or for the isolation of RNA from different organs plants were grown in soil in a greenhouse. For treatment with chemicals or pathogens seeds were sterilised and sown on MS plates [29] with vitamins (glycine 2 mg/l, nicotinic acid 0.5 mg/l, pyridoxine/HCl 0.5 mg/l, thiamine/HCl 0.1 mg/l), 2% sucrose, and 0.8% agar. To achieve uniform germination plates were stored at 4°C for 2 days and then transferred to a growth chamber (16 h light, 20°C, 8 h dark, 18°C) for 12 days. Plants were vacuum infiltrated [30] with the test solutions in an exsiccator 3 h after start of the light-period. They were then dried for 3 h in a laminar flow clean bench and put back into the growth chamber until harvest of the seedlings.

Methyl jasmonate (Serva) was used as a 100 μM solution in 0.02% acetone (v/v). Sodium salicylate (Sigma) and silver nitrate (Sigma) were used as a 1 mM solution, and ethephon (Sigma) was used at a concentration of 0.01% (w/v).

The following fungal strains were used (obtained from the Centraalbureau voor Schimmelcultures, Baarn – Delft, The Netherlands):

Fusarium oxysporum f. sp. *matthiolae* (strain 247.61)
Fusarium oxysporum f. sp. *raphani* (strain 488.76)
Alternaria brassicicola (strain 103.24
Alternaria brassicicola (strain 238.73)
Alternaria raphani (strain 114.44).

All fungi were grown on potato dextrose agar at room temperature

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for 2–3 weeks. Spores were taken up in sterile water, filtered through miracloth, and counted with a Fuchs/Rosenthal chamber. Spore suspensions were diluted to 5×10^5 spores ml^{-1}. Seedlings were grown as before and sprayed with a spore suspension (1 ml per 9 cm petri dish). Petri dishes were closed and incubated again in the growth chamber (the first 24 h in the dark) until harvest of the infected plants.

2.3. Northern blots

Plants were treated as described above. Plants grown on MS medium in Petri dishes were harvested by pouring liquid nitrogen onto the plates. Plant material was ground in liquid nitrogen and RNA was prepared as described by Melzer et al. [31].

20 μg total RNA were separated on denaturing 1.0% agarose gels as described by Ausubel et al. [32]. Ethidium bromide was included to verify equal loading of RNA. After transfer to Gene Screen membranes (NEN, Switzerland) filters were hybridised with 10^6 cpm ml^{-1} oligolabelled ^{32}P probes [33] in HYBSOL [34]).

The following probes were used:

Est 37F10T7 (*Pdf1.2*)
Est 80D5T7 (*Pdf2.3*).
PR1-, PR5-, and *Thi2.1*-specific probes were used as controls [10].

Filters were washed for 15 min at 60°C with 2×SSC, 0.1% SDS; 0.5×SSC, 0.1% SDS; and 0.1×SSC, 0.1% SDS. Filters were exposed to X-Omat-AR (Kodak) films at −80°C for 1–5 days. Probes were stripped off the membrane with a boiling 0.2% SDS solution according to the manufacturer's instructions.

2.4. Southern blots

Genomic DNA from greenhouse grown rosettes was isolated as described by Murray and Thompson [35] and purified on a cesium chloride gradient. 3 μg DNA were digested with restriction enzymes (Boehringer, Mannheim, Germany) according to the manufacturer's instructions and separated on 0.8% agarose gels. Insert DNA for cross-hybridisation experiments was prepared from the following EST clones: ATTS0830 (*Pdf2.1*), ATTS0239 (*Pdf2.2*), 80D5T7 (*Pdf2.3*), ATTS1949 (*Pdf1.1*), 37F10T7 (*Pdf1.2*). 20 ng insert DNA were loaded on a 1.5% agarose gel. The DNA was transferred to Pall Biodyne A membranes (PALL, Muttenz, Switzerland) and blots were hybridised [36] with ^{32}P-labelled cDNA probes. Filters were washed twice with 2×SSC, 0.1% SDS at 45°C, once with 0.5×SSC, 0.1% SDS at 65°C, and once with 0.1×SSC, 0.1% SDS at 65°C, and exposed for 1 h (cross-hybridisation) or 4 days (genomic blots).

3. Results

3.1. ESTs for Arabidopsis thaliana plant defensins

As of May 8, 1996, the TIGR *A. thaliana* database [37] contains sequences for five different plant defensin genes (Table 1). While there is only one EST (T04323) for one of the genes, all other genes are represented by several ESTs which have been grouped into tentative consensus sequences. The deduced protein sequences are shown in Fig. 1. An inspection of the sequences shows that they belong to two groups as is also revealed by evolutionary trees for both the nucleotide sequences (Fig. 2) and the deduced amino acid sequences (data not shown). The genes of group 1 are named *Pdf1.1* and *Pdf1.2*, while those of the second group are named *Pdf2.1*, *Pdf2.2*, and *Pdf2.3*, respectively. Inserts from two of the available ESTs, one for each subgroup, were isolated and used as hybridisation probes. Fig. 3 shows Southern blots hybridised with these two different ESTs. The cross-hybridization experiment in (A) indicates that the probes we have chosen are specific not only for one subgroup but also for the specific genes. Therefore, it is possible to differentiate the corresponding transcripts by Northern blots. The genomic blot in (B) shows that with both probes a different set of bands is recognised. Several faint bands indicate that both probes also detect other homologous genes.

Table 1
Plant defensin sequences in the TIGR *Arabidopsis thaliana* database (version 1.1, May 8, 1996)

Pdf1.1:	TC9184 (T88174, Z27258, Z29957)
Pdf1.2:	T04323
Pdf2.1:	TC9485 (R29892, Z17665)
Pdf2.2:	TC8876 (ET26747=X69139, H37692, N37912, T04082, T42542, T43215, Z18455)
Pdf2.3:	TC9117 (R84186, T20428, T21192, T43613)

3.2. Developmental expression

Northern blots were used to study the expression of the *Arabidopsis* plant defensin genes. As a first step, RNA was isolated from different organs and developmental stages. While seedlings and roots were grown in sterile culture, all other RNA probes were isolated from material which was derived from plants grown in soil in a greenhouse. Fig. 4 shows a Northern blot probed with *Pdf1.2* and *Pdf2.3*, respectively. Transcripts for the *Pdf1.2* gene were detected only in rosettes, *Pdf2.3*, on the other hand, is expressed at high levels in seedlings, rosettes, flowers, and siliques, and at slightly reduced levels in cauline leaves and stems. It was not detectable in roots.

3.3. Induction by chemicals

Several reports on related plant defensins strongly suggested a role in plant defence [16,19,21,22,24]. We have therefore tested several chemicals which are known to induce other defence related genes. Salicylate is involved in mediating the induction of SAR genes in many plant species, including *A. thaliana* [38]. Ethylene, which is set free by hydrolysis of ethephon, has been shown to induce the expression of PR1 genes in tobacco [39] and of a hevein-like gene in *A. thaliana* [40].

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         1                                      ▼                                                                          81
Pdf1.1   MAKSATIVTL IFAALVFFAA LEAPMV.VEA QKLCERPSGT WSGVCGNSNA CKNQCINLEK ARHGSCNYVF PAHKCICYFP C*
Pdf1.2   MAKFASIITL IFAALVLFAA FDAPAM.VEA QKLCEKPSGT WSGVCGNSNA CKNQCINLEG AKHGSCNYVF PAHKCICYVP C*
Pdf2.2   MKLSMRLISA VLIMFMIFVA TGMGPVTVEA .RTCESQSHR FKGTCVSASN CANVCHNEGF VGGN.CR.GF .RRRCFCTRH C*
Pdf2.3   MKLSVRFISA ALLLFMVFIA TGMGPVTVEA .RTCESKSHR FKGPCVSTHN CANVCHNEGF GGGK.CR.GF .RRRCYCTRH C*
Pdf2.1   MKFSMRLISA VLFLVMIFDA TGMGPVTVEA .RTCASQSQR FKGKCVSDTN CENVCHNEGF PGGD.CR.GF .RGRCFCTRN C*
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Fig. 1. Alignment of plant defensin precursors as deduced from the cDNA sequences. An arrowhead shows the processing site between the signal peptide and the mature plant defensin. Cysteine residues are shaded.

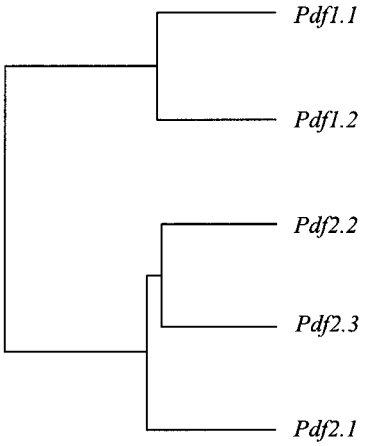

Fig. 2. Evolutionary tree showing the relationships of 5 different *Arabidopsis thaliana* plant defensin precursors.

Methyl jasmonate induces proteinase inhibitors [41] and thionins [10,42]. Silver nitrate is also an inducer of the *A. thaliana*

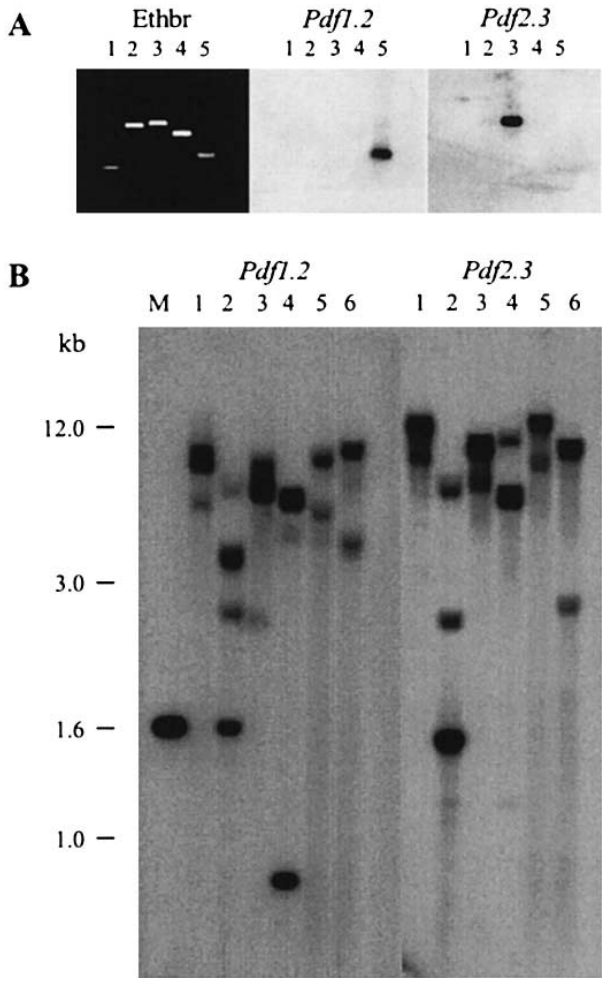

Fig. 3. Southern blots probed with plant defensin EST probes for *Pdf1.2* and *Pdf2.3*. (A) 20 ng insert DNA prepared from EST clones was separated on a 1.5% agarose gel with ethidium bromide (EthBr). 1, *Pdf2.1*; 2, *Pdf2.2*; 3, *Pdf2.3*; 4, *Pdf1.1*; 5, *Pdf1.2*. (B) Genomic DNA of the ecotype Col-2 was digested with restriction enzymes as indicated. M, marker; 1, *Bam*HI; 2, *Hin*dIII; 3, *Eco*RI; 4, *Eco*RV; 5, *Xba*I; 6, *Bcl*I.

Thi2.1 gene [10] and induces the *A. thaliana* phytoalexin camalexin [43].

Salicylate did not influence the expression of the *Pdf2.3* gene. Transcript levels remained at the same level as in the controls (Fig. 5). Expression of the *Pdf1.2* gene was also not induced. Probes for PR1 and PR5 were used as controls to show that the salicylate treatment was effective (data not shown). Methyl jasmonate induced the *Pdf1.2* gene but gave a slight reduction of the *Pdf2.3* transcript level (Fig. 5). Ethephon induced neither of the plant defensin genes as is shown in Fig. 6. Again, controls showed that the treatment was effective (data not shown). Silver nitrate did not induce the *Pdf2.3* gene but induced the *Pdf1.2* gene as did methyl jasmonate (Fig. 6).

3.4. Induction by phytopathogenic fungi

Chiang and Hadwiger [16] and Terras et al. [24] reported that plant defensins are induced after infection by phytopathogenic fungi. We have therefore tested different fungi on *A. thaliana* seedlings for an induction of plant defensin genes. The interaction of the *A. thaliana* ecotype Col-2, which was used throughout the experiments, was compatible with *F. oxysporum f.* sp. *matthiolae* and both *Al. brassicicola* strains, whereas the interaction with *F. oxysporum f.* sp. *raphani* and *Al. raphani* was incompatible. As documented in Fig. 7, none of the tested fungal isolates altered the transcript level of the *Pdf2.3* gene, but the *Pdf1.2* gene, whose expression was not detectable in seedlings by Northern blots, was induced by all fungi tested, although to different degrees. *F. oxysporum f.* sp. *matthiolae* was the most effective one, while *Al. raphani* gave only very faint signals on Northern blots.

4. Discussion

In the work presented here we made use of expressed sequence tags which have been generated for *A. thaliana* [25,26] to study the expression of plant defensins in this plant. Inspection of the databases revealed ESTs for several different plant defensin genes. By sequence comparison, these can be classified into two subgroups. We have used one EST from each group as a hybridisation probe to study the expression of *A. thaliana* plant defensin genes. On Southern blots both EST-

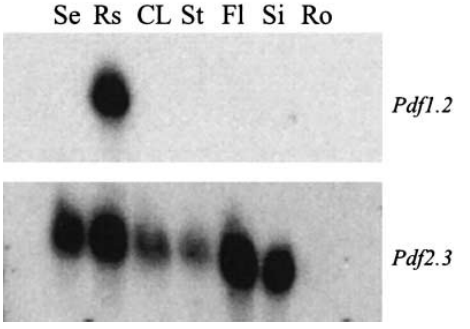

Fig. 4. Northern blots were hybridised with plant defensin EST probes for *Pdf1.2* (top) and *Pdf2.3* (bottom). 20 μg of total RNA isolated from different parts of the ecotype Col-2 were used. Seedlings were grown on MS agar medium and roots were derived from plants grown in liquid culture. All other plant organs were isolated from plants that were grown in soil in a greenhouse. Se, seedlings; Rs, rosettes; Cl, cauline leaves; St, stems; Fl, flowers; S, siliques; Ro, roots.

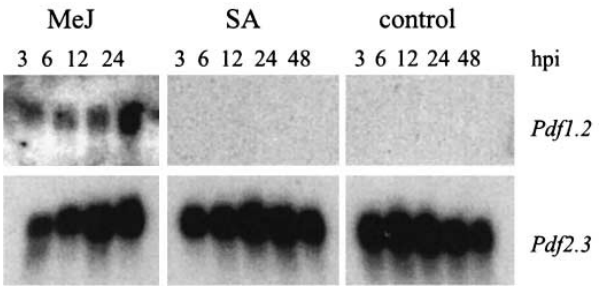

Fig. 5. Northern blots showing the effects of methyl jasmonate and salicylate on the expression of plant defensin genes. Seedlings were grown on MS agar plates and infiltrated with 100 μM methyl jasmonate (MeJ), 1 mM salicylate (SA), or water (control). Northern blots were hybridised with probes for *Pdf1.2* (top) and *Pdf2.3* (bottom). hpi, hours postinduction.

specific probes detect different strong hybridising bands. In addition to the strong signals, some other faint bands are also visible indicating that other related genes may exist in *A. thaliana*. A cross-hybridisation experiment (Fig. 3A) shows that it is possible to differentiate the expression of the different genes by Northern blots.

The Northern blot studies revealed that *Pdf1.2* and *Pdf2.3* are regulated differently. *Pdf2.3* is constitutively expressed at a high level in seedlings, rosettes, flowers, and siliques. This expression level was not further increased in seedlings by different necrotrophic fungi or by chemicals which are known to induce PR proteins [38], phytoalexins [43], or thionins [10] in *A. thaliana*. *Pdf1.2* transcripts, on the other hand, are not detected by Northern blots in untreated seedlings and the gene is neither inducible by salicylate nor by ethephon. Only two chemical inducers were found to be effective for this gene, methyl jasmonate and silver nitrate. Both of these are known to activate genes which are involved in plant defence. Methyl jasmonate is a signal transducer for proteinase inhibitors [41] and elicitor-inducible low-molecular weight metabolites [44]. Silver nitrate is a well known inducer for phytoalexins, including camalexin in *A. thaliana* [43]. In addition to these abiotic inducers, fungi of the genera *Fusarium* and *Alternaria* were able to induce this gene to different degrees. These results are in agreement with other reports which indicated a role for plant defensins in host resistance [16,19,21,22,24].

The induction by methyl jasmonate, silver nitrate, and necrotrophic fungi is reminiscent of the regulation of the *A. thaliana Thi2.1* gene [10]. *Pdf1.2* and *Thi2.1* are not induced by salicylate and ethephon, which indicates that they are regulated by a salicylate-independent signal transduction pathway which is different from that for PR proteins. It remains to be seen if the induction by fungi is due to a wound effect or to specific elicitors (or both). Doares et al. [45] found that accumulation of proteinase inhibitors in tomato leaves can be induced by elicitors such as oligogalacturonides and chitosan through the octadecanoid pathway. Why different fungi induce the *Pdf1.2* gene to such different levels is not known at the moment. For the *Thi2.1* gene we found a correlation between resistance against *F. oxysporum f. sp. matthiolae* and a higher expression level in resistant ecotypes vs. susceptible ecotypes (Epple et al., unpublished results). It might therefore be interesting to test if such a correlation does also exist for induction of the *Pdf1.2* gene by the fungi tested in this work.

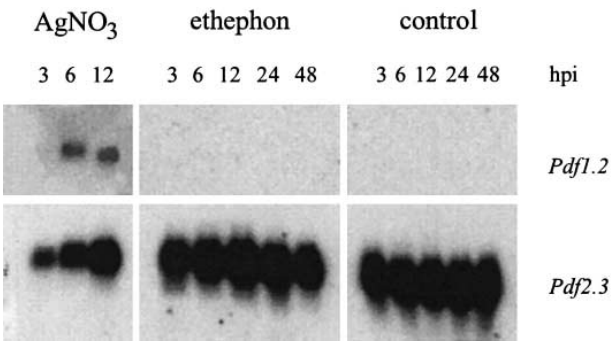

Fig. 6. Northern blots showing the effects of silver nitrate and ethephon on the expression of plant defensin genes. Seedlings were grown on MS agar plates and infiltrated with 1 mM silver nitrate ($AgNO_3$), 0.01% ethephon, or water (control). Northern blots were hybridised with probes for *Pdf1.2* (top) and *Pdf2.3* (bottom). hpi, hours postinduction.

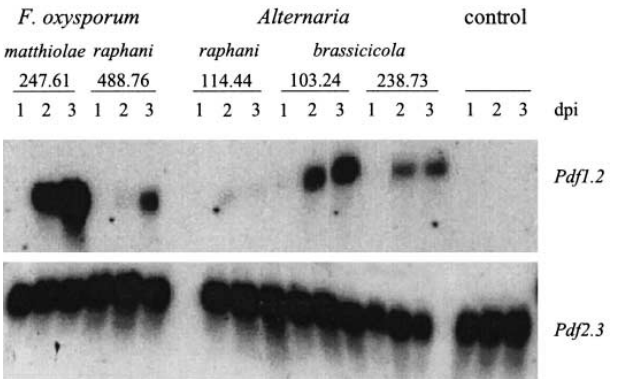

Fig. 7. *Pdf1.2* is induced by pathogenic fungi. Seedlings were grown on MS agar plates for 12 days and sprayed with a suspension of fungal spores (5×10^5 spores/ml) or water (control). Two different *Fusarium* isolates and three different *Alternaria* isolates were used (see Section 2). Total RNA was isolated after inoculation and Northern blots (20 μg) were probed with *Pdf1.2* (top) and *Pdf2.3* specific probes (bottom). dpi, days postinfection.

As recently shown by Pieterse et al. [46], biocontrol bacteria can induce systemic resistance in *A. thaliana* which is independent of salicylate. It seems possible that this resistance might be mediated by the salicylate-independent signal transduction pathway which leads to the expression of antimicrobial peptides encoded by *Thi2.1*, *Pdf1.2*, and perhaps other genes.

The *A. thaliana* thionin gene family contains two members which are differently regulated in seedlings. One of the genes (*Thi2.2*) is constitutively expressed, whereas the other gene (*Thi2.1*) is inducible by pathogenic fungi and is constitutively expressed in flowers and siliques. The members of the *A. thaliana* plant defensin multigene family are similarly specialised for constitutive and pathogen inducible expression. Moreover, in addition to the genes whose regulation has been studied in this paper, *A. thaliana* contains a seed specific plant defensin (*Pdf1.1*) which was isolated and partially sequenced [23]. Seed-specific plant defensins have also been found in several other members of the plant family *Brassicaceae* [23] and pathogen inducible plant defensins have been detected in radish [24], indicating that the plant defensin multigene family is widely distributed in *Brassicaceae*. Future studies should be aimed at cloning all *A. thaliana* plant defensin genes for a detailed analysis of their role in plant resistance.

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