

Characterisation of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins

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Abstract

We report the isolation and characterisation of a wound-induced cDNA designated AoPR1 from a suspension of mesophyll cells that had been mechanically isolated from cladodes of light-grown *Asparagus officinalis* seedlings by grinding in a mortar and pestle. The transcript abundance is up-regulated following cell separation and in chopped mesocotyl tissue from dark-grown seedlings. The expression of AoPR1 was shown by northern analysis to be located around the site of damage. Sequence analysis revealed similarity between the predicted AoPR1 polypeptide and bean PvPR1 and PvPR2 proteins, the potato pSTH2 protein, the pea PI49 protein, the parsley PcPR1-1 protein and a major pollen allergen from birch (*BetvI*). These transcripts have been shown to be induced in response to microbial attack or fungal elicitation. To our knowledge, this is the first example of a monocot cDNA belonging to this class of intracellular pathogenesis-related proteins (IPRs).

Introduction

Amongst some of the well characterised defence-related proteins are the tobacco pathogenesis-related (PR) proteins. Following infection of tobacco leaves by tobacco mosaic virus (TMV) PR proteins were shown to accumulate rapidly in the extracellular leaf spaces, with the most abundant class being the small acidic PR1 group of proteins [1, 3, 6, 9, 26]. Basic isoforms of the PR1 proteins with a molecular mass of around 16 kDa have since been identified that are targeted into the

central vacuole in TMV-infected plants [for review see 1]. PR proteins also accumulate after treatment with fungal and bacterial pathogens, chemical elicitors (such as salicylic acid) and upon mechanical wounding [1]. A functional role has been assigned to some members of the classical PR protein groups, such as the β -1,3-glucanase [16] and the chitinase activity [17]; however, the PR1 subclass of protein-encoding genes, which have been studied intensively at the nucleic acid level, still have an unknown function in the defence response.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X62103 (AoPR1 cDNA).

The tobacco acidic PR1 subgroup of PR proteins generally have a molecular mass range between 15.5 and 15.8 kDa [1, 6]. These polypeptides are all synthesised as precursor proteins with an N-terminal extension of 30 amino acids, which is cleaved during import into the endomembrane system prior to secretion into extracellular spaces. Characterisation of fungal elicitor-induced proteins and transcripts from cultured parsley cells identified proteins that were also acidic and ranged in mass from 17.5 to 19.2 kDa [24]. These polypeptides were named parsley PR1 proteins, but sequence analysis of cDNAs coding for these proteins subsequently revealed no similarity between tobacco PR1 genes and three genes coding for parsley PR1-like proteins [25]. Evidence has also been presented suggesting that the parsley 'PR1-like' proteins were not extracellular [25]. Recently, cDNAs homologous to the parsley PR1 cDNA have been cloned from potato [18], fungal elicitor-treated bean cell culture [27], birch pollen [2] and pea [9]. As in the parsley clones, in all cases there was no indication that the predicted protein product of any of these similar genes contained an N-terminal extension characteristic of a signal sequence. It has been suggested that these cDNAs represent a new category of pathogenesis-related genes and hence the designation 'PR1' is perhaps misleading [27]. Although there is no definitive proof, it is very likely that the proteins encoded by this new class of PR genes are intracellular [24, 25] and therefore, for the sake of clarity, perhaps it will be appropriate to refer to them as intracellular PR proteins (IPRs).

In previous work we have shown that asparagus mesophyll cells mechanically separated by grinding in a mortar and pestle are potentially a rich source of wound-induced transcripts [13, 19]. Several wound-induced cDNAs were isolated by differential screening of a cDNA library derived from message purified from mechanically separated *Asparagus officinalis* cells. On sequencing, the predicted amino acid sequence of one of these clones was found to be similar to the IPR group of proteins recognised previously [27]. To our knowledge, this repre-

sents the first reported cloning of a monocot cDNA of this class.

Materials and methods

Plant growth and cell isolation

Asparagus officinalis (cv. Connover's Colossal) seed was purchased from Nickerson Seeds Ltd. Six-week-old greenhouse-grown plants were used for cell isolation by grinding in a mortar and pestle as described previously [19]. These freshly isolated, 'mechanically separated' cells were then incubated in a liquid nutrient as described previously [13, 19] and RNA isolated for cDNA library construction after 1, 2 and 3 days. For studies on the wound response in intact asparagus tissue, seeds were sown on sterile vermiculite and then grown in the dark at 26 °C. Etiolated seedlings were then harvested 2 weeks after germination, and mesocotyls were then cut into various lengths and incubated on damp filter paper in Petri dishes at 26 °C in the dark until RNA isolation after 1, 2, or 3 days.

Construction and differential screening of cDNA library

Total RNA and poly (A)⁺ RNA from cells and wounded seedlings were isolated as described previously [8, 13]. 2 µg of pooled poly(A)⁺ RNA from cells 1–3 days after mechanical isolation (grinding) from asparagus seedling tissue were used to construct a λZAP II library. cDNA synthesis was carried out by a modification of the Gubler and Hoffman [12] method using a cDNA synthesis kit from Pharmacia. The cDNA was ligated into 'lambda ZAP' (Stratagene) and the phage packaged using Amersham's packaging extract. The resulting library contained 1.2×10^6 pfu. Ten thousand plaques were plated out at low density (2000 plaques per 15 cm plate) and duplicate filters lifted from the plates. One set of filters was probed with ³²P-labelled first-strand cDNA synthesised from the same mRNA used to

construct the library and the other set probed with ^{32}P -labelled first-strand cDNA synthesised from unwounded seedlings. The labelling, and removal of unincorporated nucleotides, was carried out as described [22]. Hybridisations were carried out essentially as described [22] in $6 \times \text{SSPE}$, $5 \times \text{Denhardt's solution}$, 0.5% SDS and $100 \mu\text{g/ml}$ denatured salmon sperm DNA at 65°C overnight and the filters washed finally in $0.2 \times \text{SSC}$, 0.1% SDS at 65°C . Autoradiography was carried out overnight using Amersham β -max 'Hyperfilm'. Differentially hybridising plaques were picked and repurified by another round of differential screening. The clones of interest were subcloned (according to Stratagene's protocol) by the *in vivo* excision of the Bluescript pSK(-) vector that follows super-infection of the λZAP -infected host cells with VCS-M13 helper phage.

RNA gel blots

Northern blots were carried out essentially as described [8]. Total RNA was run on formaldehyde RNA-denaturing gels. The gels were stained in ethidium bromide and blotted. Pre-hybridisation and hybridisation were carried in the same hybridisation solution as for the plaque lifts except the solution contained 50% deionised formamide. The probe was labelled by the oligo-labelling method as described [8].

DNA sequencing

The dideoxy chain-termination method [21] was used to sequence DNA from the recombinant pSK(-) clone with the Sequenase Version 2.0 kit (USB). Single-stranded template was prepared by phagemid rescue or by denaturing plasmid DNA as recommended by the USB Sequenase booklet. Computer-aided sequence analysis [5] and sequence database searches were carried out using the University of Wisconsin-released programs on a VAX. Protein sequence alignment was achieved using the CLUSTAL program on an IBM personal computer [14].

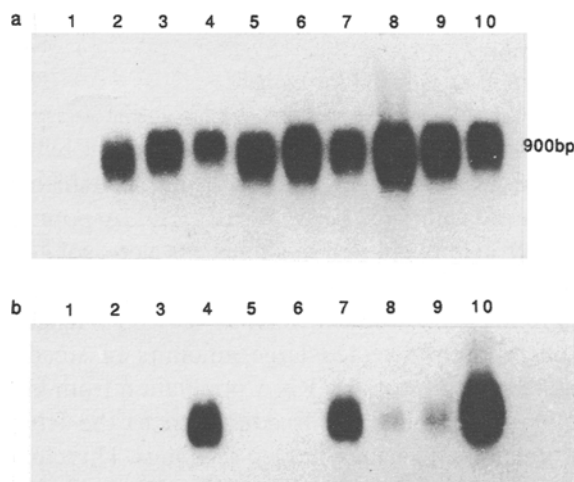


Fig. 1a. Northern analysis of transcript following cell isolation and wounding. Total RNAs were extracted from wounded or unwounded etiolated asparagus seedling tissue or mechanically isolated cells and $10 \mu\text{g}$ of each sample was run out on denaturing gels, blotted and hybridised with probe made from the AoPR1 cDNA. The RNA samples are as follows: unwounded seedlings (lane 1), cell suspension one day after isolation (lane 2), seedlings cut into 2 mm length sections one day after wounding (lane 3), seedlings cut into 10 mm length sections one day after wounding (lane 4), cell suspension two days after isolation (lane 5), seedlings cut into 2 mm length sections two days after wounding (lane 6), seedlings cut into 10 mm length sections two days after wounding (lane 7), cell suspension three days after isolation (lane 8), seedlings cut into sections 2 mm long three days after wounding (lane 9), seedlings cut into sections 10 mm long three days after wounding (lane 10). The approximate transcript size is shown in base pairs.

Fig. 1b. Northern analysis of transcript abundance at the wound site. Two week old etiolated asparagus seedling mesocotyls were cut into 1.5 cm explants and left to age as described in Materials and methods before taking the sections for analysis after 1, 2 and 3 days. Total RNA was extracted from asparagus tissue and $10 \mu\text{g}$ of each sample was run out on denaturing gels, blotted and hybridised with probe made from the AoPR1 cDNA. The samples are as follows: unwounded seedlings (lane 1), 5 mm long sections 5 mm away from the wound site one day after wounding (lane 2), 2.5 mm long sections 2.5 mm away from the wound site one day after wounding (lane 3), 2.5 mm long sections that include the wound site one day after wounding (lane 4), 5 mm long sections 5 mm away from the wound site two days after wounding (lane 5), 2.5 mm long sections 2.5 mm away from the wound site two days after wounding (lane 6), 2.5 mm long sections that include the wound site two days after wounding (lane 7), 5 mm long sections 5 mm away from the wound site three days after wounding (lane 8), 2.5 mm long sections 2.5 mm away from the wound site three days after wounding (lane 9), 2.5 mm long sections that include the wound site three days after wounding (lane 10).

Results

Isolation of the AoPR1 clone

cDNAs from a λ ZAP that differentially hybridised to a cDNA population from mechanically isolated asparagus cells versus a cDNA population from unwounded seedling cells were isolated, purified and used in northern dot blot analysis. Of several clones studied, clone 34 (200 bp in length) differentially detected large amounts of steady-state transcript in the RNA population from mechanically isolated cells compared to the RNA population from unwounded seedlings. This clone was then used to rescreen the cDNA library. From the 10 000 plaques screened, there were 170 positive clones. Ten clones were chosen at random and the insert size determined. Three of these clones contained inserts of ca. 750 bp. The size of

the transcript was estimated at 900 bp from northern gels (Fig. 1a).

Wound induction of message

One of the 750 bp cDNA inserts was used as a probe in northern analysis. As shown in Fig. 1a, the transcript is detectable in mechanically isolated asparagus cells reaching a maximum steady state one to three days after cell isolation. In chopped mesocotyl sections from etiolated seedlings, the transcript abundance is comparable to that in mechanically isolated cells and appears to follow similar time course kinetics (Fig. 1a). To determine the spatial distribution of message in relation to the wound site, total RNA was isolated from different areas of etiolated, wounded (chopped) mesocotyl and used in

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      M S S G S W S H E V A V N V A A G R M F
1  ATGAGTTCAGGGAGCTGGAGCCACGAGGTCGCTGTCAATGTCGCCGAGGACGGATGTTTC 60

      K A A M L D W H N L G P K I V P D F I A
61 AAGGCGGCAATGCTCGACTGGCACAACCTCGGCCCTAAGATTGTGCCTGACTTTATTGCCC 120

      G G S V V S G D G S V G T I R E I K I N
121 GGTGGCTCAGTGGTGTCTGGAGATGGATCTGTAGGAACCATCCGAGAGATCAAGATCAAC 180

      N P A I P F S Y V K E R L D F V D H D K
181 AATCCTGCTATACCTTTCAGCTATGTGAAGGAACGCCTGGATTTCGTAGACCATGACAAG 240

      F E V K Q T L V E G G G L G K M F E C A
241 TTCGAGGTGAAGCAGACCCTCGTGAAGGTGGAGGTTTAGGTAAGATGTTGAATGTGCC 300

      T T H F K F E P S S N G G C L V K V T A
301 ACCACTCACTTCAAGTTCGAGCCCTCGAGCAACGGTGGATGCCTCGTCAAGGTGACTGCA 360

      S Y K I L P G V A D E S A K A K E G I T
361 TCCTACAAGATTCTCCAGGTGTCGCCGATGAGAGTGCGAAGGCGAAGGAGGGAATAACC 420

      N H M K A T E A Y L L A N P T A Y V *
421 AACCACATGAAGCAACCGAAGCTTACCTCCTAGCCAACCAACTGCCCTACGTTTAAATA 480

481 TAGTGATTGTGTTTCTTTGCGTGAAGTGCTTGTGAGTTTGAATAAGGAGATTGGTTATGA 540

541 GGAAGCTTGATGGGGTCATACATAGTTAGTTTATGTTGAATGATCAGCCTTTTTTGTGTG 600

601 AAGTACTTGGGAGTTTGAATAAGGAGACTGAATATGAGAAAGATTGATGGAGTTATCGTT 660

661 CATGTTGAATGATCAGCCTTATCAGTTTGTAAACAGTGTGCAATGATCAGTCTTATCAGTT 720

721 TGTAATGGTGGCTTCAA 737

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Fig. 2. Sequence of the AoPR1 cDNA and the predicted peptide. The stop codon is represented by an asterisk.

northern analysis. This showed that after 1 or 2 days the transcript was mainly located to the wound site. However, on the third day transcript was also detectable at much lower levels in tissue distant from the wound surface (Fig. 1b).

Relationship of the cDNA to other gene sequences

As the transcript size was estimated to be 900 bp, it was assumed that the approximately 750 bp cDNA was not full-length. Following sequencing (Fig. 2), the 750 bp cDNA was found to contain a major open reading frame of 474 bp with a 263 bp 3' untranslated region containing a putative polyadenylation signal starting at position 618 but no apparent poly(A) tract. The predicted peptide amino acid sequence shown in Fig. 2 was used to screen the EMBL protein database. The sequences that showed significant similarities are a group of intracellular PR proteins found previously [27]: a pea disease resistance protein, pI49 (27% identity), a potato PR protein, pSTH2 (34% identity), a parsley PR protein, PcPR1-1 (32% identity), a birch pollen allergen *Betv1* (36% identity), and the bean elicitor-induced transcripts

PvPR1 and PvPR2 (29% identity). Because of this similarity we have named our cDNA clone AoPR1 (*Asparagus officinalis* pathogenesis-related clone 1). Sequence alignments are shown in Fig. 3.

Discussion

The initial differential screening of the cDNA library prepared from mechanically isolated asparagus cells confirmed our assertion [13] that this system is indeed a rich source of transcripts associated with the wound response. It was interesting to note that AoPR1, one of the most abundant cDNA clones in library, coded for a transcript with a predicted gene product that was similar to a group of proteins known to be strongly upregulated *in planta* by pathogen attack or by treatment of cultured cells with microbial elicitors. AoPR1 and the IPR genes from parsley and bean were all derived from mRNA purified from suspension cultured cells. However, the major differences between the procedures used to isolate the AoPR1 and the parsley and bean IPR cDNAs is that the latter clones were obtained by treatment of long-established, dividing, heterotro-

Asparagus PR1	M S S G S W S H E V A V N V A A G R M F K A A M L D W H N L G P K I V P D F I A G G S V V S G D G S V G T I R E I K I N
Parsley PR1	M G V Q K S E V * T T S S * S * E K L * * G L C * * I D T * L * Q V L * G A * K S S E T L * * * * G * * V K L V H L G
Potato STH2	M G V T * Y T L * T T T P * * P T * L * * * L V V * S D * * I * * L M * Q - V K N I E A - - - E G D * S * K K M T F V
Birch Betv1	M G V F N Y E T * T T S V I P * A * L * * * F I * * G D * * F * * V A * Q A * S S V E N I * * N * G P * * K K * S F P
Pea I49	M G V F N V E D * I T S V * * P A I L Y * * L V T * A D * * T * * V I - * A * K S I E I * * N * G A * * K K L T F V
Bean PvPR1	M G V F T F E D Q T T S P * * P A T L Y * * V A K * A D T I F * * A L * * S F K S V E I * * N * G P * * K K I S F V

Asparagus PR1	N P A I P F S Y V K E R L D F V D H D K F E V K Q T L V E G G G L G K M F E C A T T H F K F E P S S N G G C L V K V T A
Parsley PR1	D A S - * K T M * Q K V * A I * K A T * T Y S Y S I I D * D I * L G F T * S I N N * T A V * N A D * * T * * * * I
Potato STH2	E G S - * I K * L * H K I H V * * D K N L V T K Y S M I * * D V * * D K L * S I S Y D L * * A H G * * * V C * * I *
Birch Betv1	E G F - * * K * * D * V * E * * * T N * K Y N Y S V I * * * P I * D T L * K I S N E I * I V A T P D * * S I L * I S N
Pea I49	E D G - E T K H * L H K V E L * * V A N L A Y N Y S I * G * V * F P D T V * K I S F E A * L S A G P * * * S I A * L S V
Bean PvPR1	E D G - E T K F * L H K I E S I * E A N L G Y S Y S I * G * V A * P E T A * K I * F D S * L S D G P * * S * I * L S I

Asparagus PR1	S Y K I - - - L P G V A D E S A K A K E G I T N H M K A T E A Y L L A N P T A Y V
Parsley PR1	I F N T K G D A V V P E E N I K F * N D Q N L T I F * * V * * * * I * * - - - -
Potato STH2	E * H T K G D Y V L K D E D H N E G * K Q G M E L F * I V * * * * * * S V * A
Birch Betv1	K * H T K G D H E V K * E Q V K A S * * M G E T L L R * V * S * * * * H S D * * N
Pea I49	K * F T K G D A A P S E E Q L K K T D * A K G D G L F * * L * G * C * * E * D - * N
Bean PvPR1	T * H S K G D A * P N E * * L K A G * A K S D S L F * * V * * * * * * - - -

Fig. 3. Alignment of amino acid sequences of the AoPR1 homologues. The predicted asparagus AoPR1 amino acid sequence is compared [15] with the predicted primary structures of the parsley PcPR-1 protein [25], the potato pSTH2 protein [18], the birch *Betv1* allergen [2], the pea pI49 'disease response gene' protein [9] and the bean PvPR1 protein [27]. Residues showing identity to the AoPR1 predicted protein sequence are shown as an asterisk. Boxed regions show residues of absolute identity for all sequences. The conservation of residues (boxed regions) is shown by an asterisk.

phic suspension cell cultures with fungal elicitors, whereas AoPR1 was induced in freshly isolated mesophyll cells where the wound response is intense [25, 27]. In long-established suspension cultures, any wound signals would be diluted out during subculturing so that expression of PR1-like genes in these systems would predictably be very low until the addition of external elicitors. Because AoPR1 was derived from a cell system in the absence of external elicitors, it is probable that the corresponding transcript is induced during mechanical cell isolation in which endogenous elicitors such as plant cell wall fragments may be produced [4, 7, 20]. Alternatively, the breaking of apoplastic connections as a result of the shearing apart of neighbouring cells during grinding may be sufficient to induce the wound signal responsible for transcript induction. It has been shown that mRNA coding for PR proteins accumulate in freshly isolated tobacco protoplasts, which clearly have lost all intercellular connections [11]. However, in these experiments the same PR genes were induced by wounding only, so that it was difficult to determine any specific effect of cell separation on PR transcript induction.

The induction of PR protein expression is classically associated with pathogen infection of plants, however, potato pSTH-2-related transcripts were found to be induced by slicing tubers or stems as well as by the addition of arachidonic acid to tuber discs or by treatment of leaves with *Phytophthora infestans* mycelial homogenate [18]. Thus, as with AoPR1, the pSTH-2 member of the IPR group of proteins is induced by wounding. It is not known yet whether the birch, parsley or bean transcripts are induced only following the action of an elicitor or as a result of pathogen attack. *In situ* localisation of parsley PR1 mRNA has demonstrated that the transcript is highly abundant at sites immediately adjacent to the lesions undergoing hypersensitive response upon attempted fungal infection [23, 25]. This pattern of expression is similar to the AoPR1 transcript accumulation close to the wound site in chopped up asparagus seedlings, but data showing that AoPR1 transcript is induced by pathogen attack have not been obtained.

Analysis of previously described PR proteins with similarities to the predicted AoPR1 protein offers evidence that the predicted IPR proteins show evolutionary conservation. The order of the predicted proteins shown in the alignment reflects the degree of similarity among the AoPR1 protein and IPR proteins from other species, based on clustal analysis. The asparagus protein, which is derived from a monocot belonging to the family Liliaceae, is most divergent from the protein sequence consensus. Although apparent sequence similarities are evident, there may be differences between the AoPR1 gene and those of other IPR genes, reflecting the differences between monocots and dicots.

In future experiments we hope to use this monocot clone to learn more about the role of the AoPR1 protein in the wound response and to utilise the asparagus wounded cell suspension as a model to investigate the extent of similarity of the wound response in monocots compared to dicots.

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