

Short communication

Isolation of pathogen/stress-inducible cDNAs from alfalfa by mRNA differential display

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Abstract

Differential display of mRNA was used to isolate a full-length (SRG1) and a partial (SRG2) alfalfa cDNA induced during infection with the fungal pathogen *Colletotrichum trifolii*. The deduced amino acid sequences are similar to each other and resemble plant defense-related proteins and tree pollen allergens. SRG1 is a member of a gene family in alfalfa, which may also include the putative defense-related gene PR10. Unlike many defense-related genes described in similar systems, expression of SRG1-like genes does not correlate with resistance to *C. trifolii*. We speculate SRG1 is induced in response to plant stress.

Stress responses in plants are important for environmental adaptation and are associated with rapid changes in gene expression. The pathogenesis-related (PR) proteins are one class of proteins that accumulate following the stress of pathogen attack. These proteins were first identified in tobacco leaves [31], where they rapidly accumulate in the extracellular spaces following infection with tobacco mosaic virus (TMV) [2]. They also accumulate upon treatment with bacterial and fungal pathogens and chemical elicitors (such as salicylic acid) [2]. Activities have been assigned to some members of the tobacco PR group of proteins, such as the β -1,3-glucanases [16] and the chitinases [19]. The function of the PR1 class is still not known.

Another class of putative plant defense-related proteins [32] has recently been identified. These proteins include the disease resistance response [7, 13] and abscisic acid-responsive [15] proteins from pea, stress-induced proteins from soybean [8], a wound-induced protein from asparagus [33], and pathogenesis-related proteins from parsley [28], kidney bean [32] potato [21], and alfalfa [10]. These proteins are small (17–

19 kDa), like tobacco PR1 proteins, and their corresponding mRNAs accumulate in plant tissues or in cultured cells in response to various stresses. These proteins are distinct, however, because they do not display significant sequence similarity to the ‘classical’ PR proteins [13, 28] and their respective cDNAs do not encode signal sequences [28]. Because database searches do not point to any known biochemical activity, a specific function has not been assigned to any member of this protein class. Another characteristic common to these proteins is that many of their respective genes comprise gene families, which likely arose from duplication events [22].

In addition to being similar to each other, this latter group of defense-related proteins is also similar to major pollen allergens from trees of the order Fagales (birch [3], alder [4], hazel [5] and hornbeam [18]), which cause Type I human allergies. The pollen allergens are all about 17 kDa and consist of a heterogeneous mixture of different isoforms, which are probably encoded by gene families [3, 29]. The allergens are proposed to function in pollen defense responses [4]. Due to the similarities in the primary structures of the defense-related proteins and the pollen allergens, it has been suggested that these proteins display a sim-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers U42752 (SRG1) and U42753 (SRG2).

ilar, highly recognizable surface architecture involved in recognition interactions [3, 32].

In our laboratory, we are interested in identifying signaling events that occur between alfalfa (*Medicago sativa* L.) and the fungal pathogen *Colletotrichum trifolii* Bain & Essary during pathogenesis [9]. In susceptible cultivars *C. trifolii* causes anthracnose disease, which is characterized by black lesions on the stems and leaves of infected plants. *C. trifolii*-alfalfa interactions exhibit race-cultivar specificity; cv. Saranac is susceptible to both *C. trifolii* race 1 and race 2 and cv. Arc is resistant to race 1 and susceptible to race 2.

Because interactions between plants and pathogens cause specific changes in gene expression in both organisms, we sought to identify genes in either alfalfa or *C. trifolii* that are induced during disease development. Here we describe the cloning, by differential display, of a full-length (SRG1) and a truncated (SRG2) cDNA from alfalfa that are induced in *C. trifolii*-infected leaves. The deduced protein sequences are similar to each other and resemble the plant defense-related proteins and major tree pollen allergens. We further show that expression of the SRG1-like genes is induced in response to plant stress.

Isolation of differentially expressed alfalfa cDNAs

To isolate plant or fungal genes that function during disease development, differential display [14, 20] was performed on RNA from *C. trifolii* race 1 mycelium grown in culture, healthy alfalfa leaves (cv. Saranac), and alfalfa leaves infected with *C. trifolii* race 1 for 48 h (see Fig. 3 legend for general inoculation conditions). At this point, early disease symptoms were visible and mycelia was observed microscopically in and around the inoculation site. Thirty total primer set combinations were used per RNA sample. Seventeen differentially displayed cDNA tags were isolated from the infected leaf samples and tested for their differential expression in northern blots. Of these tags, three corresponded to differentially expressed genes, four did not detect any signal, and the rest were false positives that detected either signals in healthy and infected alfalfa RNA or rRNAs in all three samples. The three differentially expressed tags were isolated from different pools of infected leaf cDNAs. However, we speculated that these tags corresponded to either the same gene or members of a gene family because they displayed a number of similarities. For example, the tags were amplified using the same random decamers (E3 and E5, RAPD Primer kit, Operon Technologies,

Alamada, CA) in the modified differential display procedure [14], the size of each tag was 300 bp, and in northern blots each detected a 1 kb transcript in infected leaves with no signal in healthy leaves or in fungal mycelia. Due to these similarities, only one tag (tag G) was analyzed further.

RNA from infected plants is a mixture of plant and fungal RNA. Southern blot analysis showed tag G hybridized to DNA from alfalfa but not *C. trifolii* (data not shown). Accordingly, tag G was used to screen a fungal elicitor-induced alfalfa cDNA library, which was made from poly(A)⁺ RNA from cell cultures of alfalfa (resistant to race 1) treated with fungal elicitor for 2, 3 and 4 h. The cDNA library was prepared in λ ZAP II (Stratagene). Two clones of different sizes were recovered in pBluescript using the EXASSIST/SOLR system (Stratagene). The inserts, named SRG1 and SRG2 for stress response genes, served as probes on northern blots to confirm their differential expression. Like tag G, both SRG1 and SRG2 hybridized to a 1 kb mRNA in infected leaves and failed to hybridize to RNA from *C. trifolii* (data not shown). A faint signal was detected in healthy leaves after prolonged exposure of the blot (see Fig. 3). These results indicated that SRG1 and SRG2 corresponded to tag G and are alfalfa cDNAs induced by infection with *C. trifolii*.

Sequence and Southern blot analyses

Figure 1A shows the nucleotide and deduced amino acid sequences of SRG1 and SRG2, which were sequenced by the dideoxy-nucleotide chain termination method using the Sequenase version 2.0 DNA sequencing kit (USB). Sequence analysis was performed using the GCG package (Genetics Computer Group, Madison, WI). The SRG1 cDNA sequence is 692 bp long and contains a major open reading frame of 471 bp. It encodes a putative polypeptide of 157 amino acids with a predicted molecular mass of 16.9 kDa. The SRG2 cDNA sequence is 324 bp long and is a truncated cDNA containing only the 3' coding and non-coding portions of the gene. SRG1 and the SRG2 partial sequence are 96% identical at the nucleotide level. Only one difference in their deduced amino acid sequences was revealed, an amino acid replacement (T to I) at position 113 in the SRG1 sequence. Assuming the sequence similarity between the SRG clones is maintained in the 5'-coding region of SRG2, these cDNAs are likely members of a gene family (also see below).

A.	SRG1	CACCTTCTTAATAGTTTGTATTATTCACACATTAGTATTATAT CATCATGGGT	51
	Srg1		M G 2
	SRG1	GTATTCAACTTTGAGGATGAGACCACATCTATTGTAGCTCCTGCTAGACTT	102
	Srg1	V F N F E D E T T S I V A P A R L	19
	SRG1	TACAAAGCTCTAGTTACAGATTCTGATAACCTTATTCCAAAGGTTATTCAT	153
	Srg1	Y K A L V T D S D N L T P K V I D	36
	SRG1	GCCATCCAAAGTATTGAAATTGTTGAAGGAAACGGTGGTCCCGGAACCATC	204
	Srg1	A I Q S I E I V E G N G G A G T I	53
	SRG1	AAGAAACCTACTTTCGTTGAAGGTGGTGAACAAAGTATGATTTGCACAAA	255
	Srg1	K K P T F V E G G E T K Y D L H K	70
	SRG1	GTGGACTTAGTAGATGATGTTAACTTTGCTTACAACTACACCATAGTTGGA	306
	Srg1	V D L V D D V N F A Y N Y S I V G	87
	SRG1	GGTGGTGGACTTCCAGACACTGTGGAGAAGATCTCCCTTGAGTCTAAATTG	357
	Srg1	G G G L P D T V E K I S F E S K L	104
	SRG1	TCTGCAGGGCCACATG Ag GATCCACATGCAAAGCTTACTCTGAAATACTTC	408
	SRG2	T.....	30
	Srg1	S A G P D G G S T A K L T V K Y F	121
	Srg2	. I	10
	SRG1	ACCAAAGGTGATGCTGCACCAAGTGAAGAGGAAATCAAGGGTGGCAAGCT	459
	SRG2	..A.....T.....	81
	Srg1	T K G D A A P S E E E I K G G K A	138
	Srg2		27
	SRG1	AGGGGTGATGGTCTTTTCAAGGCTCTTGAAGGTTACGTTTGGCAATCCCT	510
	SRG2		132
	Srg1	R G D G L F K A L E G Y V L A N P	155
	Srg2		44
	SRG1	GATTACTAAAGCATATAGCTAATTCCTTGCTTTTATACATGGTGTGGT	561
	SRG2	A.	183
	Srg1	D Y *	157
	Srg2	. . .	46
	SRG1	TTGATACATTTTATTATATCATGTGTTCTTGATTGGTTGCCAATGTAATT	612
	SRG2	G.....	234
	SRG1	CTCTCTTTCCTTTTGCTCAACTGTGAGATGTGAAGTCATTTGTGT ACCTCC	663
	SRG2	C.....A.....	285
	SRG1	CTTcAATAAATTATAATAACTTCATCT (A ₁)	692
	SRG2	AT....ATTCAATTTT (A ₂)	324
B.	Srg1	MGVFNFEDETTISIVAPARLYKALVTDSDNLLPKVIDATQSIIEIVEGNGGAG	51
	PR10		..D....
DRRG49-a	V...I...V...T.....A...T.....K.....	51	
	STH-2	...TSYTH...TP...T...F...V.....LMPQVK...---AE.D.	47
	Srg1	TIKKPTFVEGGETKYDLHKVDLVDDVNFAYNYSIVGGGLPDTVRKISFES	102
	PR10	I....D....V.....L..H.....F.....	58
DRRG49-a	L....D....HV....E....VA..L.....V..F.....A	102	
	STH-2	S...MN...SPI...LK...IHV...K..LVTK...MIE..DV..G..KL..S...YDL	98
	Srg1	KLSAGPDGGSTAKLTVKYFTKGDAAPSEEEIKGGKARGDGLFKALEGYVLA	153
	PR10	N...Tl...S.....	82
DRRG49-a	N...Tl...S.....QL..TD..K.....C..	153	
	STH-2	.FE..HGN...CVC..SITE.H....YVLKD...HNE..QKQ..ME...IV..A..L..	149
	Srg1	NPIDY	157
	DRRG49-a	H...N	158
	STH-2	..SVYA	155

Figure 1. SRG cDNA sequences and amino acid sequence comparisons. A. Nucleotide and deduced amino acid sequences of SRG1 and SRG2. Identical bases or amino acids are indicated with a dot (.). Sequences shown in bold correspond to eukaryotic translation initiation [17] and polyadenylation [1] consensus sequences. Sequences corresponding to decamers E3 (CCAGATGCAC) and E5 (TCAGGGAGGT) are underlined and lower case letters indicate mismatches between the decamers and cDNA sequences. B. Amino acid sequence comparison between SRG1, the partial sequence of PR10 from alfalfa [10], DRRG49-a (formerly called p149 [7]) from pea [13] and STH-2 from potato [21]. Amino acids identical to Srg1 are indicated with a dot. Dashes (—) in STH-2 indicate a gap introduced in the sequence for optimal alignment. The threonine in SRG1 that is replaced by isoleucine in SRG2 is shown in bold.

Comparison of Srg1 with proteins in GenBank revealed similarities to several plant defense-related proteins [32], including the disease resistance response proteins (DRRG49-a, DRRG49-b and DRRG49-c/RH2 [7, 13, 25]) and the abscisic acid-responsive proteins (ABR17 and ABR18 [15]) from pea, the stress-induced proteins from soybean (SAM22 and H4 [8]), and the pathogenesis-related proteins from chickpea (capR1), kidney bean (PvPR1 and PvPR2, [32]), potato (STH-2 and STH-21 [21]) and alfalfa (the PR10 partial sequence [10]). Srg1 is also similar to the major pollen allergens from birch (Bet v I [3, 29]), hazel (Cor a I [5]) alder (Aln g I [4]) and hornbeam (Car b I [18]). Amino acid sequence comparisons between SRG1, the partial sequence of alfalfa PR10, pea DRRG49-a and potato STH-2 are shown in Fig. 2B. These proteins were chosen for comparison because Srg1 is most closely related to DRRG49-a (82% identity) and most distantly related to STH-2 (46% identity). The partial sequence reported for PR10 (83 amino acids) is deduced from a PCR product that was amplified with primers to conserved regions in DRRG49-a and DRRG49-b [10]. The PR10 amino acid sequence is 86% identical to Srg1. Though the entire sequence of PR10 is not known, it is tempting to speculate that it and the SRG clones are members of a gene family in alfalfa.

To investigate the number of genes corresponding to the SRG1 cDNA in the alfalfa genome, genomic DNA from cvs. Saranac and Arc was digested with restriction enzymes *Hind*III and *Xba*I, and the resulting fragments were hybridized with the SRG1 cDNA (Fig. 2). SRG1 hybridized to at least eight bands in the *Hind*III digests and at least seven bands in the *Xba*I digests. The complex hybridization patterns were identical in the two cultivars. These results confirm that SRG1 is a gene family member in alfalfa. As with SRG1, cDNAs encoding DRRG49-a [7], ABR18 [15], and PvPR1 [32] detect multiple bands on Southern blots of genomic DNA.

Stress induction

The expression patterns of some plant defense-related genes have been reported to correlate with disease resistance. For example, in peas DRRG49-a (pI49) expression dramatically increases within 6 h and again at 12–24 h after inoculation with *Fusarium solani* f. sp. *phaseoli* (avirulent fungal pathogen), but not after inoculation with *F. solani* f. sp. *pisi* (virulent fungal pathogen) [12]. In alfalfa, PR10 transcripts are detec-

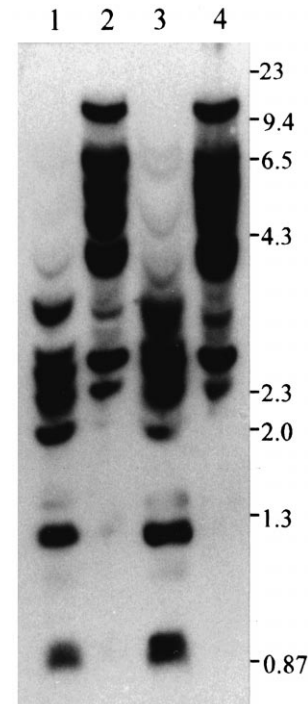


Figure 2. Southern blot analysis of SRG1-like sequences in the alfalfa genome. Genomic DNA from *M. sativa* cv. Arc and Saranac was isolated [27] and digested with either *Hind*III or *Xba*I. The DNA was fractionated on a 0.8% (w/v) agarose gel and transferred onto a nylon membrane. Ten μ g of DNA was loaded in each lane [15]. ^{32} P-labelled probes were made by PCR using primers SK (TCTAGAAC-TAGTGGATC) and KS (CGAGGTCGACGGTATCG) according to the recommended protocol in the PCR Radioactive Labeling System (BRL). Hybridizations were carried out at 65 °C in buffer containing 5 $\times$ SSPE, 5 $\times$ Denhardt's solution, 0.5% SDS and 20 μ g/ml denatured salmon sperm DNA. Blots were washed twice at high stringency (0.2 $\times$ SSPE, 0.1% SDS at 65 °C). Lane 1, Arc + *Hind*III; lane 2, Arc + *Xba*I; lane 3, Saranac + *Hind*III; and lane 4, Saranac + *Xba*I. The sizes in kb of a mixture of *Hind*III-digested λ and *Hae*III-digested Φ X 174 RF fragments are indicated.

ted 30 h after leaves are infiltrated with *Pseudomonas syringae* pv. *pisi* (avirulent bacterial pathogen) but not after infiltration with *Xanthomonas campestris* pv. *alfalfae* (virulent bacterial pathogen) [10]. In contrast, pea genes have been identified that are induced after pathogen challenge, but whose expression patterns do not correlate with the resistance response to *F. solani* f. sp. *phaseoli* [12].

To test whether induction of SRG1-like genes in alfalfa correlates with the resistance response to *C. trifolii*, SRG1 expression was followed over time in leaves (cv. Arc) inoculated with either *C. trifolii* race 1 (avirulent fungal pathogen) or race 2 (virulent fungal pathogen) (Figure 3). If SRG1 expression correlates

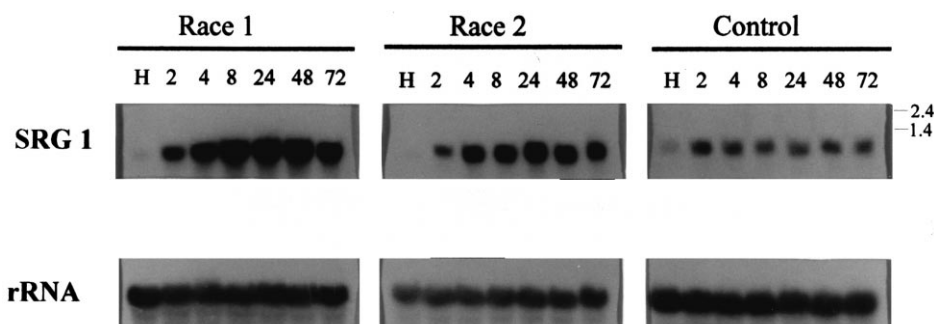


Figure 3. Induction of SRG1-like genes in alfalfa in response to avirulent and virulent races of *C. trifolii*. Fourteen-day old *M. sativa* cv. Arc plants were sprayed until runoff with a solution containing 5×10^5 to 10^6 spores from either *C. trifolii* race 1 (avirulent) or race 2 (virulent) in 0.2% soy dextrose surfactant (Helena Chemical Co., Memphis, TN). Control plants were sprayed with surfactant only, which was used to help the spore droplet 'stick' to the leaf surface. At the times indicated (in hours), leaves were excized directly into liquid N_2 and stored at $-80^\circ C$. Leaves from healthy plants (H) were harvested prior to inoculations. Total RNA was isolated [6], fractionated in 1.2% (w/v) agarose gels containing 0.2 M MOPS/0.3 M formaldehyde [26, 30], and transferred onto nylon membranes. Twenty μg of RNA was loaded in each lane. ^{32}P -labelled probes were made using the Random Primers DNA labelling system (BRL). Hybridizations were performed as described in Fig. 2. The blots were stripped in 0.2% boiling SDS and reprobed with the 1.7 kb *Bam*HI-*Xba*I fragment of pMF2, which contains a 17S rDNA clone from *Neurospora crassa* [11]. The *N. crassa* 17S rDNA clone displays sequence similarity with rDNA genes in plants and therefore strongly hybridizes to alfalfa RNA (G.M. Truesdell, unpublished data). The sizes in kb of RNA molecular weight markers (Gibco BRL) are indicated.

with resistance to *C. trifolii*, we expected that the SRG1-like genes would either be maximally expressed at an earlier time point in race 1- than in race 2-infected leaves, or expressed at significantly higher levels in race 1- than in race 2-infected leaves at the same time points. Because it was difficult to visually compare the levels of induction after each treatment in Fig. 3, the maximum level of SRG1 expression was determined for each treatment using computer imaging and analysis (Image 1.49 software, Wayne Rasband, NIH). The SRG1 signals in Fig. 3 were normalized relative to the rRNA signals and expression in healthy leaves was used as a baseline for comparison (data not shown). Importantly, normalization of the RNA signals showed that a 2 h SRG1 expression increased 4-fold in the fungal-treated samples and 3-fold in the surfactant-treated control. While expression in control leaves remained relatively constant to the 72 h time point, expression in race 1- and race 2-infected leaves increased to maximum levels at 8 h (an 8-fold induction) and 4 h (a 7-fold induction), respectively. The relatively high expression levels in the fungal-treated samples were maintained until the 72 h time point, when induction dropped to 5-fold in both samples. Since the early time points are likely key in determining resistance or susceptibility, and SRG1 was highly expressed in response to both fungal races with maximum expression at an earlier time point in response to the virulent fungal race, we speculate that the SRG1-like genes are not specific for the resistance response

to *C. trifolii*. Instead, these genes are likely induced in response to the stress associated with fungal spore inoculation. Induction in control leaves treated with surfactant is consistent with this hypothesis.

To further define the conditions that induce expression of the SRG1-like genes, northern blots were performed on RNA from senescent, u.v.-treated and mechanically wounded Arc leaves. Neither senescence nor UV treatment induced SRG1 (data not shown). However, mechanical wounding induced a high level of SRG1 expression (Fig. 4). Like SRG1, potato STH-2 [21], soybean SAM22 [8] and asparagus AoPR1 [33] are induced by wounding.

Taken together, the northern analyses indicate that external stresses, including fungal spore inoculation, surfactant and mechanical wounding, induce expression of the SRG1-like genes in alfalfa leaves. It is possible that these genes are induced by breaches in the integrity of the leaf cell wall, since microscopic studies of the alfalfa-*C. trifolii* pathosystem reveal that both virulent and avirulent races of the fungus penetrate the plant cuticle [23, 24].

Though expression of PR10, an alfalfa gene potentially closely related to SRG1, correlates with the resistance response to a bacterial pathogen [10], SRG1 induction did not correlate with the resistance response to *C. trifolii*. Our results, however, do not preclude the possibility that genes closely related to SRG1 (e.g. PR10) are specific for pathogen attack. The SRG1 probe used in the expression analyses likely hybrid-

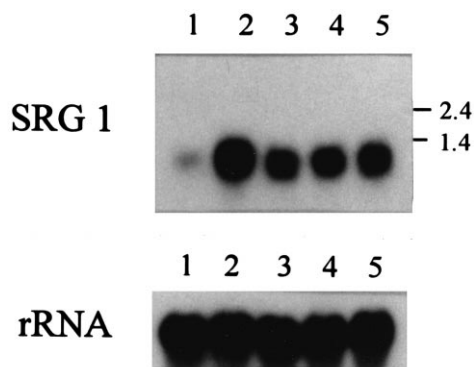


Figure 4. Induction of SRG1-like genes in wounded alfalfa leaves. Fourteen day old *M. sativa* cv. Arc plants were wounded by pinching the leaves with steel forceps. At the following times, leaves were harvested and northern blots were performed as described in Fig. 3. Lane 1, healthy leaves (no treatment); lane 2, 8 h after wounding; lane 3, 24 h; lane 4, 48 h; lane 5, 72 h. The sizes in kb of RNA molecular weight markers (Gibco-BRL) are indicated.

ized to transcripts of many closely related alfalfa genes, some of which may be specific for a resistance response, and thus possibly masked their differential expression. The differences in PR10 and SRG1 expression may also reflect differences in alfalfa's resistance to bacterial versus fungal pathogens, or may be due to the fact that PR10 expression was compared in alfalfa treated with two different bacterial species whereas SRG1 expression was compared in alfalfa treated with two closely related fungal races. Future experiments should include testing the fungal system with the PR10 probe to determine whether its expression pattern differs from SRG1.

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Note added in proof

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References

1. Birnstiel ML, Busslinger M, Strub K: Transcription termination and 3' processing: the end is in site!. Cell 41: 349–359 (1985).
2. Bol JF, Linthorst HJM, Cornelissen BJC: Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopath 28: 113–138 (1990).
3. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M: The gene coding for the major birch pollen allergen *Betv1* is highly homologous to a pea disease resistance response gene. EMBO J 8: 1935–1938 (1989).
4. Breiteneder H, Ferreira F, Reikerstorfer A, Duchene M, Valenta R, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Kraft D, Scheiner O: Complementary DNA cloning and expression in *Escherichia coli* of *Aln g I*, the major allergen in pollen of alder (*Alnus glutinosa*). J Allergy Clin Immunol 90: 909–917 (1992).
5. Breiteneder H, Ferreira F, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Rumpold H, Kraft D, Scheiner O: Four recombinant isoforms of *Cor a I*, the major allergen of hazel pollen, show different IgE-binding properties. Eur J Biochem 212: 355–362 (1993).
6. Cathala G, Savouret J, Mendez B, West BL, Karin M, Martial JA, Baxter JD: A method for isolation of intact, translationally active ribonucleic acid. DNA 2: 329–333 (1983).
7. Chiang CC, Hadwiger LA: Cloning and characterization of a disease resistance response gene in pea inducible by *Fusarium solani*. Mol Plant-Microbe Interact 3: 78–85 (1990).
8. Crowell DN, John ME, Russell D, Amasino RM: Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Mol Biol 18: 459–466 (1992).
9. Dickman MB, Buhr TL, Warwar V, Truesdell GM, Huang CX: Molecular signals during the early stages of alfalfa anthracnose. Can J Bot 73: S1169–S1177 (1995).
10. Esnault R, Buffard D, Breda C, Sallaud C, El Turk J, Kondorosi A: Pathological and molecular characterizations of alfalfa interactions with compatible and incompatible bacteria, *Xanthomonas campestris* pv. *alfalfae* and *Pseudomonas syringae* pv. *pisi*. Mol Plant-Microbe Interact 6: 655–664 (1993).
11. Free SJ, Rice PW, Metznerberg RL: Arrangement of the genes coding for ribosomal ribonucleic acids in *Neurospora crassa*. J Bact 137: 1219–1226 (1979).
12. Fristensky B, Rigglemen RC, Wagoner W, Hadwiger LA: Gene expression in susceptible and disease resistant interactions of peas induced with *Fusarium solani* pathogens and chitosan. Physiol Plant Path 27: 15–28 (1985).

13. Fristensky B, Horovitz D, Hadwiger LA: cDNA sequences for pea disease resistance response genes. *Plant Mol Biol* 11: 713–715 (1988).
14. Haag E, Rahman V: Effects of primer choice and source of *Taq* DNA polymerase on the banding patterns of differential display RT-PCR. *Biotechniques* 17: 226–228 (1994).
15. Iturriaga EA, Leech MJ, Barratt DHP, Wang TL: Two ABA-responsive proteins from pea (*Pisum sativum* L.) are closely related to intracellular pathogenesis-related proteins. *Plant Mol Biol* 24: 235–240 (1994).
16. Kauffmann S, Legrand M, Geoffroy P, Fritig B: Biological function of the pathogenesis-related proteins: four PR proteins of tobacco have 1,3- β -glucanase activity. *EMBO J* 6: 3209–3212 (1987).
17. Kozak M: Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44: 283–292 (1986).
18. Larsen JN, Stroman P, Ipsen H: PCR based cloning and sequencing of isogenes encoding the tree pollen major allergen *Car b 1* from *Carpinus betulus*, hornbeam. *Mol Immunol* 29: 703–711 (1992).
19. Legrand M, Kauffmann S, Geoffroy P, Fritig B: Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. *Proc Natl Acad Sci USA* 84: 6750–6754 (1987).
20. Liang P, Averboukh L, Pardee AB: Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucl Acids Res* 21: 3269–3275 (1993).
21. Matton DP, Brisson N: Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Mol Plant-Microbe Interact* 2: 325–331 (1989).
22. Matton DP, Prescott G, Bertrand C, Camirand A, Brisson N: Identification of *cis*-acting elements involved in the regulation of the pathogenesis-related gene STH-2 in potato. *Plant Mol Biol* 22: 279–291 (1993).
23. Mould MJR, Boland GJ, Robb J: Ultrastructure of the *Colletotrichum trifolii*-*Medicago sativa* pathosystem. I. Pre-penetration events. *Physiol Mol Plant Path* 38: 179–194 (1991).
24. Mould MJR, Boland GJ, Robb J: Ultrastructure of the *Colletotrichum trifolii*-*Medicago sativa* pathosystem. II. Post-penetration events. *Physiol Mol Plant Path* 38: 195–210 (1991).
25. Mylona P, Moerman M, Yang W, Gloudemans T, Van De Kerckhove J, Van Kammen A, Bisseling T, Franssen HJ: The root epidermis-specific pea gene RH2 is homologous to a pathogenesis-related gene. *Plant Mol Biol* 26: 39–50 (1994).
26. Rosen KM, Villa-Komaroff L: An alternative method for the visualization of RNA in formaldehyde agarose gels. *Focus* 12: 23–24 (1990).
27. Saghai-Maroo MA, Soliman KM, Jorgensen RA, Allard RW: Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci USA* 81: 8014–8018 (1984).
28. Somnich IE, Schmelzer E, Kawalleck P, Hahlbrock K: Gene structure and *in situ* transcript localization of pathogenesis-related protein 1 in parsley. *Mol Gen Genet* 213: 93–98 (1988).
29. Swoboda I, Jilek A, Ferreira F, Engel E, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breiteneder H, Pittenauer E, Schmid E, Vicente O, Heberle-Bors E, Ahorn H, Breitenbach M: Isoforms of *Bet v 1*, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* 270: 2607–2613 (1995).
30. Tsang SS, Yin X, Guzzo-Arkun C, Jones VS, Davison AJ: Loss of resolution in gel electrophoresis of RNA: a problem associated with the presence of formaldehyde gradients. *Biotechniques* 14: 380–381 (1993).
31. van Loon LC, van Kammen A: Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN'. II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology* 40: 199–211 (1970).
32. Walter MH, Liu J, Grand C, Lamb CJ, Hess D: Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. *Mol Gen Genet* 222: 353–360 (1990).
33. Warner SAJ, Scott R, Draper J: Characterization of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. *Plant Mol Biol* 19: 555–561 (1992).