

A Pistil-Specific PR-1 Like Protein of *Camellia*, Its Expression, Sequence and Genealogical Position

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Summary

A major protein endogenously expressed in *Camellia* pistils with a high level of expression at the period of anthesis was detected by two-dimensional gel electrophoresis. Their N-terminal amino acid sequences showed a high level of homology with pathogenesis-related (PR)-1 proteins from plants. Northern blot analysis demonstrated that the protein was expressed specifically in pistils. The gene coding the PR-1-like protein was cloned from tea (*Camellia sinensis*), and compared with those of other PR-1 and PR-1-like proteins. Based on sequence homology and induction, the protein was designated as *Camellia* PR-1-like (PRL-1) protein. The deduced PRL-1 protein sequence displayed the highest homology with STS14, which was earlier described as a PR-1-like protein expressed in pistils of potato. With the addition of this PRL-1 protein, we discussed the possibility of a new class of PR-1-like proteins, which are specifically induced in pistils of many plant families. The sequence of PRL-1 gene is registered as Accession: AB015047.

Key Words : *Camellia sinensis*, gene genealogy, pistil-specific protein, pathogenesis related (PR)-1 protein.

Introduction

Flowers open to receive pollen without any protective surface against potential invasion of pathogen. Despite the lack of protective surfaces, few pathogens are known to infect flowers. This may be attributed to some biochemical guard specifically evolved in the floral organs. On the other hand, pollen grains that land on a compatible stigma surface germinate, penetrate the stigma, and grow through intercellular paths in the style into the ovary. The pollen tubes are not hindered but supported by the cells of the transmitting tract. Thus, there must be two contrasting mechanisms in the flower, one for helping penetration of compatible pollen and another for rejecting pathogen and incompatible pollen.

In the above-mentioned context, it is of interest that

proteins corresponding to genes for sporophytic self-incompatibility in *Brassica* species (SRK) have a molecular structure similar to host resistance gene products (*Pto*) of tomato against a bacterial disease as reviewed by Martin *et al.* (1993). Also, the proteins involved in gametophytic self-incompatibility of Solanaceae (McClure *et al.* 1989) and Rosaceae plants (Sassa *et al.* 1993) are RNases. A ribonuclease-like protein was classified as a pathogenesis-related protein, PR-10 (Van Loon *et al.* 1994). The molecules associated with self-incompatibility from different plant families appear quite dissimilar, but the possibility that the subsequent pathways converge and share some features with pathways that mediate disease resistance remains open (Matton *et al.* 1994). Thus, a search for major proteins expressed in the pistil of flower may help us to identify plant proteins for defensive functions or self-incompatibility.

Tea, *Camellia sinensis*, exhibits gametophytic self-incompatibility in which a single multi-allelic locus, the *S*-locus, is considered to control the incompatible reaction. The *S*-genotype has been determined in several major varieties of tea (Fuchinoue 1969). We have searched for self-incompatibility-related molecules in tea by comparing 2D-PAGE profiles of major pistil proteins from different *S* genotypes. To date, we have not been able to relate *S*-genotypes to any major molecules in the pistil, but here we report a protein which was specifically expressed in the pistil as one of major proteins, and may be added to a novel class of PR-1 proteins.

Materials and Methods

Plant materials

Four *camellia* (*Camellia japonica*) cultivars; 'Bokuhan', 'Kumasaka', 'Nukifude', 'Utsusemi', two wild type cultivars 'Yabu-tsubaki' No.780, Beni No.3, and seven tea (*Camellia sinensis*) cultivars; 'Yabukita', 'Sayama-kaori', 'Kanaya-midori', 'Tama-midori', 'Yamato-midori', 'Takachiho' and 'Sayama-midori' were used as experimental materials. Flower buds of *camellia* were collected at the Subtropical Plant Institute of Kyoto University, and those of tea were sampled at the National Research Institute of Vegetable, Ornamental Plants and Tea (NIVOT) and the Tea Experiment Station of Kyoto Prefecture. The styles were dissected from the flower buds at the balloon stage, rapidly frozen

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in liquid N₂ and stored at -80 °C until use.

Preparation of protein samples and gel electrophoresis

The stored samples were processed into acetone powder according to the method of Damerval *et al.* (1986). Proteins were extracted from the acetone powder with lysis buffer (O' Farrell 1975) which contains 9.5M urea, 2% (w/v) NP-40, 5% (w/v) PVP-40, 2% Ampholine (pH 3.5-10) and 5% 2-mercaptoethanol. The supernatant was recovered by centrifugation at 15000 rpm for 10 min.

Two dimensional gel electrophoresis (2D-PAGE)

Proteins each of a standardized amount were separated by non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and by SDS-PAGE in the second dimension, as described by Sassa *et al.* (1993). Proteins in the gels were detected by silver staining using the Sil-Best Stain for Protein/PAGE (Nacalai tesque, Japan).

Analysis of amino acid sequence and homology search

Proteins separated by 2D-PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane filter according to Hirano and Watanabe (1990) and detected by Ponceau S staining (Salinovich and Montelaro 1986). PVDF membrane sections carrying major polymorphic basic proteins were cut out and placed in the upper blot cartridge of a reaction chamber in a protein sequencer (476A, Applied Biosystems, USA). Edman degradation was performed according to the standard program obtained from Applied Biosystems. Amino acid sequence homology was analyzed using the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI) non-redundant protein sequence databases (Altschul *et al.* 1990).

cDNA cloning and sequence analysis

Poly(A)⁺RNA was directly isolated from styles of 'Yamato-midori' at the balloon stage of development by using a Quick Prep Micro mRNA purification kit (Pharmacia, Sweden). Complementary DNA was synthesized from the Poly(A)⁺RNA with cDNA synthesis kit (Pharmacia). It was ligated with λ ZAPII/*Eco*RI/CIAP vector, packaged *in vitro* with Gigapack III Gold packaging extract (Stratagene, USA) and plated on *Escherichia coli* strain XL1-Blue for constructing the cDNA library.

From the poly(A)⁺RNA, cDNA was also synthesized and used as a PCR-template with an oligo-d(T) primer containing M13-20 sequence (5'-GCTCTAGACACGACGTTGTAAAACGACGGCCAGT(17)-3') and Super ScriptII Reverse Transcriptase (Gibco BRL, USA). Using the prepared cDNA, PCR was performed with a set of PCR primers, i.e., an oligonucleotide (5'-GAY/CAY/CCN/ATH/GCN/GCN/MGN/TGG/GTN/CC-3') derived from the N-terminal amino acid sequence as the forward

primer and an oligonucleotide of M13-20 sequence (3'-TGACCGGCAGCAAAATG-5') as the reverse primer. The PCR was conducted with Taq polymerase for 27 cycles of denaturation for 70 sec at 94 °C, annealing for 2 min at 46 °C and extension for 1 min at 72 °C, followed by a final extension for 3 min. The PCR product was cloned by using a TA cloning kit (Invitrogen, USA). The obtained PCR products were confirmed to contain a sequence identical with those deduced from the N-terminal amino acid sequence. After digestion with *Eco*RI and *Pst*I, the cloned product was labeled with ³²P using BcaBest DNA polymerase (Takara Shuzo, Japan) and used to screen the cDNA library.

The selected phage clones were converted to pBlue-script plasmids according to the manufacturer's protocol (Stratagene). The cloned fragments were sequenced by using a Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA). The sequences among PR-1 proteins were compared by using the Genetyx-Mac computer program (Software Development, Japan), which is based on the method of Lipman and Pearson (1985). A genealogical tree of PR-1 proteins was constructed by using Genetyx-Mac, based on the unweighted pair group maximum averages (UPGMA) method.

Northern blot analysis

Total RNA was isolated from petals, anthers, styles at the balloon stage and young leaves of cv. Yabukita by the hot-phenol method (DeVries *et al.* 1988). Twenty μ g of total RNA was run on a 1.5% agarose formaldehyde gel and transferred to Zeta Probe GT membranes (Bio Rad, USA). A radioactive probe was prepared from an insert of the cloned PCR fragment using Klenow Fragment (Takara Shuzo, Japan). Hybridizations were carried out for 20h at 42 °C in 5 \times SSC, 0.1% SDS, 5 \times Denhardt's mix, 0.5 mg/ml denatured salmon sperm DNA and 50% formamide. Filters were washed with 0.1 \times SSC, 0.1% SDS at 45 °C for 40 min and detected with Fujix BAS1000 (Fujix Photo Film, Japan).

Results

Detection of stylar proteins which show enhanced expression at anthesis

The 2D-PAGE profiles of the stylar proteins of "Yabukita", a major variety of tea in Japan obtained four days before anthesis were compared with those obtained one day before anthesis. A group of proteins which were clearly detected only by NEPHGE were identified in the basic part of 2D-PAGE (Fig. 1). They were some proteins with a size of about 18 kDa, and high pI values (around pH 9). The basic stylar proteins of other camellia (*C. japonica*) and tea (*C. sinensis*) varieties were also compared (Fig. 2, 3). In both species, a group of similar proteins showing inter-varietal polymorphism were identified. In camellia varieties, at least three types of proteins were identified (Fig. 2); and the N-terminal

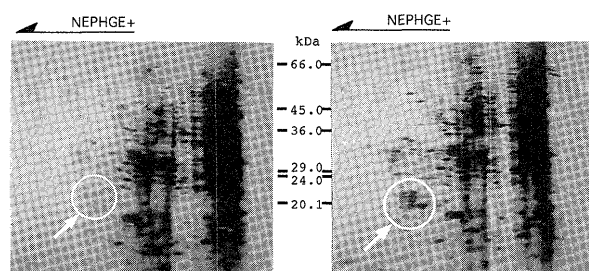


Fig. 1. 2D-PAGE profiles of pistil extract from a tea cultivar, Yabukita. Pistil proteins were separated by NEPHGE/SDS-PAGE and detected by silver staining. The left profile represents proteins extracted four days before anthesis and the right profile those extracted one day before anthesis. The major difference in temporal expression of proteins is marked with a circle and arrow.

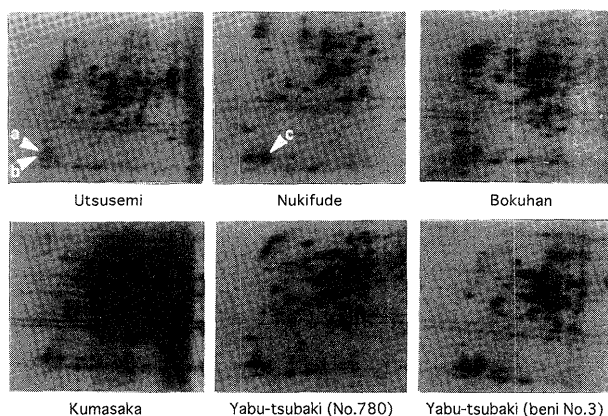


Fig. 2. 2D-PAGE profiles of pistil extracts of *Camellia japonica* cultivars. Pistil proteins were separated by NEPHGE/SDS-PAGE and detected by silver staining. The proteins for which N-terminal amino acid were sequenced are marked with arrowheads.

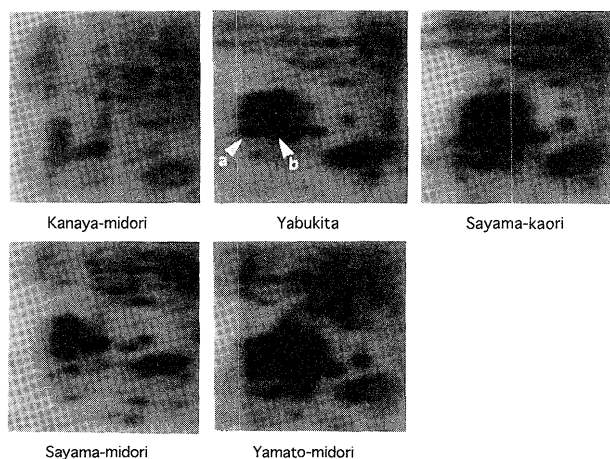


Fig. 3. 2D-PAGE profile of pistil extracts of *Camellia sinensis* cultivars. Pistil proteins were separated by NEPHGE/SDS-PAGE and detected by silver staining. The N-terminal amino acid was determined for the proteins marked with arrowheads.

amino acid sequence of two from 'Utsusemi' (a and b) and one from 'Nukifude' (c) was analyzed. In tea varieties, five types of proteins were identified (Fig. 3); and the N-terminal amino acid sequence of two from 'Yabukita' (a and b) was analyzed. 'Yabukita', 'Sayama-kaori' and 'Yamato-midori' were found to have a similar type of the protein that differed only in pI values. 'Kanaya-midori' and 'Sayama-midori' also were similar in type but with a slightly smaller size. We expected to find a relationship between the polymorphism and self-incompatibility genotypes. However, no relation was found between the migration distances of the protein and the *S*-genotypes. The three varieties, 'Yabukita', 'Sayama-kaori' and 'Yamato-midori' which showed the same patterns have been reported to have different *S*-genotypes ($S^4 S^5$, S^4 or $S^5 S^X$ and $S^2 S^3$, respectively) according to Fuchinoue (1969).

N-terminal amino acid sequence homologous to PR-1

The stilar proteins which were expressed clearly at anthesis were separated by 2D-PAGE and electroblotted onto a PVDF membrane and subjected to analysis of N-terminal amino acid sequence. The three types of proteins in camellia, i. e., the two basic ones from 'Utsusemi' and the acidic one from 'Nukifude', are hereafter referred to as 'camellia a', 'camellia b' and 'camellia c', respectively, and the representative two types in tea i. e., the common basic one and the one with a slightly larger size and middle pI value from 'Yabukita', as 'tea a' and 'tea b', respectively (See Fig. 3). The N-terminus of the proteins of 35 residues of 'camellia a' and 'camellia b', 21 residues of 'camellia c', 19 residues of 'tea a' and 16 residues of 'tea b' was determined in the (Fig. 4). These five proteins were regarded as a protein family with a common function, because they showed perfect homology with each other in the N-terminal 16 residues. Subsequently, from BLAST searches of non-redundant protein sequences, the residues following the 19th residue of N-terminal sequence of 'camellia a' and 'camellia b' were found to have significant identity with internal regions of tobacco PR-1 proteins (Fig. 4). We designated this protein family was designated as *Camellia* PR-1-like-(PRL-1) proteins.

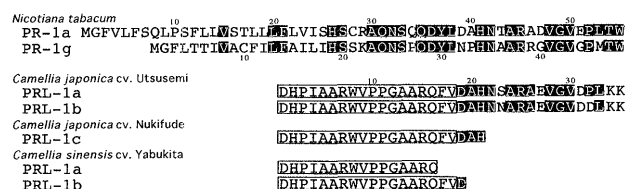


Fig. 4. Alignment of N-terminal amino acid sequences of PR-1 proteins of tobacco (*Nicotiana tabacum*) and PR-1 like protein of camellia (*Camellia japonica*) and tea (*C. sinensis*). Sequence data for *Nicotiana tabacum* are from Ohsima *et al.* (1987) and Payne *et al.* (1989).

cDNA cloning and sequence analysis of PRL-1

To prepare a probe to isolate cDNA clones encoding the *Camellia* PRL-1, PCR was performed using the cDNA which were synthesized with an oligo-d(T) primer containing the M13-20 sequence and poly(A)⁺RNA from pistils at the balloon stage of 'Yamato-midori'. M13-20 and a degenerate oligonucleotide sequence corresponding to the conserved amino acid sequence of the N-terminal region of the *Camellia* PRL-1 protein were used as primers in PCR. A cDNA library was screened using the PCR product as a probe. The nucleotide sequences of cDNA clones encoded an amino-acid sequence that completely matched the N-terminal common sequence of the *Camellia* PRL-1 (Fig. 5). The cDNA clone for the PRL-1 was about 700 bp in size and contained an open reading frame encoding a protein of 191 amino acids. The deduced amino acid sequence of *Camellia* PRL-1 is shown in Fig. 5. The estimated molecular mass(Ms) was 17.7 kDa, and corresponded to that estimated to be 18 kDa by 2D-PAGE. The N-terminal of mature PRL-1 was preceded by a hydrophobic signal peptide region of 36 amino acid residues.

Northern blot analysis for the specificity of expression

10	20	30	40	50	60
GTCCACCTTCTCACAAAATTTCCCTATAAATAACCAATGTCTCTACAAAACAGTCATAAGC					
			M	S	Y
			K	T	V
			I	S	
70	80	90	100	110	120
CAACTACAAGTCCAAGCAATGGCCAAAGTCATACTACCAAGTCTCTCTCTAGTCATATGC					
Q	L	Q	V	Q	A
M	A	K	V	I	L
P	V	L	L	L	V
I	C				
130	140	150	160	170	180
CACAGCTCAACCCATCTCCTAGCTGATCACCCTTGCAGCCCGGTGGGTGCCTCCCGGC					
H	S	S	T	H	L
L	A	D	H	P	I
A	A	R	W	V	P
P	G				
190	200	210	220	230	240
GCTGCCGACAGTTCGTAGACGCACACAACCTCCGCTCGAGCGGAAGTCGGAGTCGACCT					
A	A	R	Q	F	V
D	A	H	N	S	A
R	A	E	V	G	V
D	P				
250	260	270	280	290	300
CTGAAATGGAGCTACAGTCTGGCCAAACCGCGCATCCAGGCTCGTCCCTACCAAAAAAAC					
L	K	W	S	Y	S
L	A	N	A	A	S
R	L	V	R	Y	Q
K	N				
310	320	330	340	350	360
TACATGCATTTCGAGTTTCGCCGACATGACGGTCAGCTGACGACGACCAACCAATG					
Y	M	H	C	E	F
A	D	M	T	G	Q
L	Q	Y	G	S	N
Q	M				
370	380	390	400	410	420
TGGAGCGATTACTCAGCGAAGCCGCCGCTGAGGTGGTGAATACTGGGTGAATAGTGGG					
W	S	D	Y	S	A
K	P	R	E	V	V
E	Y	W	V	N	S
G					
430	440	450	460	470	480
AAGAAACACTATAGATATACGCACAACTATTTGTGTGAGGAATCAGAATTGTGGGCTTAT					
K	K	H	Y	R	Y
T	H	N	Y	C	V
R	N	Q	N	C	G
P	Y				
490	500	510	520	530	540
AAGCAGGTGGTGTGGGAGAAAGACGAGATGGTGGGTTGTGCACAAGGTGTGTGCGGGAAC					
K	Q	V	V	W	E
K	T	E	M	V	G
C	A	Q	G	V	C
G	N				
550	560	570	580	590	600
AACAATGGGAGTCTTAGCATATGCTTTTACTATCCTCATCTGGAAATCTGGGAGGACAA					
N	N	G	S	L	S
I	C	F	Y	Y	P
H	P	G	N	L	G
G	Q				
610	620	630	640	650	660
CGCCCTTACTGATCAAGAGTGTTTTCGTATGTATGTATGGGTGTGTGTGCTGTGTGG					
R	P	Y	*		
670	680	690	700	710	
CAACCATGTTGTCAACCTTAAAAAGCCATGTATGTTGTCTATGGAGAG					

Fig. 5. Nucleotide and deduced amino acid sequences of the cDNA of the PRL-1 of tea. Deduced amino acid residues are shown under the nucleotide sequence. Putative signal peptides are shown in *italics*; DDBJ/EMBL/GenBank accession number is AB015047.

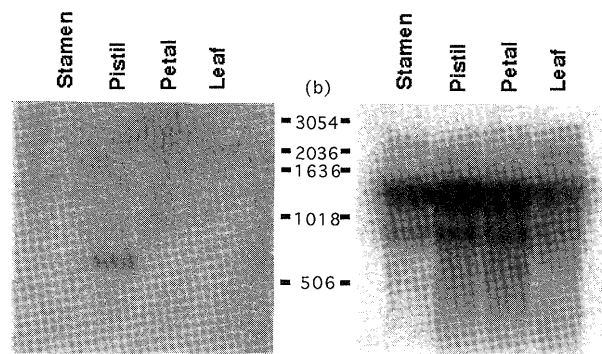


Fig. 6. Northern blot analysis of total RNAs extracted from different organs; stamen, pistil, petal and leaf of tea (cv. Yabukita). Extraction from floral tissues was performed one day before anthesis. A PRL-1 insert (left) and 28S ribosomal RNA sequence from *Ipomoea trifida* (right: control) were used as a probe and each lane contained 20 μ g of total RNA.

To analyze the expression of *PRL-1* gene in different organs of tea, northern blot analysis was performed by using an insert sequence of the cDNA clone as a probe. As shown in Fig. 6, the transcript of *PRL-1* was specifically accumulated in the pistils and no signal was detected in the stamen, petal and leaf.

Comparison of the amino acid sequences of the PRL-1 and the other members of PR-1 family

The amino acid sequence of *Camellia* PRL-1 determined in the present study was compared with those of nine members of the PR-1 family and one particular PR-1-like protein developmentally expressed in styles, STS14 (Fig. 7A). Among these eleven proteins, there was no identity in the putative signal peptides but many common residues were recognized in the regions corresponding to mature peptides, where all the five cysteine residues were conserved. The amino acid sequence identity among them was calculated (Fig. 7B). Among the members of PR-1 expressed in organs other than the flower, amino acid sequence identity ranged from 46.3% (between *S. nigra* PR-1 and *M. truncatula* PR-1) to 77.9% (between *Z. mays* PRms and *H. vulgare* PR-1), but these members had a low level of identity with *Camellia* PRL-1, from 32.9% (with *N. tabacum* PR-1b) to 40.0% (with *M. truncatula* PR-1). It is interesting that a protein of *S. tuberosum* STS 14 which has spatial and temporal expression patterns similar to PRL-1 showed the highest sequence identity, 45.8%, with PRL-1. Sequence identity between STS14 and the members of PR-1 family except PRL-1 was low ranging from 38.6% (with *Z. mays* PRms) to 43.3% (with *A. thaliana* PR-1).

A genealogical tree of these 11 proteins was constructed by the unweighted pair group maximum averages (UPGMA) method (Fig. 8). From this analysis, the pistil-specific PR-1-like proteins and other members of the PR-1 family were classified into different classes.

A

<i>C.sinensis</i> PRL-1	1:-----MS---YKTV-I-----SQLQVQA-MAKVI-LPVLLL--V--ICHSSTHLLAD	37
<i>S.tuberosum</i> STS14	1:MFVLSTAMACLVYIYIYIYDEEKKRELKVRNKMNTNLLFFQFLLLTASSLTHISAQTVPP	60
<i>N.tabacum</i> PR-1b	1:-----MPSF-FLVSTL-L	11
<i>N.tabacum</i> PR-1g	1:-----MG-F-LTTIVACF	11
<i>L.esculentum</i> P14	1:-----MGLFNISLLLTCL	13
<i>A.thaliana</i> PR-1	1:-----MNEMSFFGY-SFIVVALFFDLT-QAYRH	26
<i>B.napus</i> PR-1a	1:-----MKVTNCSRLLLILA	27
<i>S.nigra</i> PR-1	1:-----MAHNHWCNLFVSVALVC	29
<i>Z.mays</i> PRms	1:-----MEASNKLAVL--LLWL	15
<i>H.vulgare</i> PR-1	1:-----MQTP-KLAIL--LA-LA	13
<i>M.truncatula</i> PR-1	1:-----MSFRCFSFALFLLLLTFISHVS	22
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<i>C.sinensis</i> PRL-1	39:HPIAARW--VPPG-AAQOFVDAHNSARAQVGVDPKWSYSLANAASRLVRYQKNYMHCE	94
<i>S.tuberosum</i> STS14	61:PPPPPTSAATPPSRAAQEELDAHNKARSEVGVGPIITWSPMLAKETSLVRYQKRDQNCSE	120
<i>N.tabacum</i> PR-1b	12:LFL-IISHSSHAQNSQDYLDAHNTARADVGVPEITWDNGVLAAYAQNYV--SQLAAD	67
<i>N.tabacum</i> PR-1g	12:ITFAILLHSSKAQNSPDYLNPHNAARQVGVGPMITWDNRLAAYAQNY--ANQRIGD	68
<i>L.esculentum</i> P14	14:MVLAIF-HSCEAQNSPDYLAHNDARAQVGVGPMISWDANLASRAQNY--ANSRAGD	69
<i>A.thaliana</i> PR-1	27:TPAQPPKANANGDVKPEQLTVVHNKARAMVGVGPMVNETLAAYAQSY--AHERARD	83
<i>B.napus</i> PR-1a	28:ALVGALVHPSKAQSDPDYVNAHNOARQAVGVGPMVQMDGTLAAYAQNY--ADRLRGD	71
<i>S.nigra</i> PR-1	30:VVALVMVQYSVAQNSPDYVDAHNAARSAVNVGCVTWDSEVLAFAAQY--AASRAGD	73
<i>Z.mays</i> PRms	16:MAAATAVHPSYSSENSPDYLTPONSARAQVGVGPMVTSKLOQFAEKY--AAQRAGD	72
<i>H.vulgare</i> PR-1	14:MAAA-MVNLSQAQNSPDYVSPHNAARSAVGVGAVSWSTKLOQFAEKY--ANQRIND	69
<i>M.truncatula</i> PR-1	23:ASYIPNKKSFKRSRKFNOELIPONIRAAVGLRPLVWDDKLTHTYAQWY--ANQRRND	79
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<i>C.sinensis</i> PRL-1	96:ADMTQQLQYGSNOMW-SDYSAKPPREVVEYVWNSGKRHYRYTHNYCVRNONGCPYKQVW	153
<i>S.tuberosum</i> STS14	121:ANLSNG-KYGGNOLW-ASGTVVTPRMAVDSWV-AEKKFYNNENNSCTGDDKCCVYTOIVW	177
<i>N.tabacum</i> PR-1b	68:LVHSHG-QYGENLAQGS-GDFMTAAKAVEMWV-DEKQYMDHDSNTCAQGVCGHYTOVW	125
<i>N.tabacum</i> PR-1g	69:MIHSHG-PYGENLA-AAFPQLN-AAGAVKMTV-DEKRFYDINSNSCVGG-VCGHYTOVW	124
<i>L.esculentum</i> P14	70:LIHS-G-A-GENLAKG-GGDF-TGRAAVQLWV-SERPSTNYATNOCVGGKKCRHYTOVW	124
<i>A.thaliana</i> PR-1	84:MKHSLG-PFGENLAAG-WGT-MSGPVATPYWM-TERENYDSDNTCGDGVCGHYTOIVW	139
<i>B.napus</i> PR-1a	72:LIHSDG-PYGENLA-GSSADFSGVSAVNLWVN-EKANYNHDSNTCNGE--CLHYTOVW	125
<i>S.nigra</i> PR-1	75:LVHSCDPYGENLAFSGFEL-TGRNAVDMWV-AERNDYNPNTNTCAPGKVCGHYTOVW	131
<i>Z.mays</i> PRms	73:LQHSQG-PYGENIFWGSAGFDWKAVDARSWV-DEKQYNYATNSCAAGKVCGHYTOVW	130
<i>H.vulgare</i> PR-1	70:LQHSQG-PYGENIFWGSAGADWKASDAVNSWV-SEKKDYDYGSTNTCAAGKVCGHYTOVW	127
<i>M.truncatula</i> PR-1	80:LEHSNG-PYGENIFWGS-GVGWNPAAQVSAWV-DEKQFYNYWHNSCVDGEMCGHYTOVW	136
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<i>C.sinensis</i> PRL-1	155:EKTEMVGAQGVCGNNNCSLSICFYYPHPCNLGCGRPY-----	191
<i>S.tuberosum</i> STS14	178:KKSIELGCAQRTCYEGPATLTVCFYNP-PGNVICCKPY-----	214
<i>N.tabacum</i> PR-1b	126:RNSVRVGCAR-VKCNNGCYVVSCHNYDE-PGNVICGSPY-----	160
<i>N.tabacum</i> PR-1g	125:RNSVRLGCAR-VRSNNGWFFITCNHYDE-PGNVICGPFGLDLEEQPFDKLELPTDV	177
<i>L.esculentum</i> P14	125:RNSVRLCGGRARC-NNGWFFISCHNYDE-VGNWICGOPY-----	159
<i>A.thaliana</i> PR-1	140:RDSVRLGCASVRCKNDEYIWWITCSYDE-PGNYICGOPY-----	176
<i>B.napus</i> PR-1a	126:RKSVRICCGKARC-NNGTIISCHNYDE-RCNYVNEKPY	161
<i>S.nigra</i> PR-1	132:RNSVRICGARVRC-NNGAWFITCNYS-PGNYACGOPY	167
<i>Z.mays</i> PRms	131:RAITTSICGARVVCNDRGVFITCNHYE-RCNIIAGMKPY-----	167
<i>H.vulgare</i> PR-1	128:RASTSIICGARVVCNDRGVFITCNHYE-RCNIIAGMKPY-----	164
<i>M.truncatula</i> PR-1	137:GSTTKVGCASVCSDDKCTFMTCNHYDE-PGNYICGOPY-----	173

Fig. 7. Comparison of the derived amino acid sequence of PRL-1 with PR-1 and PR-1 like proteins from plants. A. Multiple alignment of the sequences. Deduced transit and signal peptides are shown in *italics*. Gaps are marked by *dashes*. *Black boxes* indicate residues where the genes show the identical sequence with PRL-1. Amino acid residues which are conserved in all cases are marked with *asterisks*.

Because of the sequence similarity, these PR-1-like proteins form a new class in the PR-1 family that is distinct

from PR-1 proteins expressed in organs other than the flower.

B

	PR-1 like protein	PR-1 family proteins								
	ST STS14	NT PR-1b	NT PR-1g	LP P-14	AT PR-1	BN PR-1a	SN PR-1	ZM PRms	HV PR-1	MT PR-1
CS PRL-1	45.8	38.5	32.9	33.3	34.0	32.6	34.3	35.4	36.8	40.0
ST STS14		41.1	40.4	39.0	43.3	39.7	40.4	38.6	39.4	42.4
NT PR-1b			65.2	60.1	60.1	59.4	62.6	52.9	57.1	50.7
NT PR-1g				65.7	62.5	58.1	62.9	54.6	61.4	53.3
LP P-14					59.9	59.9	64.0	51.4	55.0	46.7
AT PR-1						79.3	63.3	55.0	57.1	50.4
BN PR-1a							59.7	52.1	56.4	48.1
SN PR-1								58.9	61.7	46.3
ZM PRms									77.9	59.6
HV PR-1										55.1

Fig. 7. (continued) B. Sequence similarities excluding transit and signal peptides in percent similarity, calculated by the method of Lipman and Pearson (1985) using the computer program Genetyx. Sequence data included in the alignment and calculation are as follows: *Solanum tuberosum* STS14 (Van Eldik *et al.* 1996); *Nicotiana tabacum* PR-1b (Pfitzner *et al.* 1987); *N. tabacum* PR-1g (Payne *et al.* 1989); *Lycopersicon esculentum* P14 (Tornerio *et al.* 1993); *Arabidopsis thaliana* PR-1 (Uknes *et al.* 1992); *Brassica napus* PR-1a (Hanfrey *et al.* 1996); *Sambucus nigra* PR-1 (Coupe *et al.* 1997); *Zea mays* PRms (Casacuberta *et al.* 1991); *Hordeum vulgare* PR-1 (Muradov *et al.* 1993); *Medicago truncatula* PR-1 (Szybiak-Strozycka *et al.* 1995).

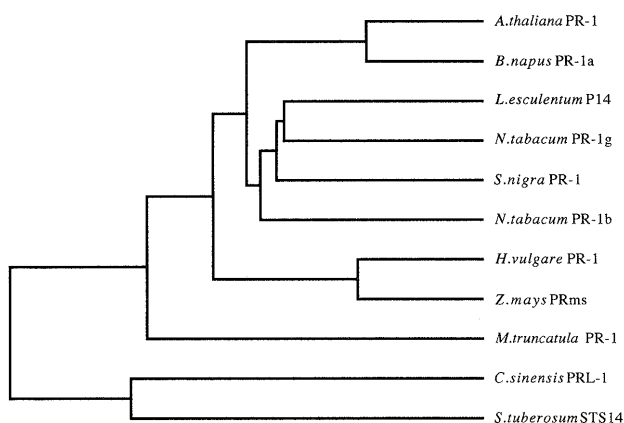


Fig. 8. Genealogical tree of the mature PR-1 and PR-1 like proteins in plants. The genealogy was constructed using the unweighted pair group maximum averages (UPGMA) method on the basis of the alignment showed in the Fig. 7A. Horizontal branch length reflects the difference between the proteins. Designations are as in Fig. 7.

Discussion

We identified a group of major proteins which were endogenously expressed in *Camellia* pistils with a high level of expression at anthesis. This group of proteins with a relatively high pI of around pH 9 and low Ms values of 18 kDa were found in 2D-PAGE profiles of protein extracts from one day before anthesis in all the varieties of *Camellia* examined with inter-varietal polymorphism. Their spatial expression was confirmed to be specific to the pistil by northern blot hybridization.

Thus, this group of proteins was considered to play a role in the anthesis-specific events in pistils. However, no relationship between these proteins and self-incompatibility genotypes was found.

The entire amino acid sequence of one of these proteins was determined based on the nucleotide sequence of the corresponding cDNA clone. In the 2D-PAGE profiles and N-terminal amino acid sequence there were a group of proteins with slightly different pI and Ms values. At least two different N-terminal sequences were found in a camellia variety, Utsusemi. However, only one whole sequence was obtained from the sequence analysis of the cDNA clones in the cDNA library constructed from the pistil Poly(A)⁺RNA. Perhaps the number of sequenced clones was insufficient.

The endogenously expressed pistil-specific protein showed the highest homology with STS14, a PR-1-like protein found in pistils of *Solanum tuberosum* (Van Eldik *et al.* 1996) as shown in Fig. 7, where the predicted protein sequence is presented in comparison with the sequences of STS14 and nine other PR-1 proteins of tobacco, *Arabidopsis thaliana*, barley, maize, tomato and *Medicago truncatula*. The protein from *Camellia* pistils showed the highest homology with the sequence of STS14 over the entire sequence (Fig. 7). Although this PR-1-like protein has a sequence homology with PR-1 proteins which are induced by pathogens as well as stress conditions such as those provoked by pathogens, this protein is endogenously induced in the healthy pistil even without infection. Therefore, this protein can not be called a pathogenesis-related protein according to the nomenclature by Van Loon *et al.* (1994). Thus, this protein was designated as *Camellia* PR-1-like (PRL-1).

protein.

The present findings indicate that a group of proteins homologous to PR-1 proteins expressed in pistils at anthesis may be distributed in many plants families, and that these pistil-specific PR-1-like proteins form a new class in the PR-1. In this context it is interesting that there are some other PR-like genes which are also expressed in stigma or styles. A type of PR-2 protein, Sp41 has been reported to be localized in the transmitting tissue of tobacco pistils (Sessa and Flur 1995).

The direct function of defense-related proteins in pistil has not yet been established in reproductive physiology. There are a number of events in the process from pollination to fertilization, and a series of proteins may be involved in guiding pollen tubes as suggested from the fact that pollen tubes of *Arabidopsis* can grow within the cortical tissue at very early stages of pistil development (Kandasamy *et al.* 1994). There is a possibility that the PRL-1 identified to be a major protein at anthesis may be one such molecule guiding the pollen tube during fertilization. Further research is needed to determine their function in the pistils.

Although the PR-1 proteins have been identified as major proteins induced by fungal or viral invasion, their functions as well as enzymatic activity are not yet clear. Recently, PR-1 proteins have been reported to have an anti-fungal function against *Phytophthora infestans* (Niderman *et al.* 1995). In an alignment study, PR-1 from various plants and vespid venom antigen 5 showed significant similarity to a part of specific granule proteins of 28 kDa (SGP28), which was purified from exocytosed material from human neutrophils and found to be highly similar to the deduced amino acid sequences of the cDNA of the testis-specific human gene TPX-1 and of an sperm coating glycoprotein (SCG) from mouse (Kjeldsen *et al.* 1996). This alignment study also indicated that an amino-acid sequence, GHYTQVVW is well preserved throughout the PR-1 like proteins of plants and animals, and suggested an important function of this sequence. A similar sequence, GPYKQVVW, was also observed in the residues 145–153 of PRL-1 (Fig. 7 A). Cysteine is abundant in the sequence just before and after those residues. The cysteine-rich sequence is indicated by the alignment study as a characteristic feature of several natural antibiotic peptides. The fact that PRL-1 and STS14 from the pistil of potato have a homology with PR-1 proteins indicates that they may have a function to protect the pistils against pathogen attack or to help penetration by pollen of the extracellular matrix in the style.

From the view point of gene induction, salicylic acid-induced expression of PR-1 genes has been confirmed and related to some promoter regions (Hagiwara 1993). Since the pistil-specific PR-1-like proteins are expressed during the course to anthesis, their *cis* elements may show specificity for such an endogenous induction.

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