Isolation and Characterization of cDNAs Expressed in the Early Stages of Flavonol-Induced Pollen Germination in Petunia[1]

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Petunia (*Petunia hybrida*) pollen requires flavonols (Fl) to germinate. Adding kaempferol to Fl-deficient pollen causes rapid and synchronous germination and tube outgrowth. We exploited this system to identify genes responsive to Fls and to examine the changes in gene expression that occur during the first 0.5 h of pollen germination. We used a subtracted library and differential screening to identify 22 petunia germinating pollen clones. All but two were expressed exclusively in pollen and half of the clones were rare or low abundance cDNAs. RNA gel-blot analysis showed that the steady-state transcript levels of all the clones were increased in response to kaempferol. The sequences showing the greatest response to kaempferol encode proteins that have regulatory or signaling functions and include S/D4, a leucine-rich repeat protein, S/D1, a LIM-domain protein, and D14, a putative Zn finger protein with a heme-binding site. Eight of the clones were novel including S/D10, a cDNA only expressed very late in pollen development and highly up-regulated during the first 0.5 h of germination. The translation product of the S/D3 cDNA shares some features with a neuropeptide that regulates guidance and growth in the tips of extending axons. This study confirmed that the bulk of pollen mRNA accumulates well before germination, but that specific sequences are transcribed during the earliest moments of Fl-induced pollen germination.

Pollen develops within the anther and requires the products of both sporophytic gene expression, arising from the tapetal layer of the anther wall, and gametophytic gene expression, arising from the vegetative and generative nuclei. Pollen is released from the anther in a dehydrated and metabolically quiescent state. When it falls on a receptive stigma, pollen rapidly hydrates and activates the stored RNA, protein, and bioactive small molecules that allow rapid germination and tube outgrowth. Pollen tube growth is extremely rapid (1 cm/h in maize) and highly polar; growth is restricted to a small area at the tip of the tube (for review, see Taylor and Hepler, 1997).

Flavonols (Fl) are plant-specific compounds that are required for pollen germination and tube growth in maize and petunia (*Petunia hybrida*; Mo et al., 1992; Taylor and Jorgensen, 1992; Ylstra, 1994). Fl-deficient plants are self-sterile because the pollen fails to germinate (Mo et al., 1992; Taylor and Jorgensen, 1992) or to produce a functional tube (Ylstra, 1994; Pollak et al., 1995). These defects are conditional: When Fl-deficient pollen was placed on wild-type stigmas, the pollen germinated and seeds were produced (Taylor and Jorgensen, 1992; Vogt et al., 1994; Ylstra, 1994), a phenotype defined as conditional male fertility (CMF) by Taylor and Jorgensen (1992). The bioactive compound from the stigma exudate was identified as kaempferol, a Fl aglycone (Mo et al., 1992; Vogt et al., 1994). CMF pollen can be complemented (pollen rescue) with exogenously added Fls. The response is rapid (tube outgrowth detected within 5 min), sensitive (maximum germination at 0.4 μM), and specific for Fl aglycones (Mo et al., 1992; Ylstra, 1994; Vogt et al., 1995).

It is generally accepted that the mRNA required for pollen germination is present in mature pollen. Several observations contribute to this view, including the rapidity of germination, the fact that most of the pollen sequences isolated to date are expressed before germination (for review, see Taylor and Hepler, 1997), and early studies showing that maize and *Tradescantia* pollen germinated in the presence of actinomycin D, a RNA synthesis inhibitor (for review, see Mascarenhas, 1993). Significantly, the early studies also demonstrated that mRNA synthesis did occur during the first moments of germination and tube outgrowth. One study estimated that during the 1st h of pollen tube growth, approximately 50% of protein synthesis used newly transcribed mRNA templates (Mascarenhas and Mermelstein, 1981). However, attempts to determine if the newly synthesized mRNA was unique to germination yielded inconclusive results because the experimental approach

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lacked the sensitivity to detect rare or moderately rare transcripts (for review, see Mascarenhas, 1993).

Of the estimated 20,000 genes that are expressed during pollen development, about 10% are considered to be pollen-specific (Willing and Mascarenhas, 1984; Willing et al., 1988). Many of the pollen-specific genes are transcriptionally activated following pollen mitosis I; some of these mRNAs are stored to be translated later, during germination and early tube growth (Mascarenhas, 1990). Most gene isolation efforts have focused on the developing pollen grain (for review, see Taylor and Helper, 1997) and little is known about the identity of genes that are expressed at germination. Here, we report on experiments to determine whether the genetic program that exists during pollen maturation is identical to that used during germination and early tube growth. We exploited the rapid and synchronous in vitro germination response of CMF pollen to exogenous kaempferol (Mo et al., 1992) to isolate a group of petunia germinating pollen (PGP) cDNAs, including eight novel sequences that responded to a Fl signal during the earliest moments of pollen germination.

RESULTS

Isolation of cDNAs Expressed in Kaempferol-Induced Germinating Pollen

Before embarking on this study, we confirmed that mRNA synthesis takes place during the first 0.5 h of kaempferol-induced pollen germination by measuring the incorporation of [^{3}H]uridine into nascent, poly(A$^+$) transcripts (Y.Y. Mo and L.P. Taylor, unpublished data). Our selection scheme as outlined in Figure 1 was designed to maximize the identification of genes expressed during early germination in response to kaempferol, and to minimize the isolation of sequences involved in maintaining tube growth. After the construction and screening of a subtracted cDNA library of +Fl-enriched clones, differential screening #1 was performed to enrich for cDNAs expressed during the first 0.5 h in Fl-induced germinating pollen. From this screen, 72 subtracted and differentially screened (S/D) clones were selected that hybridized with the [+Fl 0.5 h] probe from germinating pollen and not with the [−Fl 0.5 h] probe from hydrated but non-germinating pollen. To isolate sequences that represent rare transcripts (Hodge et al., 1992), subtracted cDNA clones that hybridized with neither the [+Fl 0.5 h] nor the [−Fl 0.5 h] cDNA probe were selected and designated non-hybridizing (S/NH). In addition, differential screening of the +Fl cDNA library with the [+Fl 0.5 h] and [−Fl 0.5 h] probes generated 38 differentially screened (D) clones.

To search for transcripts that specifically responded to Fl, a second differential screening was devised that relied on the fact that in addition to kaempferol, CMF pollen requires boron to germinate (Fig. 1, differential screening #2). By excluding boron in the form of boric acid (Bo) from the germination medium (GM), the effect of kaempferol on pollen gene expression could be determined in the absence of germination. A total of 110 differentially expressed S/D and D cDNA clones were differentially hybridized to cDNA probes from kaempferol-treated pollen in GM lacking Bo [+Fl 0.5 h, −Bo] and from kaempferol-treated germinating pollen [+Fl 0.5 h]. A total of 14 S/D and D clones were identified that were expressed at high levels in kaempferol-induced, non-germinating pollen. Together with the eight S/NH clones, they comprise the 22 PGP cDNA clones that were analyzed in detail (Table I).

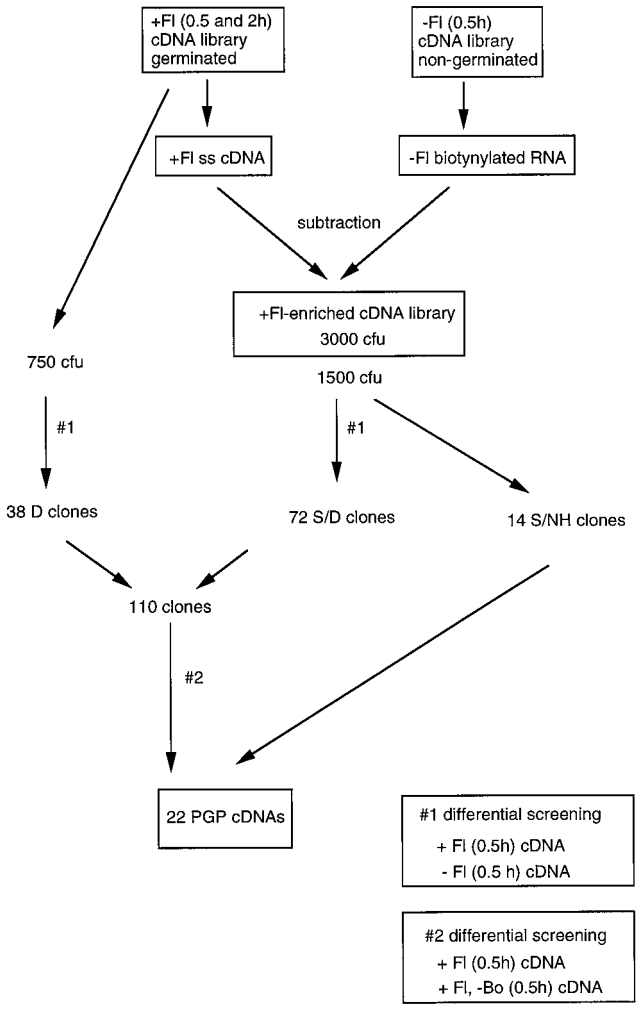

Figure 1. Isolation scheme to recover PGP cDNAs differentially expressed in kaempferol-induced germinating pollen.

Differential Expression of PGP cDNA Clones during Pollen Germination

RNA gel-blot analysis was used to confirm that the expression of all the PGP cDNA clones increased during the first 0.5 h of Fl-induced germination. The results presented in Figure 2 show that a significant

Table I. *PGP Clones*

Clone	Sequence Identity[a]	Fold Kaempferol Increase[b]	Fold Germination Increase[c]	cDNA Insert (p = partial)	Transcript	Abundance[d] (1 = high, 4 = low)	First Report in Pollen	GenBank Accession No.
				bp	*kb*			
S/D1	LIM domain	9.0	0.5	897	0.9	2	No	AF049917
S/D2	Novel	3.3	0.8	650	0.8	3	Yes	AF049918
S/D3	Novel (neuromodulin); E, A, K rich	1.9	0.9	591	0.6	1	Yes	AF049919
S/D4	LRR, PGIP	7.6	0.6	1,346	1.35	3	Yes	AF049920
S/D5	Arabidopsis protein	2.3	1.9	604	2.0	4	Yes	AF049921
S/D6	ER lumen protein receptor	3.3	0.9	927	0.95	2	Yes	AF049922
S/D7	Novel; S, A, T rich	3.7	0.7	868	0.9	2	Yes	AF049923
S/D8	Cytochrome *c* oxidase	2.0	0.9	521	0.5	3	Yes	AF049924
S/D9	Novel	1.6	1.6	625p	1.2	4	Yes	AF049925
S/D10	Novel; S rich	3.9	0.9	665p	1.25	4	Yes	AF049926
S/D11	AGP-like; A, S, P rich	3.0	0.6	647	0.65	1	Yes	AF049927
S/D12	Arabidopsis ovary EST	1.5	0.9	782	0.8	1	Yes	AF049928
D13	Pectinesterase	ND[e]	ND	423p	ND	ND	No	AF049929
D14	Heme binding, yeast protein	5.2	1.4	1620	1.65	4	Yes	AF049930
S/NH15	Ascorbate oxidase	4.4	1.6	725p	2.2	1	No	AF049931
S/NH16	Novel	ND	ND	620	ND	4	Yes	AF049932
S/NH17	Protein transport to ER	6.7	0.4	342p	0.6, 0.8	4	Yes	AF049933
S/NH18	Novel	3.6	2.1	443p	1.7	2	Yes	AF049934
S/NH19	Polygalacturonase	3.8	1.5	416p	1.2	2	No	AF049935
S/NH20	Profilin	4.1	1.1	629	0.65	4	No	AF049936
S/NH21	Zn metallothionein	2.3	3.3	773	0.75	3	No	AF049937
S/NH22	Novel, S rich	4.8	0.8	790p	1.0	4	Yes	AF049938

[a] The cut off for probability of homology occurring by chance, P(N) was $<10^{-10}$. [b] Calculated as the ratio of the [−Bo, +F1] hybridization signal to the [−F1] signal from Fig. 2. [c] Calculated as the ratio of the [+F1] to the [−Bo, +F] hybridization signal from Fig. 2. [d] Autoradiogram exposed for less than 2 h = 1; 2 to 12 h = 2; 12 to 24 h = 3; and 24 to 72 h = 4. [e] ND, Not determined.

increase was measured for S/D4, S/D6, S/NH15 and S/NH20, whereas S/D3, S/D8, S/D11 and S/D12 showed a moderate increase in steady-state transcript levels (Fig. 2, compare [-Fl 0.5 h] lane with [+Fl 0.5 h] lane). For some cDNAs, transcript abundance was also determined after 2 h of germination (Fig. 2, [+Fl 2 h] lane) when the pollen tube length is four times the pollen diameter. This time point is still considered early in pollen tube growth, since in petunia, sperm cells require more than 24 h to reach the ovule. S/D1, S/D3, S/D4, S/D7, S/D11, and S/NH17 transcripts continued to accumulate 2 h after germination suggesting that they may also be involved in tube growth or post-germination interactions.

In addition to Fls, some of the PGP transcripts may respond to other signals during the burst of metabolic activity that accompanies germination. Therefore, to specifically measure the effect of Fls on the steady-state levels of PGP mRNAs, we compared transcript accumulation in two populations of non-germinating pollen: one supplemented with kaempferol and the other without. Bo was excluded from the kaempferol-supplemented GM to prevent germination. Figure 2 shows that all of the PGP cDNA clones showed a substantial increase in transcript accumulation in response to kaempferol treatment (compare [−Fl 0.5] and [−Bo, +Fl 0.5 h] lanes).

There are reports that boron may interact with Fls (for review, see Stanley and Linskens, 1974). To exclude the possibility that the Fl-response in the previous analysis was compromised by a lack of Bo, we repeated the experiment in the presence of Bo and withheld calcium from the GM to prevent germination [−Ca, +Fl 0.5 h]. All but one of the tested cDNAs showed comparable levels of kaempferol upregulation in the absence of either calcium or Bo. The exception, D14, showed a 20-fold increase of mRNA in the absence of calcium compared to expression in the absence of Bo. Although both calcium and Bo are required for pollen germination, it is well established that intracellular free calcium plays a key role in the regulation of pollen tube growth (for review, see Taylor and Hepler, 1997), whereas the function of boron is unknown.

We calculated the kaempferol effect and the germination effect on the steady-state levels of PGP mRNAs from densitometric scans of the hybridization signals shown in Figure 2, after correcting for loading differences. The kaempferol effect was determined as the ratio of the [−Bo, +Fl] signal to the [−Fl] signal. The values reported in Table I indicate that our cloning strategy successfully selected for transcripts that respond to a Fl signal during the first 0.5 h of pollen germination. The strongest response to kaempferol, a 5- to 9-fold increase, was measured for five PGP transcripts (S/D1, S/D4, D14, S/NH17, and S/NH22). Nine additional clones (S/D2, S/D6, S/D7, S/D10, S/D11, S/NH15, S/NH18, S/NH19, and S/NH20)

showed a 3- to 4.4-fold induction by kaempferol treatment. The ratio of the [+Fl] signal to the [−Bo, +Fl] signal measured the change in PGP transcript accumulation that accompanied germination. A ratio of less than one for the germination effect indicates that virtually all of the increase in steady-state mRNA for these clones can be attributed to kaempferol. In addition to a kaempferol effect, clones, S/D5, S/D9, S/NH18, and S/NH21 also showed a significantly increased expression in response to germination.

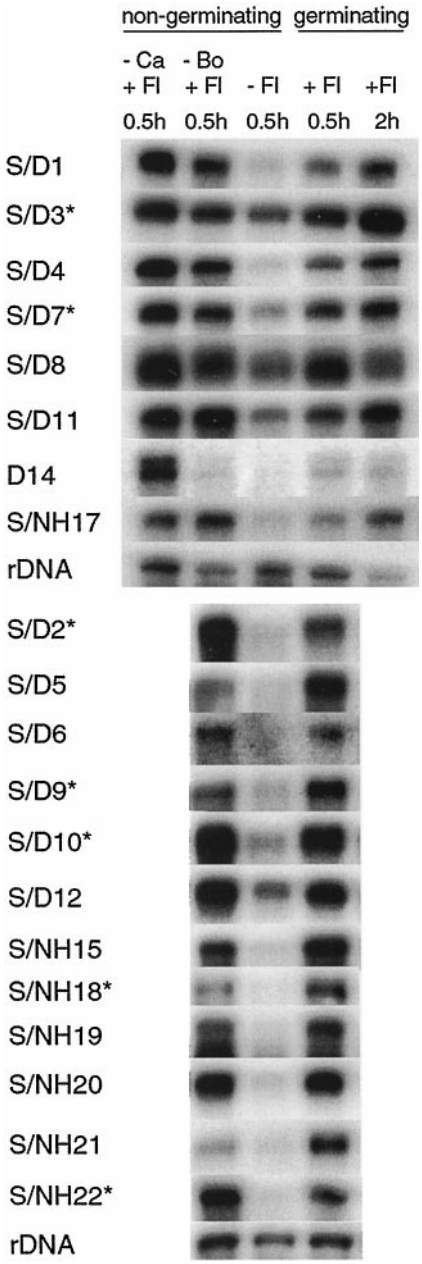

Figure 2. Differential expression of PGP transcripts in germinating and non-germinating pollen. RNA gel-blot analysis of 1 μg of poly(A^+)-enriched RNA isolated from CMF pollen incubated in GM supplemented as follows: [−Fl], No added kaempferol; [+Fl 0.5 h] and [+Fl 2 h], 0.5 μM kaempferol for 0.5 h and 2 h; [−Bo, +Fl] and [−Ca, +Fl], 0.5 μM kaempferol for 0.5 h lacking Bo or calcium. Novel sequence cDNA clones are marked with an asterisk.

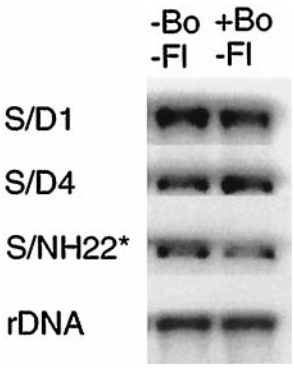

Figure 3. Effect of boron (as Bo) on hydrated pollen. RNA gel-blot analysis of 1 μg of poly(A^+)-enriched RNA isolated from CMF pollen incubated in GM for 0.5 h and supplemented as follows: [−Bo, −Fl] and [+Bo, −Fl].

To confirm that the Bo in the GM of the noninduced [−Fl] pollen sample did not affect expression of the PGP genes, three of the cDNAs showing the greatest response to kaempferol (Table I) were hybridized to RNA extracted from hydrated pollen, with or without Bo in the GM. In Figure 3, the hybridization signal shows that S/D1, S/D4, and S/NH22 are expressed at the same levels in the two pollen populations (compare lanes [−Bo, −Fl] and [+Bo, −Fl]). Thus, we feel confident that the increase reported in Table I accurately represents a response to kaempferol alone.

The abundance of PGP transcripts varied considerably: RNA gel blots were exposed for 15 min (S/D12) to over 4 d (S/D6; Table I). S/NH16 gave a faint signal in kaempferol-treated pollen with or without Bo treatment, but no signal was detected after 4 d of exposure in the absence of both kaempferol and germination (data not shown). From the intensity of the hybridization signals and the cold plaque selection (Hodge et al., 1992), we propose that classes 3 and 4 in Table I represent rare to moderately rare transcripts.

Tissue and Stage-Specific Expression of PGP Transcripts

The pattern of PGP gene expression during pollen development, and the tissue-specificity of each cDNA clone, was determined by RNA gel-blot analysis as shown in Figure 4. Total RNA was extracted from both wild-type (V26) and Fl-deficient (CMF) anthers at different stages of development and from different floral and vegetative tissues. None of the PGP cDNAs isolated from germinating pollen hybridized to RNA from young leaves. Two clones, S/NH17 and S/NH19, were expressed in the pistil, and S/NH17 and D14 transcripts were detected in the corollas.

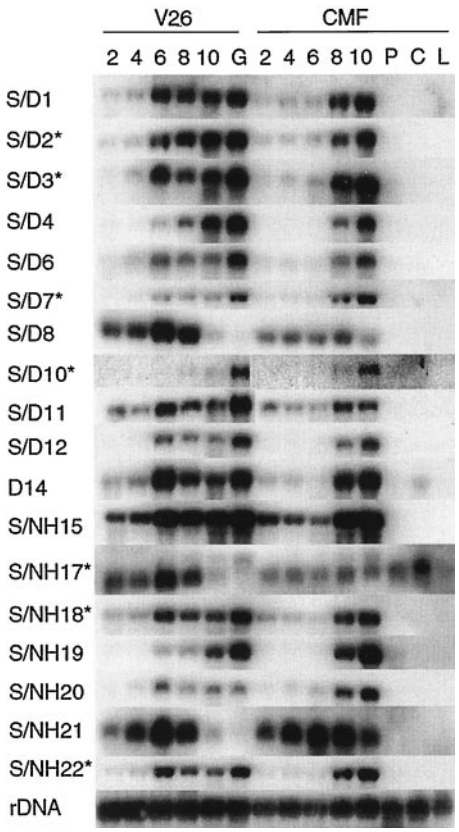

Figure 4. Stage- and tissue-specific expression of PGP transcripts. Total RNA was extracted from wild-type (V26) and mutant (CMF) petunia anthers at developmental stages 2 to 10 and from V26 pollen germinated in vitro for 0.5 h (G). RNA from CMF tissues: P, pistil from stage 9 flowers; C, corolla; L, young leaves. Novel sequence cDNA clones are marked with an asterisk.

A stated objective of this study was to determine if unique transcripts were expressed during germination that were not present earlier in development. Figure 4 shows that, with one exception, all of the PGP transcripts accumulated well before germination. The exception, S/D10, was transcribed at barely detectable levels in mature, wild-type anthers (V26 at S10). Upon pollen release from the anther, a 100-fold increase in S/D10 mRNA was measured in the first 0.5 h of pollen germination (Fig. 4, V26). Compared to the expression pattern of all the other pollen genes examined to date, S/D10 shows the most dramatic effect of germination upon transcript accumulation. S/D10 is represented in the low abundance class of transcripts, and sequence analysis (following section) revealed that it encodes a novel protein.

Two patterns of PGP transcript accumulation during pollen maturation and germination were evident. The most common pattern, exhibited by clones S/D1, S/D2, S/D4, S/D6, S/D7, S/D12, D14, S/NH18, S/NH19, S/NH20, and S/NH22, showed no, or very low, levels of transcript accumulation early in development. Beginning at stage 6 (stage 8 for S/D4 and stage 10 for S/NH19), expression increased dramatically and continued increasing until maturity. Petunia pollen undergoes pollen mitosis I around stage 6, thus these sequences would be classified as late genes as defined by Mascarenhas (1990). PGP clones S/D8, S/D11, S/NH15, S/NH17, and S/NH21 exhibited a second pattern that was characterized by an early onset of gene expression (stage 2, corresponding to uninucleate microspores in wild-type plants). Transcripts S/D11 and S/NH15 continued to increase through development and germination, but the expression of S/D8, S/NH17, and S/NH21 decreased dramatically in the final stages of maturity and in germinating wild-type pollen. However, in CMF pollen, expression of these cDNAs followed a biphasic pattern: it declined in maturing pollen similar to wild type, but increased upon Fl treatment and germination (Fig. 2). Clone S/NH17 detected two transcripts that differed in their temporal and tissue-specific expression. The smaller one showed pollen-specific expression and reached its maximum accumulation during germination. The larger S/NH17 transcript was detected in the pistil, the corolla, and immature pollen. It was not expressed at germination.

CMF anthers showed delayed accumulation of PGP cDNA transcripts when compared to wild type (V26). The difference is unlikely to be a manifestation of Fl-deficiency in the CMF pollen, since prior comparisons did not detect asynchronous development of the two lines (Taylor and Jorgensen, 1992). It is most likely due to environmental effects; it has been reported that the correlation between microspore development and bud length is easily altered by external factors (Thurling and Chay, 1984). The CMF plants were grown in a cooler environment than V26 and for an equivalent bud length, the pollen lagged two developmental stages behind wild type.

The developmental expression pattern shown in Figure 4 was performed with RNA isolated from anthers and it is possible that expression in the early stages of development could originate from the tapetal layer of the anther wall. The tapetum begins a programmed cell death in stage 2 anthers and microscopic inspection reveals it is absent by stage 4. In addition, petunia pollen can be easily separated from the anther beginning at stage 6. Therefore, RNA gel blots were prepared with RNA extracted from pollen isolated at stages 6, 7, 8, 9, and 10 and hybridized with selected PGP clones. Precisely the same pattern of expression was obtained as in Figure 4 (data not shown), suggesting that the hybridization signal in

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  1  MTNSQRDIQR AQEIFKEEMG YLRYVLGIQI IYTMCTSTSL SMAMENDSAI
 51  APSSSQDDSE MAPSSSQDDI AMAPSSSQDD SAMAPSSSQD DHIVLCVRDC
101  MRICMKLDDA TSTECEEACR TGCRPLLARK EISALHSDDN SSMILTDKNN
151  PTNKPGEFI
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Figure 5. The amino acid sequence of S/NH22 contains 8-mer repeats (underlined) that are predicted to form a disordered loop.

the early stages (S2 and S4) originates from the pollen and not the tapetum.

Sequence Analysis of PGP cDNAs

Novel Sequences

Eight PGP sequences (S/D2, S/D3, S/D7, S/D9, S/D10, S/NH16, S/NH18, and S/NH22) showed no similarity or limited similarity to other sequences or sequence motifs associated with known functional domains as indicated in Table I. These represent a set of novel pollen-expressed clones that may play a critical role in Fl-mediated pollen germination. Some of the proteins encoded by the novel PGP cDNAs feature a high proportion of a specific amino acid or repeated patterns of amino acids (Table I). We performed secondary structure and disorder predictions on the novel clones to determine whether the translation products had a propensity to assume particular conformations that might suggest a functional role. The results of this analysis revealed features of two clones (S/NH22 and S/D3) that suggest a possible role during germination.

The S/NH22-deduced peptide sequence consists of 159 amino acids with a high proportion of Ser (15%), a predicted M_r of 16,367, and a pI of 4.1. A hydropathy plot does not indicate a signal sequence. As shown in Figure 5, starting at residue 51 are four copies of a perfect 8-mer repeat composed of negatively charged and polar residues. Each repeat is separated by a three-amino acid spacer that is itself highly conserved. Analysis of S/NH22 with the PONDR program, which detects a local propensity for ordered or disordered regions within a protein (Romero et al., 1997), indicated that the region from residue 38 to 94, which includes the 8-mer repeats, is very highly disordered, i.e. not folded into a three-dimensional structure. This region also has low sequence complexity, another indicator of disorder, and is predicted to assume a loop configuration. Highly disordered regions are proposed to confer functional advantages on a protein, including the

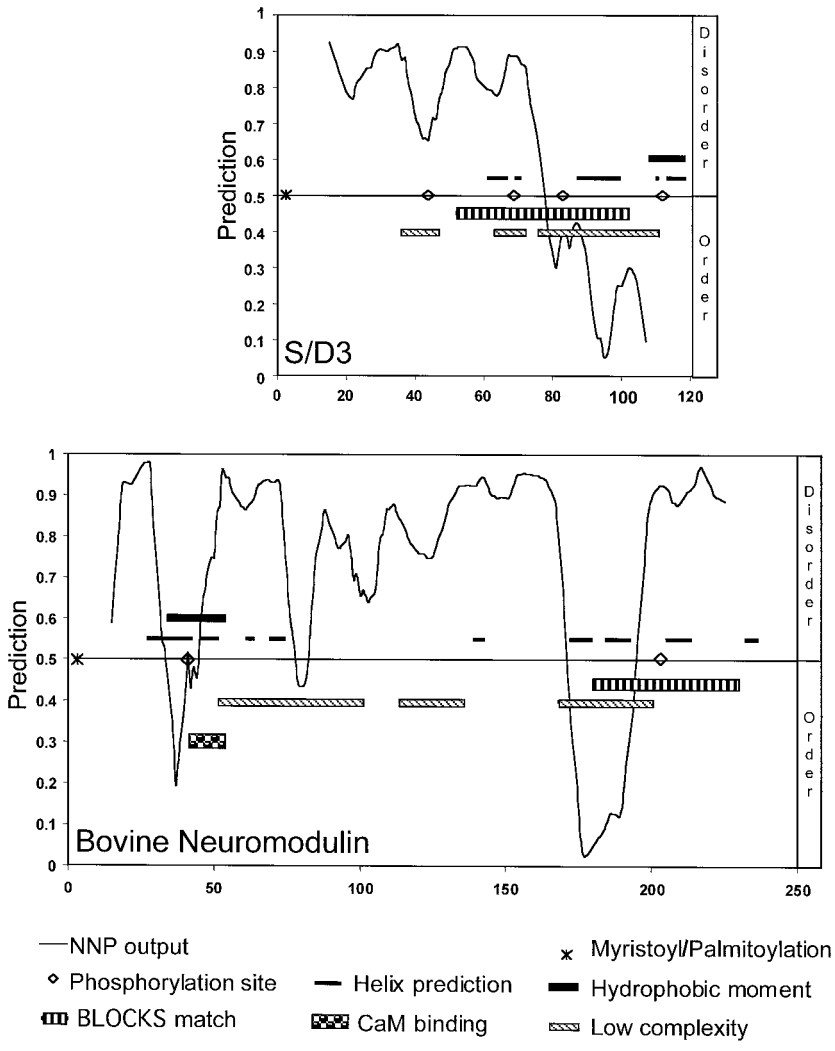

Figure 6. Summary of the sequence and secondary structure analysis of S/D3 showing possible functional motifs and regions of similarity shared with neuromodulin. Numbers on the *x* axis indicate amino acid residues. Values above the midpoint of the *y* axis indicate a high propensity for disorder and values below the midpoint suggest ordered regions. NNP, Neural network predictor.

ability to bind multiple target proteins (Wright and Dyson, 1999).

S/D3 encodes a small acidic protein of 121 residues that originally showed no high scoring matches to any sequences in the non-redundant National Center for Biotechnology Information BLAST database. Using the Blocks Searcher Program (Henikoff and Henikoff, 1994), which compares sequence blocks instead of pairwise combinations, we detected a significant match (Block E value of 2.3×10^{-5}) with neuromodulin, an acidic protein of 239 residues that is present in the motile growth cone of elongating axons of vertebrates. Using the Align Program (www.blocks.fhcrc.org) with a BLOSUM 50 matrix and gap penalties of $-14/-4$, we identified a region of 37% identity within 73 residues (18–88 in S/D3 and 72–140 in neuromodulin) between S/D3 and neuromodulin.

Neuromodulin is a membrane-anchored, calmodulin (CaM)-binding protein (Zuber et al., 1989) that is regulated by protein kinase C (Chakravarthy et al., 1999). An extensive comparison of the two proteins is summarized in Figure 6 and shows that the predicted S/D3 protein shares the following prediction characteristics with neuromodulin: (a) a high fraction of disorder; (b) protein kinase C phosphorylation sites; (c) casein kinase II phosphorylation sites; (d) a fatty acid membrane attachment site in the first seven residues of the amino terminus; and (e) three regions of low sequence complexity. In addition, the proportion of particular acidic (Asp and Glu) and basic (Lys) amino acids is similar in S/D3 (29% and 14%) and neuromodulin (22% and 12%; data not shown).

Neuromodulin has a CaM-binding domain (CaMBD; residues 41–51) termed the IQ motif that mediates CaM binding at low Ca^{2+} concentrations (Rhoads and Friedberg, 1997). CaMBDs have been predicted using the criteria of hydrophobic moment, mean hydrophobicity, and the ability to form a basic amphiphilic helix (O'Neil and DeGrado, 1990). Secondary structure prediction programs indicated a helical region and a high hydrophobic moment in the carboxyl terminus of S/D3. However, the helical residues did not form a strong basic amphiphilic helix and no other CaMBD signature sequences (e.g. IQ motif) were present. Because CaMBDs show wide sequence variation and no general motif is absolutely predictive of CaM binding (O'Neil and DeGrado, 1990), it will be necessary to functionally test the S/D3 protein for CaM binding.

Known Sequences

PGPs cDNAs 9, S/D4, S/D6, S/D8, S/D11, 236, D14, S/NH15, S/NH17, S/NH19, S/NH20, and S/NH21 showed high levels of identity with genes of known function and were grouped according to a possible role in pollen development, tube growth, and/or pollen-stigma interaction. Among the cDNAs sharing similarity with known sequences, a few were known to be expressed in pollen (S/D1, 236, S/NH15, S/NH19, S/NH20, and S/NH21), whereas for the majority this is the first reported detection in pollen (Table I).

PGP cDNAs Encoding Protein or Nucleic Acid-Binding Proteins

Several PGP sequences have structural characteristics indicating a possible regulatory or signaling role. S/D4 is a full-length cDNA that encodes a putative peptide of 353 amino acids, containing an N-terminal hydrophobic sequence with characteristics of a signal peptide followed by 10 Leu-rich repeats (LRRs). LRRs are found in a number of diverse proteins from mammals, yeasts, and plants, and the LRR domains are proposed to mediate protein-protein interactions (Kobe and Deisenhofer, 1994). The 24-amino acid consensus sequence of the 10 LRRs in S/D4 most closely matches the canonical extracytoplasmic LRR found in the tomato resistance genes, *Cf*-9 and *Cf*-2 (Jones and Jones, 1997).

The S/D4 amino acid sequence shares 42% similarity with a pear polygalacturonase-inhibiting protein located in fruit cell walls. Polygalacturonase-inhibiting proteins are soluble, extracellular matrix glycoproteins defined by their inhibitory activity toward fungal endopolygalacturonase substrates. The S/D4-deduced peptide is also similar (43%) to the FIL2 gene product, another LRR protein (Steinmayr et al., 1994). FIL2 is expressed in both female and male reproductive organs of *Antirrhinum* including the filament, connective, epidermis, and endothecium of the anther. It is not expressed in pollen.

S/D1 encodes a protein containing a LIM domain, a conserved Cys-rich motif composed of two adjacent zinc fingers, which is present in a structurally diverse group of proteins and is considered to be a protein-binding interface (Dawid et al., 1995, 1998). The deduced amino acid sequence of S/D1 consists of 195 residues including two LIM motifs, and it shares 76% similarity with pLIM-1, a sunflower pollen-specific protein (Baltz et al., 1992). The sequence comparison of the deduced S/D1 peptide and pLIM shows a high degree of homology between the LIM domains, but S/D1 lacks the pentapeptide repeat in the C terminus of the protein and resembles more the animal LIM proteins, CRP and MLP (Dawid et al., 1995). Therefore, S/D1 is a second pollen-specific cDNA encoding a LIM protein. pLIM-1 has been localized to the germination pore in mature sunflower pollen (Baltz et al., 1999).

The D14-deduced protein sequence contains a putative heme-binding site of the cytochrome *c* type, and two zinc fingers: one imperfect C2H2 type and one perfect C3HC4 type. Zinc fingers are known DNA-binding motifs, and recent reports suggest that they also mediate protein-protein interactions (Mackay and Crossley, 1998). D14 shows 44% identity to a *Schizo-*

saccharomyces pombe protein of unknown function (Table I). Interestingly, the D14 transcript was highly expressed in non-germinating pollen that was hydrated in a calcium-deficient medium but barely detected in GM containing calcium. In vitro pollen tube growth requires an inward directed flux of Ca^{2+} from the medium, in addition to the intracellular gradient at the tip (Taylor and Helper, 1997; Franklin-Tong, 1999). Thus, D14 may represent a new class of genes responsive to fluctuations in calcium levels.

PGP Involved in Protein Processing in the Secretory Pathway

S/D6 encodes a protein of 215 amino acids sharing 81% similarity with the endoplasmic reticulum (ER) protein retaining receptor from Arabidopsis. ER protein retaining receptors recognize soluble proteins containing a C-terminal endomembrane retention signal, typically K/H/RDEL in plants, and returns them from the cis-Golgi to the ER. S/NH16 is a partial cDNA with a deduced amino acid sequence that shows similarity to a subunit of the Sec61 transport protein. The *sec*61 gene product is a component of the apparatus that catalyzes protein translocation into the ER (Corsi and Schekman, 1996). To our knowledge, this is the first report of pollen isoforms of secretory pathway proteins. Protein-processing through the endomembrane system is crucial for pollen tube growth since brefeldin A, a fungal toxin known to interfere with membrane trafficking, stops both germination and tube growth in tobacco (Rutten and Knuiman, 1993).

AGP-Like Sequences

S/D11 is a full-length cDNA that encodes a protein of 113 amino acids showing 28% sequence similarity with two putative arabinogalactan proteins (AGP) expressed in *Brassica* pollen (Gerster et al., 1996). Characteristically, AGPs have a small protein core rich in Ser, Ala, and Pro residues, and is heavily modified by a large carbohydrate component. The S/D11 predicted peptide has a molecular mass of 10.8 kD, and is mainly composed of Ala (27.4%), Ser (15.9%), and Pro (12.0%) residues. It has a potential signal sequence but lacks the hydrophobic C terminus that is found in all other AGPs (Qiu et al., 1997). AGPs have been identified in pollen and the extracellular matrix of the stigma and style. Yariv reagent, which specifically interacts with AGPs, blocks tip growth of lily pollen tubes (for review, see Knox, 1999).

DISCUSSION

Gene Expression during the Early Moments of Fl-Induced Germination

We designed a selection scheme to enrich for sequences expressed during the first 0.5 h of Fl-induced germination and identified a new group of pollen-specific sequences, including eight novel genes. The time point selected represents the minimum amount required for petunia pollen to rehydrate and resume metabolic activity leading to detectable tube outgrowth. The question posed at the onset of this study, whether the same genetic program active during pollen maturation operates during germination, was answered by finding that virtually all of the PGP clones expressed in the first 0.5 h of germination were also expressed in developing pollen. However, the steady-state transcript patterns of the PGP clones during the maturation program and germination did show a qualitative difference. One novel PGP clone, S/D10, was highly up-regulated at germination. It encodes a Ser-rich protein with no similarity to known proteins or functional motifs and is an obvious candidate for functional testing by a gene-silencing approach. There may be additional transcripts preferentially expressed during early germination and a different selection scheme might detect them. For example, ephemeral transcripts that function before 0.5 h would not have been present in our libraries.

A structure-based search of one of the novel clones raised the possibility that S/D3 and neuromodulin might play similar roles in cell elongation (of pollen tubes and axons respectively). S/D3 lacks an obvious CaMBD, but its other similarities to neuromodulin suggest that the two proteins may share some functions. Neuromodulin is a multifunctional protein; various functional elements responsible for its different effects have been mapped (Gamby et al., 1996). It interacts with several second messenger systems suggesting it may mediate multiple signal transduction pathways in the growth cone, modulating the parameters of axonal growth (Gamby et al., 1996). Its largely disordered structure (Zhang et al., 1994) could permit binding to several different target macromolecules (Wright and Dyson, 1999). Alternatively, the similarities between S/D3 and neuromodulin may simply be due to functioning in a high Ca^{2+} environment.

Do the PGP cDNAs Suggest How Fls May Induce Pollen Germination?

Although we targeted genes expressed early in germination, the selection scheme was biased toward early transcripts responding to kaempferol. The success of our approach is reflected in the fact that all of the PGP clones were up-regulated by kaempferol. The mechanism of Fl action has not been elucidated, but a structural role in wall formation has been ruled out using a radioactive Fl tracer (Xu et al., 1996). The sensitivity and specificity of the germination requirement, as well as the rapid response to added Fls suggests they may function as signal molecules. The most compelling model in this regard is the communication between leguminous roots and soil-bacterium

of the genus *Rhizobium*, which is mediated by flavonoids (Fisher and Long, 1992).

A prime objective is to identify the cellular components that interact with, and transduce, the Fl signal. Attempts to exploit the vast amount of information that exists on the interaction of dietary Fls with animal proteins have been frustrated by the number of targets. A partial list shows that Fls, usually in the aglycone form, inhibit protein kinase, phospholipase, and lipoxygenase activity, act as potent antioxidants, and have anticarcinogenic effects (Middleton and Kandaswami, 1993). Thus, it is difficult to draw parallels with pollen germination.

What conclusions can we draw about the action of Fls in pollen germination from the present study? The PGP cDNAs with identity to known sequences provide no overt clues, although the two PGP clones showing the greatest response to kaempferol, S/D1 and S/D4, encode proteins that are known to function in signal transduction pathways in other systems. Extracellular LRR domains present in some animal receptor proteins are the site of ligand binding and they bind non-proteinaceous ligands as well as peptides (Kobe and Deisenhofer, 1994). In higher plants, LRR proteins are involved in cell-cell communications, including host-pathogen interaction, plant morphogenesis, and floral meristem development (for review, see Jones and Jones, 1997), as well as hormone signaling (Li and Chory, 1997). In addition to S/D4, other cDNAs encoding extracellular LRR proteins expressed in pollen include protein kinases (Mu et al., 1994; Muschietti et al., 1998) and Pex-1, an extensin-like protein, (Rubinstein et al., 1995). To date, no ligand has been identified that interacts with these LRRs. We are testing the role of S/D4 in germination by abolishing gene expression in the pollen. Preliminary analysis of the transgenic pollen expressing an antisense copy of S/D4 cDNA indicates that pollen function is impaired.

Given the rapidity of the response to added Fl, perhaps pollen germination is mediated by a post-transcriptional mechanism. This would be in accord with early experimental findings that suggested that pollen germination was more dependent on translation than transcription (for review, see Mascarenhas, 1993). In this case, we envision that some of the Fl-regulated transcripts might function as downstream elements in a pathway that is set in motion by binding of the Fl ligand to a receptor. All possible scenarios must take into account the fact that Fl-treated CMF pollen and wild-type pollen are exposed to Fls at very different stages of development. Wild-type pollen accumulates Fls long before germination and they are rapidly conjugated to pollen-specific Fl diglycosides (Vogt and Taylor, 1995; Miller et al., 1999). In this regard, it is interesting that many of the PGP transcripts were up-regulated in CMF pollen when it was exposed to kaempferol (Fig. 2), but the same transcripts were not increased in germinating V26 pollen (Fig. 4). Perhaps during the initial interaction between Fls and pollen, a pathway is set in motion and a stored signal is generated that is activated upon pollen hydration. Evidence for this type of regulation exists in maturing maize seeds carrying the *c1*-p allele of a Fl regulatory gene, where light generates a long-term signal that is activated at rehydration and germination (Chen and Coe, 1978; Kao et al., 1996).

MATERIALS AND METHODS

Plant Material

Two petunia (*Petunia hybrida*) lines were used in this study: inbred V26, designated wild type, and a CMF mutant (Taylor and Jorgensen, 1992). Growth conditions and staging criteria for anthers and pollen were according to Pollak et al. (1993).

In Vitro Pollen Germination

Pollen was extracted from stage 9 anthers into GM and adjusted to a density of about 2×10^5 grain/mL (Mo et al., 1992). For CMF pollen germination, GM was supplemented with kaempferol and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 5 μM. The non-germinating sample was not supplemented, but an equal volume of DMSO was added to the GM. Forty-milliliter pollen suspensions were incubated at room temperature in a 50-mL sterile tube and placed on a rotary shaker at 150 rpm. The pollen was collected after 0.5 or 2 h of incubation by centrifugation at 3,000 rpm for 5 min, and the pellet was flash-frozen in liquid nitrogen and stored at −70°C until needed.

Construction of cDNA Libraries

cDNA was synthesized using the SuperScript Plasmid System (Gibco-BRL, Rockville, MD) from poly(A^+) RNA extracted from mature CMF pollen (stage 9–10) imbibed in GM without kaempferol [−Fl] for 0.5 h, and from CMF pollen germinated in 0.5 μM kaempferol [+Fl] for 0.5 and 2 h (40% and 60% contribution, respectively). The [−Fl] cDNA was ligated into pSport2 and electroporated into Electromax DH12S *Escherichia coli* (Gibco-BRL), whereas the [+Fl] cDNA was cloned into pSport1 and transformed into Electromax DH10B cells (Gibco-BRL).

Subtraction of the two libraries was performed as described by Gruber et al. (1993). Briefly, single-strand DNA was prepared from the [+Fl] library, and RNA from the [−Fl] library was transcribed in the presence of biotinylated dCTP. Two rounds of hybridization were carried out in 50% (v/v) formamide at 42°C for 38 h. The biotinylated RNA/DNA hybrids were removed using streptavidin followed by chloroform extraction. The resulting single-strand DNA was dialyzed, repaired with *Taq* polymerase, and electroporated into Electromax DH10B cells (Gibco-BRL). The titer of the subtracted library was 3×10^3 colony forming units/mL.

Differential Screening

1,500 colonies resulting from the subtraction were plated at low density and replica were lifted onto nylon membranes. In addition, 750 colony forming units from the [+Fl] library were also differentially screened to increase chances of isolating cDNA clones expressed preferentially in kaempferol-induced germinating pollen. cDNA probes were synthesized from 3 μg of poly(A^+) RNA isolated from CMF pollen incubated for 0.5 h in GM [−Fl, 0.5 h], and from pollen germinated for 0.5 h in GM supplemented with 0.5 μM kaempferol [+Fl, 0.5 h]. Single-stranded cDNA was synthesized using the SuperScript Plasmid System (Gibco-BRL) in the presence of 70 μCi of [α^{32}P]dCTP and oligo(dT) primer. Probe integrity was confirmed by alkaline agarose gel electrophoresis, and the specific activity was determined by liquid scintillation counting. Filters were incubated for 5 h in hybridization solution (5× SSPE, 5× Denhardts, 0.5% [v/v] SDS, 0.1 mg/mL salmon sperm DNA, and 50% [v/v] formamide) at 42°C. Single-strand cDNA probes (1×10^5 cpm/mL) were added to fresh hybridization solution lacking salmon sperm DNA and incubated for 27 h at 42°C. Filters were washed at 65°C for 20 min each in 2× SSPE and 0.1% (v/v) SDS twice; 1× SSPE and 0.1% (v/v) SDS; and finally 0.5× SSPE and 0.1% (v/v) SDS. Filters were exposed to RX film (Fuji, Tokyo) for 2 and 4 d at −70°C. Differentially expressed clones, defined as those not hybridizing to the [−Fl] probe, but giving a signal with the [+Fl] probe, or giving a stronger signal with the [+Fl] probe compared to the [−Fl] probe, were selected.

To differentiate between cDNAs expressed in response to kaempferol and those expressed in response to germination, a second differential screening was performed. Colonies were dotted onto plates, replicated onto nylon membrane, and hybridized to single-stranded cDNA probes synthesized from 2 μg of poly(A^+) RNA extracted from either CMF pollen supplemented with kaempferol, but lacking Bo to prevent germination [−Bo, +Fl], or kaempferol-treated CMF pollen germinated for 0.5 h [+Fl 0.5 h]. Clones showing a more intense signal with the [−Bo, +Fl] probe than with the [+Fl, 0.5 h] probe were selected.

RNA Isolation and Gel-Blot Analysis

Total RNA was extracted from germinated and ungerminated pollen, anthers at various developmental stages, corollas (stage 4–6), stigma/style from stage 9 flowers, and young leaves using Trizol (Gibco-BRL) reagent according to the manufacturers instructions. Pollen was disrupted using zirconium beads (four times for 100 s each) and a bead-beater (BioSpec Products, Bartlesville, OK). Poly(A^+)-enriched RNA was isolated using oligo(dT) resin in the form of Dynal beads for library construction and PolyA Spin mRNA Isolation Kit (New England Biolabs, Beverly, MA) for differential screening and RNA-gel analysis.

For detecting differential expression (Figs. 2 and 3), poly(A^+)-enriched RNA was isolated from CMF pollen incubated in GM with the following additions or omissions from the DMSO solvent: [−Fl], none; [+Fl, 0.5 h] and [+Fl, 2 h], 5 μM kaempferol for 0.5 and 2 h, respectively; [−Bo, +Fl] and [−Ca, +Fl], 5 μM kaempferol, but lacking Bo or calcium for 0.5 h. The [−Ca, +Fl] treatment also included 10 mM EGTA to chelate any calcium present in the solution. For testing a possible Bo effect, poly(A^+)-enriched RNA was extracted from pollen incubated for 0.5 h in GM with or without Bo. The developmental and tissue RNA blots (Fig. 4) were prepared with 10 μg of total RNA and the RNA-gel blots shown in Figures 2 and 3 used 1 μg of poly(A^+)-enriched RNA.

RNA was size-fractionated in formaldehyde gels, transferred to nylon membranes, and hybridized with random primed probes (10^6 cpm/mL) as described above. The blots were sequentially hybridized with the PGP clones and finally with an rDNA probe; although the RNA preparation is enriched in poly(A^+), it contains residual total RNA. Autoradiograms were exposed for periods ranging from 15 min to 72 h, as listed in Table I, in order to get comparable hybridization signals. The autoradiograms were scanned and hybridization signals were quantified using the NIH Image program (National Institutes of Health, Bethesda, MD). The resulting values were corrected for loading differences by standardization to the rDNA probe signal. The experiments shown in Figures 2 and 3 were repeated at least two times with independent isolates of RNA each time.

DNA Sequence Analysis

Both strands of the cDNA were sequenced with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE-Biosystems, Foster City, CA) and extension products were analyzed with a DNA sequencer (PE-Biosystems). To determine the identity of the PGP clones, nucleic acid and protein databases were searched using BLASTP and BLASTN algorithms within the GCG program (Devereux et al., 1984). Additional identity searches used the Blocks Searcher Program (Henikoff and Henikoff, 1994). Secondary structure predictions used three methods, GOR IV (Garnier, 1996) PHD, HNN (Guermeur, 1999), and NNPREDICT (Kneller et al., 1990), and the predicted regions of helix are in agreement by all three methods. Putative myristoylation, palmitoylation, and phosphorylation sites were identified using PROSITE (Bairoch, 1997). Calculations of mean hydrophobic moment and helical hydrophobicity were determined using the GCG software package (Genetics Computer Group, Madison, WI; Devereux et al., 1984).

Sequence complexity estimates used informational entropy as described by Wootton and Federhen (1996). PONDR (predictor of native disordered regions), version XL1, a neural network predictor of protein disorder, was trained using balanced sets of ordered and disordered (e.g. not completely folded) amino acid sequences as described by Romero et al. (1997) and Garner et al. (1998). Some of the characteristics of disordered sequences are lack of aromatic residues, lack of Cys, unbalanced charge, a high Ser content, a strongly negative value for hydropathy, and a high value for the flexibility index (Romero et al., 1997; Xie et al., 1998).

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