

# Diversity of Arabidopsis Genes Encoding Precursors for Phytosulfokine, a Peptide Growth Factor<sup>1</sup>

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Phytosulfokine- $\alpha$  (PSK- $\alpha$ ), a unique plant peptide growth factor, was originally isolated from conditioned medium of asparagus (*Asparagus officinalis*) mesophyll cell cultures. PSK- $\alpha$  has several biological activities including promoting plant cell proliferation. Four genes that encode precursors of PSK- $\alpha$  have been identified from Arabidopsis. Analysis of cDNAs for two of these, *AtPSK2* and *AtPSK3*, shows that both of these genes consist of two exons and one intron. The predicted precursors have N-terminal signal peptides and only a single PSK- $\alpha$  sequence located close to their carboxyl termini. Both precursors contain dibasic processing sites flanking PSK, analogous to animal and yeast prohormones. Although the PSK domain including the sequence of PSK- $\alpha$  and three amino acids preceding it are perfectly conserved, the precursors bear very limited similarity among Arabidopsis and rice (*Oryza sativa*), suggesting a new level of diversity among polypeptides that are processed into the same signaling molecule in plants, a scenario not found in animals and yeast. Unnatural [serine-4]PSK- $\beta$  was found to be secreted by transgenic Arabidopsis cells expressing a mutant of either *AtPSK2* or *AtPSK3* cDNAs, suggesting that both *AtPSK2* and *AtPSK3* encode PSK- $\alpha$  precursors. *AtPSK2* and *AtPSK3* were expressed demonstrably not only in cultured cells but also in intact plants, suggesting that PSK- $\alpha$  may be essential for plant cell proliferation in vivo as well as in vitro. Overexpression of either precursor gene allowed the transgenic calli to grow twice as large as the controls. However, the transgenic cells expressing either antisense cDNA did not dramatically decrease mitogenic activity, suggesting that these two genes may act redundantly.

The sporophytic phase of seed plants encompasses embryonic and postembryonic development. Following fertilization, the zygote undergoes cell division to produce the embryo. Higher plants elaborate much of their architecture postembryonically through development initiated at the tips of shoots and roots: Cell division is activated in the shoot and root apical cells, generating the shoot and root meristems. It is known that these apical meristematic cells can remain in the cell cycle to generate new cells for growth and development throughout the plant's life, but the molecular mechanisms are not yet well understood.

In animal systems, cells release and recognize extracellular signaling molecules such as hormones and growth factors. Oligopeptides play a major role in cell-cell communication, whereas mitogenic peptides contribute to cell cycle control, reactivation of the cell cycle being dependent on such extracellular signal molecules (Meyerowitz et al., 1998). Several peptide signals recently were isolated from plants. For example, systemin from tomato regulates the synthesis of defensive proteins in plant tissues (Pearce et al., 1991; McGurl et al., 1992). In Arabidopsis, CLAVATA3

(CLV3), a small extracellular protein, acts as a ligand in a stem cell-restricting signal transduction pathway that regulates the balance between cell proliferation and differentiation at the shoot meristem (Clark et al., 1997; Fletcher et al., 1999; Brand et al., 2000; Trotochaud et al., 2000). However, only one kind of mitogenic oligopeptide, the phytosulfokines (PSKs), has been identified so far.

PSKs were originally isolated from conditioned medium (CM) of asparagus (*Asparagus officinalis*) mesophyll cell cultures and their structures determined as a sulfated pentapeptide, PSK- $\alpha$  [Tyr(SO<sub>3</sub>H)-Ile-Tyr(SO<sub>3</sub>H)-Thr-Gln], and a C-terminal-truncated tetrapeptide, PSK- $\beta$  [Tyr(SO<sub>3</sub>H)-Ile-Tyr(SO<sub>3</sub>H)-Thr] (Matsubayashi and Sakagami 1996). PSK- $\alpha$  is the most active compound although its amount in CM is less than 20% of that of PSK- $\beta$ . PSK- $\beta$  recently was shown to be an enzymatic derivative of PSK- $\alpha$  (Yang et al., 1999b). The latter strongly stimulates proliferation of plant cells in low-density cultures, the minimum concentration exhibiting mitogenic activity being  $1.0 \times 10^{-9}$  M. Although PSK- $\beta$  contributes the major form in CM, it exhibits activity at a lower concentration of  $1.0 \times 10^{-8}$  M (Matsubayashi and Sakagami 1996).

PSK- $\alpha$  has several other biological activities in addition to promoting plant cell proliferation. For example, it enhances chlorophyll synthesis in etiolated cotyledons of cucumber (Yamakawa et al., 1998a), as well as the growth and chlorophyll content of Arabidopsis seedlings under high nighttime temperature conditions (Yamakawa et al., 1999). PSK- $\alpha$  also promotes adventitious root formation by hypocotyls of

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cucumber (Yamakawa et al., 1998b) and adventitious bud formation in snapdragon (*Antirrhinum majus*) (Yang et al., 1999a). Furthermore, it stimulates both tracheary element differentiation of isolated mesophyll cells from zinnia (Matsubayashi et al., 1999) and somatic embryogenesis in carrot cultures (Kobayashi et al., 1999; Hanai et al., 2000a).

We previously cloned the *OsPSK* gene encoding a precursor of PSK- $\alpha$  from monocot rice (*Oryza sativa*; Yang et al., 1999b, 2000a). Expression was found not only in cultured cell but also in intact plants (Yang et al., 1999b). In intact plants, transcripts of the *OsPSK* gene are concentrated in shoot and root meristems in which cells proliferate vigorously (Yang et al., 1999b). PSKs have also been found in dicots including *Arabidopsis* (Yang et al., 2000a), and the amino acid sequences of both PSK- $\alpha$  and PSK- $\beta$  are perfectly conserved among different species (Yang et al., 2000a).

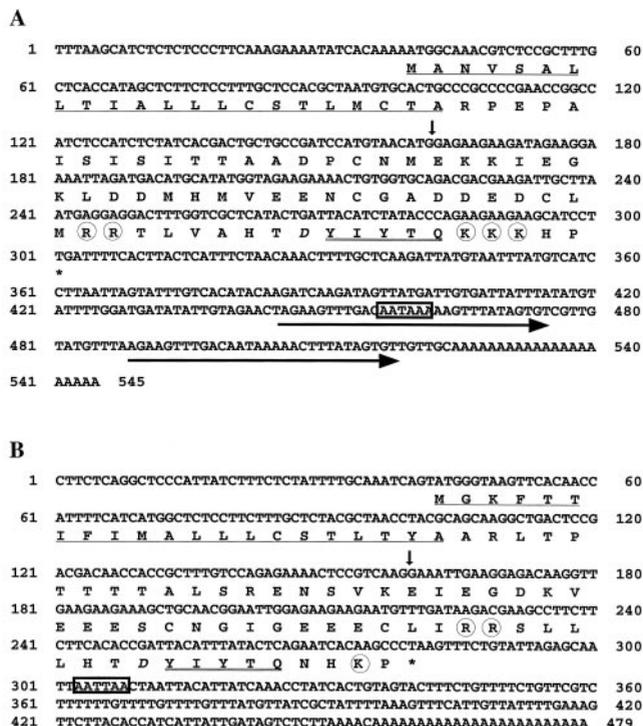
The flowering plant *Arabidopsis* is an important model system for identifying genes and determining their functions, because of its small body, short generation time, and small genome size. In the present article, we describe the functional and comparative analysis of two PSK precursor genes, *AtPSK2* and *AtPSK3*, in *Arabidopsis*. Both precursors exhibit di-basic processing sites flanking PSK, analogous to animal prohormones. Although the PSK domain including the sequence of PSK- $\alpha$  is perfectly conserved, the two precursors bear very limited similarity. This structure suggests a new level of diversity among polypeptides that are processed into the same signaling molecule in plants, a scenario not found in animals and yeast.

## RESULTS

### Characterization of Arabidopsis Genes for Precursors to PSK

Although PSK peptides had been detected in *Arabidopsis* cell cultures (data not shown), our attempts to clone *Arabidopsis* PSK precursor gene(s) using a rice PSK precursor cDNA (*OsPSK*) as a probe were unsuccessful, suggesting that *Arabidopsis* PSK precursor gene(s) may share very low similarity with the rice *OsPSK* gene. To identify *OsPSK* homologous gene(s), the *Arabidopsis* expressed sequence tags (EST) were searched with the BLAST program using the amino acid sequence of PSK- $\alpha$ . Several putative homologs were found to encode proteins that contain the PSK sequence. Their sequences were compared and showed to correspond to two genes that we designated as *AtPSK2* and *AtPSK3*, respectively, based on their chromosomal location.

*AtPSK2* cDNA (EST no. 120C15T7) is 524 bp in length with polyadenylation motifs (AATAAA) that present 19 and 62 bp upstream from the polyadenyl tail (Fig. 1A). Two imperfect 35-bp repeats were noted in the 3'-untranslated region (Fig. 1A). The



**Figure 1.** Nucleotide and deduced amino acid sequences of *AtPSK2* and *AtPSK3* cDNAs. The deduced amino acid sequences with single-letter abbreviations are shown below the nucleotide sequences of *AtPSK2* cDNA (A) and *AtPSK3* cDNA (B). The positions of introns are shown with vertical arrows. The repeats are indicated with horizontal arrows. The most likely sequences for a polyadenylation signal are boxed. The potential N-terminal signal sequences are underlined, and the PSK- $\alpha$  sequence is underscored with double lines. The Asp residues near PSK- $\alpha$  are printed in italic, and the putative bordering processing sites are circled. The nucleotide sequences reported in this paper have been deposited in the GenBank, EMBL, and DDBJ nucleotide sequence databases (accession nos. AB029344, AB052752, AB029820, and AB050627).

*AtPSK2* cDNA contains an open reading frame of 261 bp that can code for a precursor to PSK with 87 amino acids (Fig. 1A) and a predicated molecular mass of 9.6 kD. In contrast, *AtPSK3* cDNA (EST no. 91K2T7) has no repeats and is shorter than *AtPSK2* cDNA. The precursor deduced from *AtPSK3* cDNA (79 amino acids with a predicated molecular mass of 8.9 kD) is also smaller than that of *AtPSK2*. (Fig. 1B).

To analyze the genomic structure of the *AtPSK* genes, we isolated clones containing the entire *AtPSK2* or *AtPSK3* genes. The *AtPSK2* gene was shown to consist of two exons (162 and 362 bp) that perfectly match the cDNA sequence interrupted by an intron of 148 bp with a well-conserved GT-AG intron border sequence. The first exon consists of the 5'-non-coding region and a coding region for the 22-amino acid N-terminal signal peptide. The second exon was found to consist of the 3' non-coding region and a coding region for the remaining residues of the precursor including the 5-amino acid PSK- $\alpha$  sequence. *AtPSK3*, like *AtPSK2*, consists of two exons

and one intron, but the intron (265 bp) of *AtPSK3* is longer than that of *AtPSK2*.

**Structures of the PSK Precursors Encoded by *AtPSK2* and *AtPSK3***

As expected, the *AtPSK2* has a potential hydrophobic N-terminal signal sequence (residues 1–22), and the mature precursor is predicted to be 65 amino acids in length (Fig. 1A). The 5-amino acid PSK- $\alpha$  sequence occurs only once (amino acids 78–82) within the *AtPSK2* close to its C terminus, as shown in Figure 1A. There is an Asp residue immediately N terminal to the first Tyr of PSK- $\alpha$  in the –1 position (Fig. 1A), suggesting the Tyr residues in the PSK- $\alpha$  sequence can be sulfated (Hanai et al., 2000b). It is interesting that two Arg (amino acids 69–70) and three Lys (amino acids 83–85) residues exist (Fig. 1A), suggesting that putative processing sites bordering PSK- $\alpha$  of the Arabidopsis PSK precursor conform to the consensus sequence for endoproteolytic processing sites flanking bioactive peptides in animal pro-hormone precursors (Harris, 1989), and that PSK- $\alpha$  can be proteolytically processed from the Arabidopsis PSK precursor.

The *AtPSK3* also was found to contain a potential N-terminal signal sequence (amino acids 1–21) and one copy of the PSK- $\alpha$  sequence (amino acids 71–75) at its C terminus (Fig. 1B). The Asp residue is conserved in the –1 position relative to the first Tyr of PSK- $\alpha$ . Also, two Arg (amino acids 62–63) and one Lys (amino acid 28) residues were found to border the PSK- $\alpha$  sequence (Fig. 1B).

A search for homologies to *AtPSK2* and *AtPSK3* genes in the Arabidopsis Database (AtDB) indicated that the *AtPSK2* gene is located on chromosome II, within the region contained in bacterial artificial chromosome clone T20K9, and the closest genetic marker is mi238, a recombinant inbred marker that exists at 39.92 cM (Liu et al., 1996). The bacterial artificial chromosome clone T16K5 harbored the *AtPSK3* gene indicating that *AtPSK3* is located on chromosome III close to MS–3–1 (Reiter et al., 1992), a recombinant inbred marker that exists at 72.75 cM.

**Similarity among Arabidopsis and Rice Precursors to PSK**

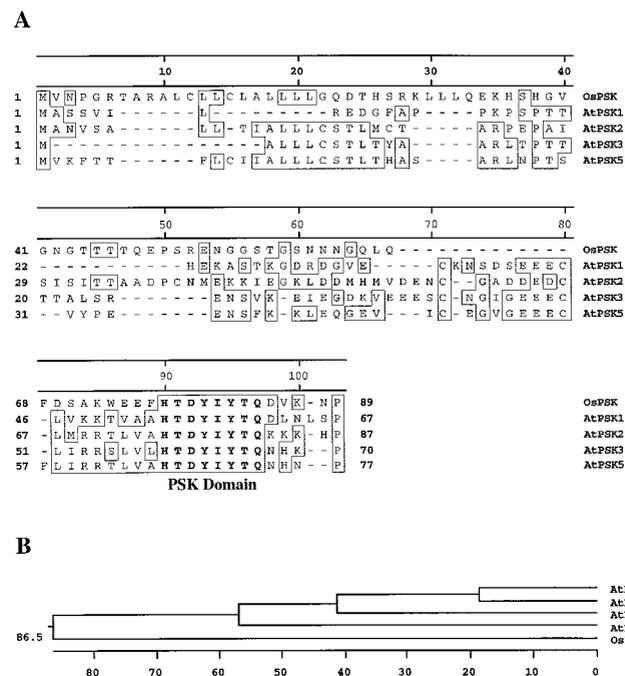
Two more PSK precursor genes recently were found in the AtDB by a BLAST homology search using the sequence of the PSK domains. These genes were designated as *AtPSK1* (accession no. AC027656.4) and *AtPSK5* (accession no. AB018108.1), respectively. The *AtPSK1* gene is located on chromosomes I, close to mi348 (23.7 cM), whereas the *AtPSK5* gene is present in the south part of chromosome V, close to mi335 (131.1 cM). Both the *AtPSK1* and *AtPSK5* genes also have a single intron and the predicted amino acid sequences contain N-terminal

signal peptides. Therefore, the Arabidopsis genome appears to possess a gene family encoding PSK precursors.

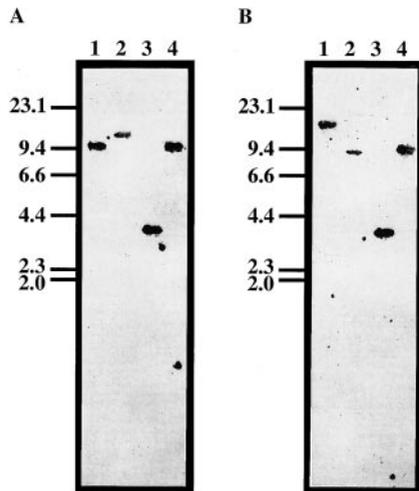
The amino acid sequences of the Arabidopsis and rice PSK precursors were aligned using the Clustal W (1.7) Multiple Sequence Alignment provided by the GenomeNet CLUSTALW Server (Thompson et al., 1994). It is quite unexpected that these precursors do not share significant similarity throughout the whole length, although the PSK- $\alpha$  sequence and three amino acid residues immediately N terminal to it are perfectly conserved (Fig. 2). We refer to this eight-amino acid region as the PSK domain. In addition, a very limited similarity was found for N-terminal signal peptides.

**Copy Numbers of the PSK Precursor Genes**

Copy numbers of the *AtPSK2* and *AtPSK3* genes in the Arabidopsis genome were investigated by genomic Southern-blot analyses. Wild-type DNA was probed with the full-length cDNAs and hybridizations were performed under low- and high- stringency conditions. As results, only one band was detected under either low or highly stringent conditions in the DNA fragments derived from single digestion of the four different restriction enzymes (Fig. 3, A and B), indicating that both *AtPSK2* and *AtPSK3* are single-copy genes.



**Figure 2.** Amino acid sequence comparison of Arabidopsis and rice PSK precursors. The alignment (A) and phylogenetic tree (B) were generated using the MegAlign program. Single-letter abbreviations for the amino acid residues are used. Gaps are shown as dashes (-). Identical amino acid residues are boxed. The amino acid sequence of the PSK domain is in bold.



**Figure 3.** DNA gel blot showing the presence of *AtPSK2* and *AtPSK3* genes. Total DNA (10  $\mu$ g) isolated from Arabidopsis culture cells was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3), or *Xba*I (lane 4) and subjected to DNA gel-blot analysis using  $^{32}$ P-labeled *AtPSK2* (A) or *AtPSK3* cDNA (B) at 50°C. Marker lengths are indicated on the left in kilobases.

#### Expression of PSK Precursor cDNAs in Transgenic Cells

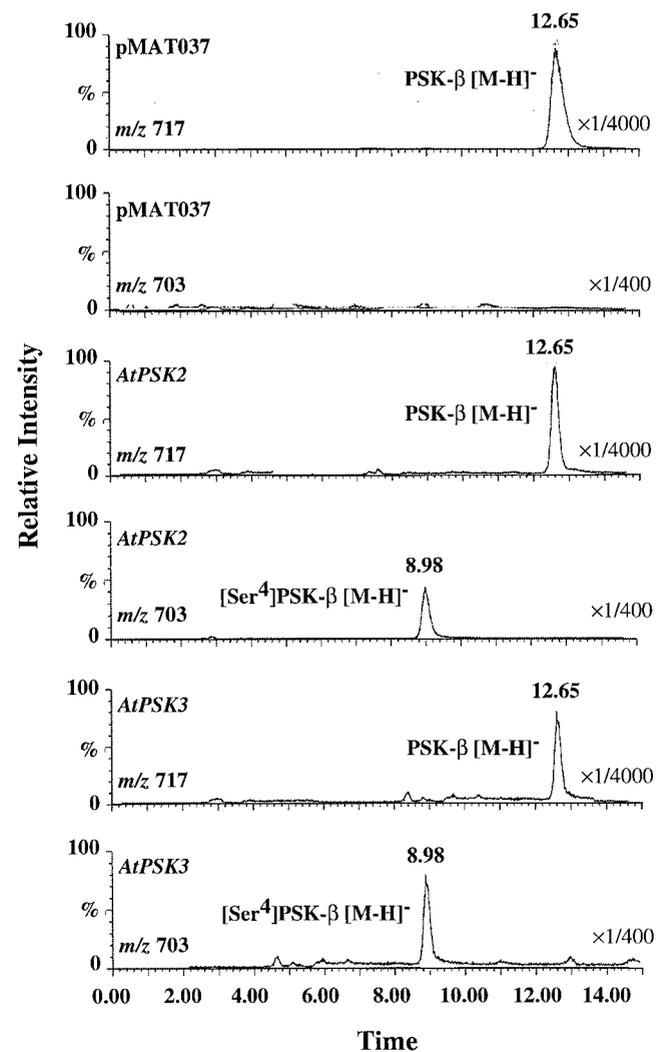
To examine whether *AtPSK2* and *AtPSK3* code for precursors to PSK, we transformed 28-d-old Arabidopsis roots with artificially mutated *AtPSK2* or *AtPSK3* cDNAs in sense orientation, which were designed to produce [Ser-4]PSK- $\alpha$  [Tyr(SO<sub>3</sub>H)-Ile-Tyr(SO<sub>3</sub>H)-Ser-Gln] instead of PSK- $\alpha$ . The chimeric genes harboring the mutated cDNAs in frame were placed under the control of the constitutive cauliflower mosaic virus 35S promoter incorporated within the binary vector pMAT037 (Matsuoka and Nakamura, 1991). B5 medium (Gamborg et al., 1968) supplemented with 0.5 mg L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.05 mg L<sup>-1</sup> kinetin (KIN) was used to induce calli; transgenic calli were selected on basis of kanamycin resistance. Transgenic calli were obtained at a frequency of 79%. DNA gel-blot hybridization analyses confirmed the presence of transgenes; around 70% of the transformants had a single locus insertion (data not shown).

Transgenic cells were transferred to liquid callus-inducing medium after 1 month. Two weeks after the onset of subculture, PSK- $\alpha$  and its analogs in the CM by the transgenic cells were purified and analyzed by liquid chromatography/mass spectrometry (LC/MS). Although only PSK- $\beta$  was detected with transgenic cells containing the pMAT037 vector alone, both PSK- $\beta$  and [Ser-4]PSK- $\beta$ - $\alpha$  [Tyr(SO<sub>3</sub>H)-Ile-Tyr(SO<sub>3</sub>H)-Ser] peaks were present in eluates derived from the CM of transgenic cells harboring either the mutated *AtPSK2* or *AtPSK3* cDNAs (Fig. 4).

Transgenic cells harboring the mutated cDNAs were quantified for their secretion of PSK analogs into CM. Amounts of the PSKs in CM were measured

by LC/MS based on peak heights without an internal standard. Transgenic cells containing the binary vector alone served as controls. The amount of [Ser-4]PSK- $\beta$  produced by the transgenic cells with the mutated cDNA was one order of magnitude higher than that of PSK- $\beta$  derived from the endogenous gene (Table I).

We also introduced wild-type cDNAs in the sense and antisense orientations into Arabidopsis cells to examine the effects of supernumerary or suppressed expression of the PSK precursor genes on cell proliferation. Amounts of PSK precursor mRNA were higher in the sense but lower in the antisense transgenic cells than in the controls (Fig. 5). Overexpression of the PSK precursor genes allowed the trans-



**Figure 4.** Mass chromatograph-obtained LC/MS analyses. The transgenic cells harboring the empty vector, the mutated *AtPSK2* cDNA, or the mutated *AtPSK3* cDNA were cultured in B5 liquid medium for 2 weeks to prepare CM. PSK- $\alpha$  and its analogs containing in the CM were concentrated by two steps of column chromatograph and subjected to LC/MS analysis with selected ion monitoring at *m/z* 703 ([M-H]<sup>-</sup> of [Ser-4]PSK- $\beta$ ) and *m/z* 717 ([M-H]<sup>-</sup> of PSK- $\beta$ ). The peaks eluting at 8.9 and 12.65 were [Ser-4]PSK- $\beta$  and PSK- $\beta$ , respectively.

**Table 1.** PSK production by transgenic *Arabidopsis* cells

Transgenic cells (0.8 g) harboring pMAT037 alone or mutated cDNAs were incubated in 100 mL of fresh B5 liquid medium supplemented with 0.5 mg L<sup>-1</sup> of 2,4-D and 0.05 mg L<sup>-1</sup> of KIN at 25°C in the light with rotary shaking at 120 rpm. After 2 weeks of culture, PSK-β and its analog concentrations in the CM were quantified by LC/MS. PSK concentrations are presented in μM. Averages of three independent experiments are given with the SD. PSK-α was not detectable by this assay because its amount was beyond the detection limit.

Construct	PSK-β	[Ser <sup>4</sup> ]PSK-β
PMAT037	413 ± 30	0
[Ser <sup>4</sup> ]PSK <i>AtPSK2</i> cDNA	387 ± 41	3,750 ± 40
[Ser <sup>4</sup> ]PSK <i>AtPSK3</i> cDNA	350 ± 36	4,785 ± 50

genic calli to grow twice as large as the controls (Fig. 6). However, the transgenic cells in which expression of the PSK precursor genes was suppressed by the antisense cDNA did not dramatically decrease mitogenic activity (data not shown), suggesting that these two genes may act redundantly.

#### Expression of the *AtPSK2* and *AtPSK3* Genes

RNA-blot analysis with RNA extracted from suspension culture cells revealed the *AtPSK2* gene to be continuously expressed, suggesting provision of a supply of PSK-α, which allows the cells to proliferate rapidly. The transcripts increased gradually after cells were transformed to fresh medium and maximal expression values were observed after 7 to 10 d, followed by a decrease (Fig. 7). *AtPSK3* showed an expression pattern similar to that of *AtPSK2*, although maximal expression started earlier (Fig. 7).

*AtPSK2* transcripts also accumulated in 28-d-old *Arabidopsis* plants and were most abundant in roots (Fig. 8). *AtPSK3* was also expressed most abundantly in roots, but transcripts in stems including shoot apices were greater in this case than with *AtPSK2* (Fig. 8). These findings suggest that the PSK-α molecule is also produced and has physiological functions in intact plants.

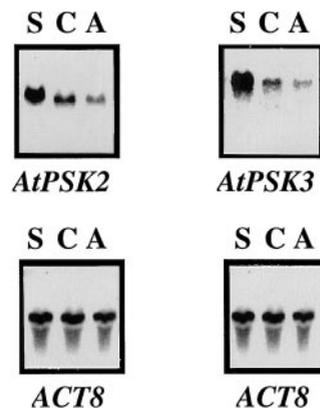
#### DISCUSSION

PSK-α, the first sulfated peptide found in plants, is a unique signal strongly promoting proliferation of plant cells at low concentrations that may be universally distributed in the plant kingdom (Yang et al., 2000a). We previously identified *OsPSK*, a PSK-α precursor gene in rice (Yang et al., 1999b, 2000b), and in this article have described the molecular cloning and characterization of *AtPSK2* and *AtPSK3* genes that encode PSK precursors in *Arabidopsis*.

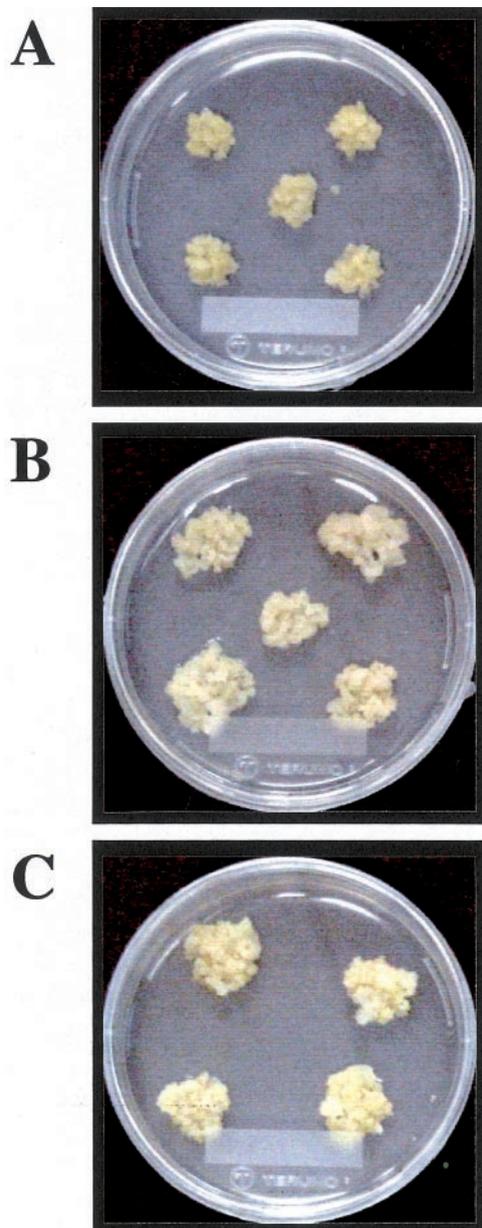
*AtPSK2* or *AtPSK3* are found as single copies with two exons and one intron, on chromosomes II and III, respectively. The first exon of *AtPSK2* contains a coding region for about half of the PSK precursor including the N-terminal region that might serve as a

signal peptide involved in secretion, a common feature of animal active peptide hormones, and the second exon has a coding region within which the 5-amino acid PSK-α sequence occurs only once, close to the C terminus, as reported for rice *OsPSK* (Yang et al., 2000b). *AtPSK3* also shares these characteristics. Although the GT-AG intron border sequences are well conserved in both *Arabidopsis* and rice PSK precursors, the introns of the *Arabidopsis* PSK precursor genes (148 bp for *AtPSK2* and 256 bp for *AtPSK3*) are only 13% to 22% identical to the *OsPSK* intron (1,150 bp). This difference may reflect characteristics of *Arabidopsis*: The size of the genome is the smallest of those for higher plants. Although *OsPSK* cDNA has 16 GA repeats in the 5'-untranslated region (Yang et al., 1999b), and *AtPSK2* cDNA contains two 35-bp repeats in the 3'-untranslated region, *AtPSK3* cDNA has no such repeats (Fig. 1, A and B), and these possible functions remain to be clarified. *AtPSK2* consists of 87 amino acids with a predicated molecular mass of 9.6 kD, similar to those of rice PSK precursor (89 amino acids and 9.8 kD; Yang et al., 1999b). On the other hand, *AtPSK3* is composed of 79 amino acids with a predicated molecular mass of 8.9 kD and thus is smaller than both the rice PSK precursor and *AtPSK2*.

A comparison of *Arabidopsis* and rice PSK precursors at the amino acid sequence level uncovered little similarity between *AtPSK2* and *AtPSK3* and only limited resemblance to rice PSK precursor. However, it is noteworthy that an eight-amino acid (HTDY-IYTQ) region is perfectly conserved, and some similarity was also observed among the three N-terminal peptides (Fig. 2). The conserved eight-amino acid region includes the PSK-α sequence itself and the Asp residue (Fig. 2) that has been proved to be of importance for Tyr sulfation at -1 to the first Tyr of



**Figure 5.** Changes in *AtPSK2* and *AtPSK3* transcripts in transgenic cells. Twenty-microgram aliquots of total RNA extracted from sense transgenic cells (lane S), control (lane C), or antisense transgenic cells (lane A) cultured for 14 d were separated on 1.2% (w/v) agarose gels, transferred to nylon membranes, and allowed to hybridize with <sup>32</sup>P-labeled full-length *AtPSK2* or *AtPSK3* cDNAs, as described in "Materials and Methods." The blots were reprobbed with an *Arabidopsis* actin cDNA to indicate equal loading of RNA.



**Figure 6.** Comparison of growth of control and transgenic calli. Control or transgenic cells were transplanted into fresh B5 medium containing  $0.5 \text{ mg L}^{-1}$  of 2,4-D,  $0.05 \text{ mg L}^{-1}$  of KIN, and 0.8% (w/v) agar. Photographs were taken 2 weeks after culture at  $25^\circ\text{C}$  under 16-h-light/8-h-dark cycle. This experiment was repeated for three times and four or five lines of each construct were shown. A, Transgenic calli harboring empty pMAT037 alone. B, Transgenic calli harboring the sense *AtPSK2* chimeric gene. C, Transgenic calli harboring the sense *AtPSK3* chimeric gene.

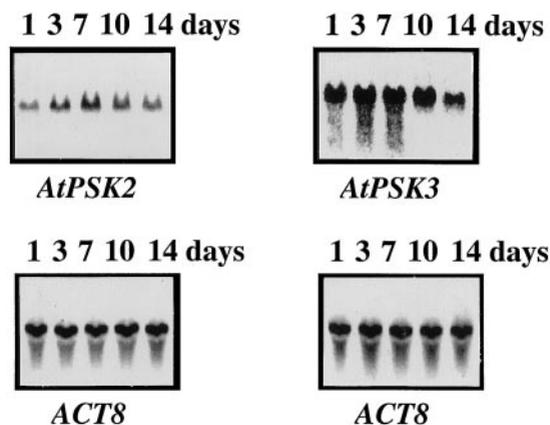
PSK (Hanai et al., 2000b). Therefore, this region, which we have termed the "PSK domain," may be the most important for posttranslational modification of the PSK precursors and the production of PSK- $\alpha$ .

The similarity at the nucleic acid level among *Os-PSK*, *AtPSK2*, and *AtPSK3* genes is so low that they do not cross-hybridize even under low-stringency conditions. *AtPSK1* and *AtPSK5* genes recently were

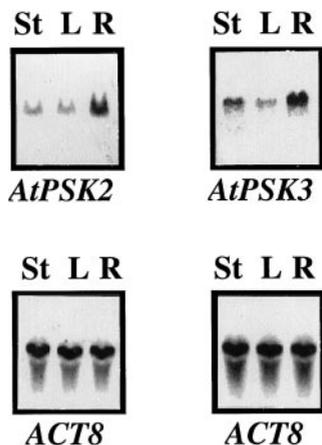
found in the AtDB by a BLAST homology search using the sequence of the PSK domains. The *AtPSK1* gene is located on chromosome I, whereas the *AtPSK5* gene is present in the south part of chromosome V. It is interesting that these genes bear very low similarity. Our data presented here suggest a new level of diversity among polypeptides that are processed into same signaling molecules in plants, a scenario not found in animals and yeast. Furthermore, several rice EST clones (C743, D15509, and S15186) may also encode proteins that contain the PSK domain but share very limited similarity, suggesting that the divergence observed outside the PSK domain is a more general feature of (at least two) plant PSK precursor gene families.

Polyploidy occurs widely in plants and it is proposed to be a key factor in plant evolution (Wendel, 2000). The evolution of Arabidopsis apparently involved a whole-genome duplication, followed by subsequent gene loss and extensive local gene duplications, giving rise to a dynamic genome (The Arabidopsis Genome Initiative, 2000). It is interesting that *AtPSK2* and *AtPSK3* genes are located in the large duplicated segments of chromosomes II and III, suggesting a derivation from the same ancestor but divergence after the duplication event. However, the long period of time over which genome stabilization has occurred has provided ample opportunity for divergence of functions of genes during through duplication. In many cases, the number of copies of a gene and its counterpart differ (for example, one copy on one chromosome and multiple copies on the other). As described above, Arabidopsis PSK precursor genes are separately located on chromosomes I, II, III, and V.

Protein Tyr *O*-sulfation is one of the posttranslational modifications that occurs with many secretory



**Figure 7.** Persistent expression of *AtPSK2* and *AtPSK3* in cultured Arabidopsis cells. Twenty micrograms of total RNA extracted from Arabidopsis cells cultured for 1 (lane 1), 3 (lane 3), 7 (lane 7), 10 (lane 10), or 14 (lane 14) d were separated on 1.2% (w/v) agarose gels, transferred to nylon membranes, and allowed to hybridize with  $^{32}\text{P}$ -labeled full-length *AtPSK2* or *AtPSK3* cDNAs, as described in "Materials and Methods." The blots were reprobbed with an Arabidopsis actin cDNA to indicate equal loading of RNA.



**Figure 8.** Tissue specificity of *AtPSK2* and *AtPSK3* expression. Twenty micrograms of total RNA prepared from stems (lane S), leaves (lane L), and roots (lane R) of 28-d-old Arabidopsis plants were separated on 1.2% (w/v) agarose gels, transferred to nylon membranes, and allowed to hybridize with  $^{32}\text{P}$ -labeled full-length *AtPSK2* or *AtPSK3* cDNAs, as described in "Materials and Methods." The blots were reprobed with an Arabidopsis actin cDNA to indicate equal loading of RNA.

and membrane proteins in animal cells (Huttner, 1984). It has been shown to affect the biological activity of tyrosylproteins (Niehrs and Huttner, 1990) and deletion of the sulfate groups of Tyr-1 and Tyr-3 results in compounds with 0.6% and 4% of the activity of PSK- $\alpha$  (Matsubayashi et al., 1996). Unsulfated PSK- $\alpha$  has neither competitive ability for ligand binding nor mitogenic activity (Matsubayashi et al., 1997). Sulfated tyrosines are located within acidic regions of tyrosylproteins and all sites that have been characterized in animals have associated aspartic or Glu, with three or more acidic residues usually found between -5 and +5 in tyrosylproteins (Dorner and Kaufman, 1990). We previously demonstrated the existence of an Asp at -1, a Glu at -5, and an Asp at position +5 relative to the first Tyr, as well as aspartic acids at -3 and +5 positions around the second Tyr residue (Yang et al., 1999b). Amino acid substitution tests confirmed that the acidic amino acid residues adjacent to the Tyr residues of rice PSK precursor to be essential for Tyr sulfation, and that the Asp at -1 to the first Tyr is the most important determinant in rice PSK precursor (Hanai et al., 2000b). In Arabidopsis PSK precursors, the PSK- $\alpha$  sequence also exists within the acidic region, and an Asp at -1 to the first Tyr is conserved in the PSK domain (Fig. 2), suggesting that the Tyr residues in Arabidopsis PSK precursors could be similarly sulfated.

Peptide hormones and other biologically active peptides are generally synthesized as inactive higher  $M_r$  precursors that must undergo a variety of post-translational processing steps to yield the active forms (Harris, 1989). It is considered that the primary processing recognition sequence for endoproteolysis

of animal prohormone precursor proteins is a pair of basic amino acid residues (Lys-Arg, Lys-Lys, or Arg-Arg) that bracket the peptide hormone (Harris, 1989). It is interesting that a doublet of basic amino acids (Arg-Arg) was conserved in the Arabidopsis PSK precursors close to the N terminus of PSK- $\alpha$  (Fig. 1), suggesting that the initial cleavage event with Arabidopsis PSK precursors may be similar to that of animal prohormones. Consensus sequences for Trypsin were found at amino acid residues 69 to 70 and 83 to 85 in the *AtPSK2*, and 62 to 63 as well as 78 in the *AtPSK3*, flanking PSK- $\alpha$  on both sides (Fig. 1, A and B). However, there is no such recognition site in the amino acid sequence of rice PSK precursor (Yang et al., 1999b), suggesting that the endoproteolytic enzymes may differ between monocots and dicots. Details of the processing procedure are now under investigation.

Our transgenic experiments demonstrated that the *AtPSK2* and *AtPSK3* cDNAs could produce PSK- $\alpha$  in transformed Arabidopsis cells. The finding of [Ser-4]PSK- $\beta$  in the CM prepared from transgenic cells harboring the mutated *AtPSK2* or *AtPSK3* cDNAs simultaneously confirmed that PSK- $\beta$  is an enzymatic derivative of PSK- $\alpha$  (Yang et al., 1999b). The fact that the amount of [Ser-4]PSK- $\beta$  produced by mutated *AtPSK2* or *AtPSK3* cDNAs was more than PSK- $\beta$  produced by the endogenous gene might result from a higher level of expression of foreign genes and no influence to the modification and/or processing efficiency by the amino acid substitution. It is also plausible that the degradation speed of [Ser-4]PSK- $\beta$  is slower than that of natural PSK- $\beta$  in Arabidopsis cultures.

Our previous data indicated that rice Oc cells produce more PSKs than any other plant cells tested. We could clearly detect PSK- $\alpha$  as well as PSK- $\beta$  from only 40 mL of CM derived from Oc cells by LC/MS analysis (Yang et al., 1999b). PSK- $\alpha$  is the active principle of cell proliferation activity in CM; however, the amount of PSK- $\beta$  is much more than that of PSK- $\alpha$  in all investigated cultures. The total amount of PSKs in Arabidopsis cultures were found to be almost two orders less than that produced by rice Oc cells, explaining why PSK- $\alpha$  was not detectable by LC/MS in the present study. It is likely that the degradation of PSK- $\alpha$  in the Arabidopsis culture is much faster than in rice Oc culture or alternatively, the PSK degradation system in rice Oc culture is unusually weak.

We found that *AtPSK2* and *AtPSK3* transcripts remained at a high level in cultured Arabidopsis cells with maximum values observed 7 to 10 d after transplanting (Fig. 7). Hitherto, the PSK- $\alpha$  peptide has not been detected in any intact plants with chemical and immunological methods. We previously detected *Os-PSK* transcripts in rice seedlings by reverse transcriptase-PCR (Yang et al., 1999b), and here, we could detect transcripts of both the *AtPSK2* and

*AtPSK3* genes in Arabidopsis plants by northern hybridization (Fig. 8), indicating that these PSK precursor genes may also function in whole plants.

*AtPSK2* and *AtPSK3* transcripts accumulated in all tissues tested (Fig. 8), suggesting that these two genes may act redundantly. *AtPSK1* and *AtPSK5* genes are also expressed in a similar pattern (data not shown). Such pairs of genes allow functional stabilization in case one becomes switched off. To clarify the mode of action of *AtPSK2* or *AtPSK3* genes in intact plants, it is of importance to make transgenic plants in which they are inactivated, both individually and in combination.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Seeds of Arabidopsis ecotype Columbia (collection no. Col-0) were purchased from Lehle Seeds (Round Rock, TX), sown on vermiculite, and grown in a greenhouse at 25°C under continuous illumination from daylight fluorescent tubes providing 150 mmol m<sup>-2</sup> s<sup>-1</sup> (FL40SBRN, Toshiba, Tokyo). Plants 28 d after germination were used as the source of RNA for northern-blot analysis.

### Establishment of Arabidopsis Cell Suspensions

Arabidopsis seeds (collection no. Col-0) were surface sterilized for 5 min in 70% (v/v) ethanol, washed three times with sterile distilled water, transferred to 5% (w/v) NaOCl for 10 min, rinsed five times with sterile distilled water, and placed on 150 × 25-mm petri dishes containing Murashige and Skoog medium (Murashige and Skoog, 1962) to germinate. Plants were grown at 25°C in a 16-h-light/8-h-dark cycle. The same growth room conditions were used for tissue culture procedures. Roots were collected from 28-d-old plants and cultured on B5 medium (Gamborg et al., 1968) supplemented with 0.5 mg L<sup>-1</sup> 2,4-D and 0.05 mg L<sup>-1</sup> KIN. Calli obtained from the explants were subcultured on the same medium at regular intervals of 2 weeks.

### Genomic DNA-Blot Analysis

Genomic DNA was extracted from the calli using the cetyl-trimethyl-ammonium bromide method (Murry and Thompson, 1980). Aliquots of 10 μg of DNA were digested with restriction endonucleases, separated on 0.8% (w/v) agarose gels, and transferred to Biodyne nylon filters (Pall, New York) in 20× SSC. Hybridization was executed in a solution of 5× SSC, 0.5% (w/v) SDS, 5× Denhardt's solution, and 500 μg mL<sup>-1</sup> of salmon sperm DNA at 50°C and 65°C using the <sup>32</sup>P-labeled full-length cDNA prepared with a Random Primed DNA Labeling Kit (Takara, Shiga, Japan). Washing after hybridization was performed with 2× SSC at 25°C for 15 min, three times, and then 2× SSC containing 0.1% (w/v) SDS at 50°C and 65°C for 15 min, three times.

### Construction and Screening of Genomic Library

Genomic DNA (50 μg) was partially restricted with *Sau3AI* to generate *Bam*HI-compatible fragments ranging in size from 9 to 23 kb. The genomic fragments (0.3 μg) were inserted into the *Bam*HI sites of EMBL3 vectors that had been digested with *Bam*HI and *Eco*RI (Stratagene, La Jolla, CA) and packaged with Gigapack III Gold Packaging Extract (Stratagene) to construct a genomic library. Two independent cDNAs encoding PSK precursors were found in the Arabidopsis EST database in June 1999 by a BLAST homology search using the sequence of the PSK domains. The EST clones (accession nos. 120C15T7 and 91K2T7) were ordered from the DNA Stock Center of the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The inserts were purified, sequenced, and then used to screen the genomic library by plaque hybridization. Hybridization was performed as described above.

### DNA Sequence Analysis

The inserts of positive phages were subcloned into pBS SK<sup>-</sup> plasmids (Stratagene). *Escherichia coli* strain JM109 was used as the host for the plasmids. Deletion clones were generated for DNA sequencing with a Kilo Sequencing Kit (Takara) according to the protocol recommended by the manufacturer. The plasmid DNA templates were amplified with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) in accordance with the manufacturer's protocols.

### Construction of Chimeric Genes

A 22-mer primer (5'-TCTTCTGGGAATAGATGTAATC-3') based on the nucleic acid sequence of the *AtPSK2* cDNA and a 24-mer primer (5'-CTGAGAATAAATGTAATCGG-TGTG-3') based on the nucleic acid sequence of the *AtPSK3* cDNA were synthesized and used to obtain the mutated cDNAs that were designed to produce unnatural [Ser-4]PSK-α instead of PSK-α. Mutations were introduced with an LA PCR In Vitro Mutagenesis Kit (Takara) and confirmed by sequencing. The pMAT037 (Matsuoka and Nakamura, 1991) was employed as a binary vector for Arabidopsis root transformation. Original or mutated cDNAs were digested with *Sac*I and *Hind*III or *Kpn*I and inserted in frame into the same sites of the vectors to construct chimeric genes harboring the cDNA in sense or antisense orientations, respectively. Expression of the chimeric genes was driven by the promoter of the cauliflower mosaic virus 35S transcript.

### Transformation of Arabidopsis Roots

Constructs were transformed into *Agrobacterium tumefaciens* (C58C1Ri<sup>6R</sup>) by triparental mating (Van Haute et al., 1983), and *A. tumefaciens*-mediated transformation of roots of Arabidopsis Columbia ecotype was essentially as described (Valvekens et al., 1988). Transgenic microcalli were generated by incubating cocultivated root explants for 3

weeks on the callus induction medium (B5 + 0.5 mg L<sup>-1</sup> 2,4-D and 0.05 mg L<sup>-1</sup> KIN) supplemented with 50 mg L<sup>-1</sup> kanamycin sulfate (Life Technologies, Grand Island, NY) and 750 mg L<sup>-1</sup> vancomycin hydrochloride (Wako, Osaka). The individual regenerating microcalli were transferred to fresh medium to ensure that all transformants were due to independent T-DNA insertions. To detect the introduced genes, genomic DNA was isolated from transgenic calli, digested with *Bam*HI, and allowed to hybridize with the labeled cDNA as described above. Arabidopsis cells transformed with the binary vector alone served as controls.

### Purification of PSKs from CM

CM was prepared from 14-d-cultured calli by filtration (Advantec no. 2) and stored at -20°C until use. Eighty-milliliter aliquots of each CM were buffered by adding Tris to a final concentration of 20 mM, adjusted to pH 8.0 with 6.0 N HCl, and then applied to a DEAE Sephadex A-25 column (1.7 × 8 cm, Pharmacia, Piscataway, NJ) which was first equilibrated with 20 mM Tris-HCl buffer at pH 8.0. The column was washed with 50 mL of equilibration buffer and eluted successively with 50 mL of buffer containing 0, 400, 800, then 1,200 mM KCl at a flow rate of 60 mL h<sup>-1</sup>. Trifluoroacetic acid (TFA) was added to a final concentration of 0.1% (v/v) into the last two fractions containing PSKs, and then the samples were applied to a Sep-Pak Vac column (12 cc, C18, Millipore, Tokyo) after equilibration with 0.1% (v/v) TFA. The column was washed with 30 mL of the same buffer at a flow rate of 60 mL h<sup>-1</sup> and eluted with 30 mL of 30% (v/v) acetonitrile containing 0.1% (v/v) TFA. The fraction containing PSKs was collected and lyophilized for subsequent analyses.

### LC/MS Analysis

Mass spectra were obtained using a Fisons VG platform quadrupole mass spectrometer with electrospray ionization interfaced to a Jasco PU 980 HPLC system. The fraction containing PSKs was dissolved in 200 μL of water and separated on a reverse-phase HPLC column (Develosil ODS-HG-5, 4.6 × 250 mm, Nomura Chemicals, Seto, Japan) with 10% (v/v) acetonitrile containing 0.1% (v/v) TFA at 1.0 mL min<sup>-1</sup>. The HPLC eluate was split 1:9 (v/v) so that 100 μL min<sup>-1</sup> flowed to the mass spectrometer during the separation. The pseudomolecular ions of PSKs were scanned every 1.9 s with selected ion monitoring at *m/z* 703 ([M-H]<sup>-</sup> of [Ser-4]PSK-β) and *m/z* 717 ([M-H]<sup>-</sup> of PSK-β). The semiquantitative amounts of the PSK-β and [Ser-4]PSK-β in CM were measured based on the peak heights without an internal standard.

### Expression Analysis of the *AtPSK2* and *AtPSK3* Genes

Total RNAs were isolated from Arabidopsis roots, leaves, shoots, or culture cells incubated for 1, 3, 7, 10, or 14 d as reported (Chomczynski, 1993). Twenty-microgram aliquots were denatured at 65°C for 5 min in 50% (v/v) formamide, 1× MOPS [3-(*N*-morpholino)-propanesulfonic

acid] (200 mM MOPS, 10 mM EDTA, and 50 mM NaOAc, pH 7.0) and 1.5% (v/v) formaldehyde. The RNAs were then fractionated by electrophoresis on a 1.2% (v/v) agarose gel containing 2.2 M formaldehyde, and subsequently transferred to Biodyne nylon membranes (Pall) in 20× SSC and then allowed to hybridize with <sup>32</sup>P-labeled cDNA as described above.

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