sion in *Drosophila* embryos has revealed the existence of unknown molecular mechanisms in this well-studied prototype system for embryonic development (*26*).
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29. The backbone plasmid into which the library was cloned contains elements (in this order) as follows: the *kan*^{R} gene surrounded by Dra III sites, the promoter P$^\lambda$_ followed by the *gfpmut3* (*21*) allele of GFP, the transcriptional terminator T1, the SC101* origin of replication (*23*), and the ampicillin resistance gene *bla*. This plasmid was digested with Dra III, and the larger fragment containing P$^\lambda$_-*gfp*-T1-SC101*-*bla* was gel-purified and used in the ligation reaction for constructing the library. The Dra III sites were designed such that Dra III digestion exposed cohesive ends compatible with the 5′ and 3′ ends of the assembled networks. The library DNA was transformed into *E. coli* strains by electroporation with the use of standard protocols.
30. Plasmid pOd O_{id} 70.5 O_1 was a gift of B. Müller-Hill; source plasmids for $P_L$lacO1, $P_L$tetO1, and SC101* origin of replication were kindly provided by H. Bujard. We would like to thank the Silhavy lab for strain CLC90, A. Beavis for great help and expertise with FACS, and people for helpful discussions as follows: U. Alon, P. Cluzel, S. Da Re, B. Houchmandzadeh, R. Kishony, T. Lecuit, A. J. Levine, A. C. Maggs, J. Merrin, I. Mihalcescu, A. W. Murray, N. Questembert-Balaban, B. I. Shraiman, T. J. Silhavy, M. G. Surette, S. Tavazoie, J. M. G. Vilar. M.B.E. is supported by the Burroughs-Wellcome Fund and the Seaver Institute. This work was partially supported by a grant from the National Institutes of Health.

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An LRR Receptor Kinase Involved in Perception of a Peptide Plant Hormone, Phytosulfokine

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The sulfated peptide phytosulfokine (PSK) is an intercellular signal that plays a key role in cellular dedifferentiation and proliferation in plants. Using ligand-based affinity chromatography, we purified a 120-kilodalton membrane protein, specifically interacting with PSK, from carrot microsomal fractions. The corresponding complementary DNA encodes a 1021–amino acid receptor kinase that contains extracellular leucine-rich repeats, a single transmembrane domain, and a cytoplasmic kinase domain. Overexpression of this receptor kinase in carrot cells caused enhanced callus growth in response to PSK and a substantial increase in the number of tritium-labeled PSK binding sites, suggesting that PSK and this receptor kinase act as a ligand-receptor pair.

The relative growth rate of plant cells in culture strictly depends on the initial cell density, even if sufficient amounts of auxin and cytokinin are supplied, indicating that additional factors play a role in cell proliferation (*1, 2*). One such factor is the 5–amino acid peptide phytosulfokine (PSK), which has sulfated Tyr residues [Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln] (*3*). Together with auxin and cytokinin, PSK induces plant cells to dedifferentiate and reenter the cell cycle at nanomolar concentrations (*3, 4*). PSK is processed from the COOH-terminal region of ~80–amino acid precursor proteins ubiquitously expressed in the leaf, apical meristem, hypocotyl, and root of seedlings, as well as in suspension cells in culture (*5*). It has been proposed that mature PSK is secreted from individual cells in response to changes in the levels of auxin and cytokinin (*4*) and that it functions as an autocrine-type growth factor to regulate cellular dedifferentiation and proliferation in plants.

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Evidence for the existence of high-affinity binding sites for PSK has been provided by binding assays with radiolabeled PSKs (*6, 7*). The observed binding is saturable, reversible, and localized in plasma membrane fractions. In addition, photoaffinity cross-linking analysis has shown that the putative receptors for PSK in rice plasma membrane are 120- and 160-kD glycosylated proteins (*8*). In the present study, we performed purification, molecular cloning, and functional expression of the PSK receptor to gain further insight into the molecular basis of signal transduction triggered by PSK.

For the purification of membrane proteins that specifically interact with PSK, we used microsomal fractions derived from the carrot cell line NC, which has been found to contain a relatively high concentration of high-affinity PSK-binding proteins: ~150 fmol per mg of microsomal proteins, with a dissociation constant (K_d) of 4.2 ± 0.4 nM, as determined by a [^{3}H]PSK binding assay (*9*) (Fig. 1, A and B). This K_d value is consistent with physiological concentrations of PSK in carrot suspension cultures (*10*), and it is also consistent with the PSK concentration that induces a 50% cell division of dispersed mesophyll cells (*3*). Photoaffinity labeling of NC membrane proteins with a photoactivatable PSK analog (*8*) indicated that a 120-kD protein and a minor 150-kD protein specifically interact with PSK (Fig. 1C). Both proteins contain ~10 kD of N-linked oligosaccharide chains that can be cleaved by treatment with peptide *N*-glycosidase F (PNGase F) (Fig. 1C).

We purified these PSK-binding proteins from the microsomal fractions of NC cells by Triton X-100 solubilization and specific ligand-based affinity chromatography using a [Lys^5]PSK-Sepharose column containing a long spacer chain between the ligand and matrix (*9*) (fig. S1). Elongation of the Lys^5 side chain of [Lys^5]PSK does not interfere with its binding affinity and specificity (*11*). Proteins specifically eluted by PSK were further purified by hydroxyapatite column chromatography and concentrated by ultrafiltration (*9*). SDS–polyacrylamide gel electrophoresis (PAGE) and Nile red staining of the proteins in the fractions eluted by PSK showed specific recovery of a major 120-kD protein and a minor 150-kD protein (Fig. 1D). Both of these proteins were absent in the fractions eluted by [2-5]PSK, a synthetic analog of PSK with no biological or binding activities (*12*) (Fig. 1D). PNGase F treatment of these two proteins decreased their apparent sizes to 110 and 140 kD, respectively, suggesting that they are identical to the proteins we detected in photoaffinity cross-linking experiments (Fig. 1D; see also Fig. 1C).

Four independent purifications were performed, yielding 50 μg of the major 120-kD protein from 4800 mg of microsomal proteins, with an overall recovery rate of 40%. The protein was digested with TPCK-trypsin (TPCK, tosyl phenylalanyl chloromethyl ketone), and peptide fragments thus generated were separated by reversed-phase high-performance liquid chromatography (HPLC) (*9*) (Fig. 1E). We analyzed the fragments of the 120-kD protein contained in 15 independent peaks, using a protein sequencer and MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spec-

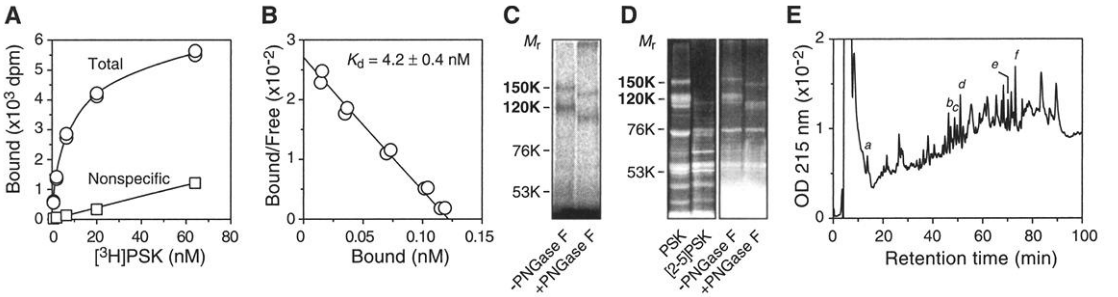

Fig. 1. (**A**) [^{3}H]PSK binding to microsomal fractions of carrot NC cells (dpm, disintegrations per minute). (**B**) Scatchard plot of the binding data in (A). (**C**) Photoaffinity labeling and PNGase F treatment of the PSK-binding proteins. M_r, relative molecular mass; +PNGase F indicates treatment with PNGase F. (**D**) SDS-PAGE analysis of the affinity-purified proteins. Proteins eluted by PSK or [2-5]PSK from the [Lys^5]PSK-Sepharose column were further purified by hydroxyapatite column chromatography, concentrated by ultrafiltration, and analyzed by SDS-PAGE. PNGase F treatment of the PSK-eluted proteins indicated that the 120- and 150-kD proteins both contain ~10 kD of N-linked oligosaccharide chains. (**E**) Reversed-phase HPLC profile of the tryptic digest of the purified 120-kD protein. Each peak was collected and sequenced by a protein sequencer and MALDI-TOF MS. The peptide sequences of peaks *a* to *f* are as follows (*20*): *a*, LSESVAK; *b*, SSVSLGLDDVNESGR; *c1*, EFQAEVETLSR; *c2*, QLHVLNLK; *d*, ALEGFMR; *e*, ENAVEEPSPDFPFFK; and *f*, VLNVYENSFHGLIPASLCNNLPR. Peak *c* contains two peptide fragments. OD 215 nm, optical density at 215 nm.

trometry), and obtained the complete amino acid sequences of seven internal peptides from six peaks (Fig. 1E, peaks *a* to *f*). Using the same procedure, we found three internal peptide sequences in the minor 150-kD protein (*13*). The three internal sequences of the 150-kD protein were identical to the *c1*, *c2*, and *f* sequences of the 120-kD protein (Fig. 1E, legend), which suggests that the peptide backbone of the 150-kD protein is identical to that of the 120-kD protein. Of the seven internal peptides of the 120-kD protein, amino acid sequences of three peptides (*c1*, *e*, and *f*) were used to synthesize 10 degenerate oligonucleotides, which were used as nested primers in polymerase chain reaction (PCR) amplification of carrot NC first-strand cDNAs. Of the six primer pairs tested, a specific PCR product was obtained only with the primer set based on peptides *e* and *f* (*9*).

Using the PCR product as a hybridization probe, we screened the carrot NC cDNA library and isolated a 3.5-kb cDNA clone. Analysis of the amino acid sequence of the longest open reading frame (3.1 kb) revealed an in-frame stop codon (TGA) at the –21 to –19 position, followed by the first ATG and the entire coding sequence. The cDNA encoded a 1021–amino acid protein, with a deduced molecular mass of 112 kD, that had features found in several hormone receptors in plants and animals (Fig. 2A). It contained an NH_2-terminal hydrophobic signal sequence, extracellular leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic kinase domain (Fig. 2B). Seventeen potential N-linked glycosylation sites were also found, as predicted by the results of deglycosylation experiments. The calculated molecular mass of this protein, without the signal sequence, was 109.5 kD, which is in good agreement with the mass estimated by SDS-PAGE analysis of the deglycosylated protein. The major extracellular domain of this protein contained 21 tandem copies of a 24–amino acid LRR (fig. S2A); it has been suggested that this string of LRRs plays a key role in protein-protein interactions (*14*). In addition, a 36–amino acid island was detected in the 18th LRR. An island domain has also been found among the extracellular LRRs of the brassinosteroid receptor BRI1 and has been shown to be critical for its function (*15*). The cytoplasmic region of the predicted amino acid sequence contains all 12 subdomains found in almost all eukaryotic serine-threonine kinases (fig. S2B) (*16*). The kinase region of this protein shares substantial sequence identity with those of the known plant hormone receptors BRI1 and CLV1 (*17*) (fig. S2B).

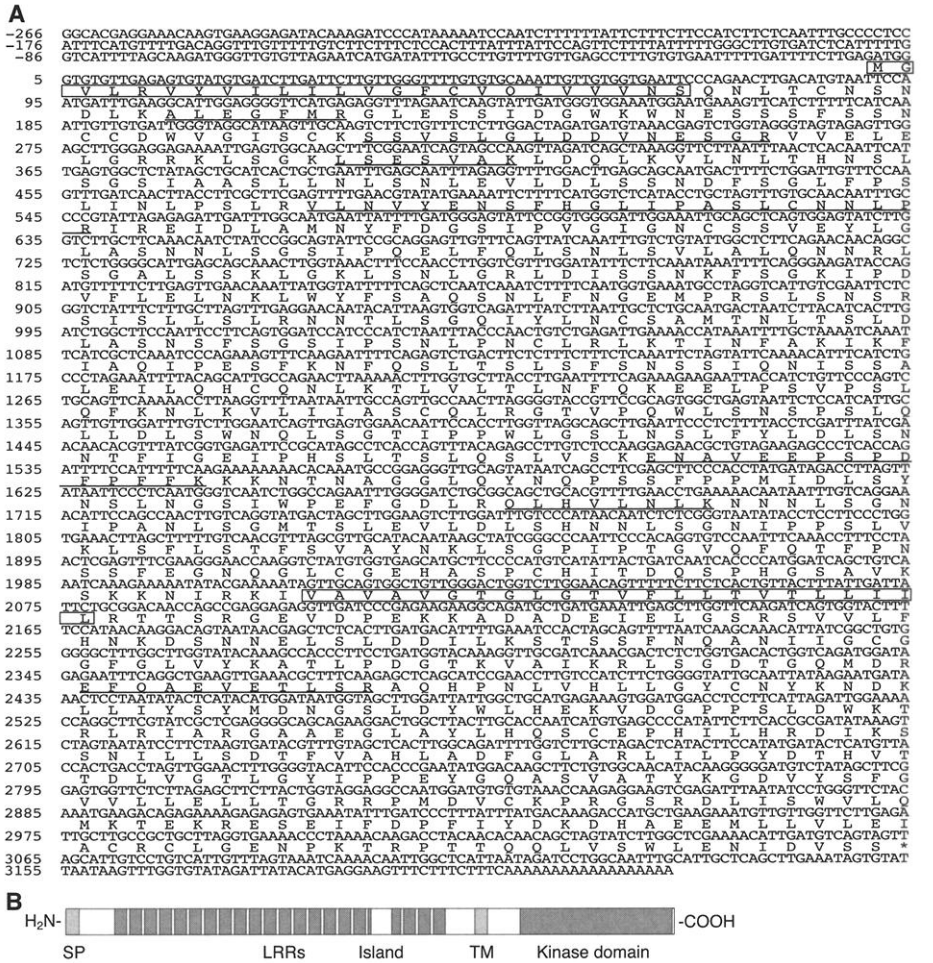

Fig. 2. (**A**) Nucleotide sequence of cDNA encoding the 120-kD protein, as well as the deduced amino acid sequence (GenBank accession number AB060167) (*20*). The regions corresponding to a possible signal peptide and a predicted transmembrane domain are boxed. Sequences found in tryptic peptides are underlined. (**B**) Schematic of the 120-kD receptor kinase. The diagram shows the signal peptide (SP), extracellular LRRs, a 36–amino acid island, a transmembrane domain (TM), and a cytoplasmic kinase domain.

Southern blot analysis of Xba I and Hind III digests of carrot NC genomic DNA with the full-length cDNA of this protein indicated that a single gene encodes this protein (fig. S3). We also used Northern blot analysis to examine the expression pattern of the corresponding gene and detected a single species of mRNA of ~3.5 kb in NC cells (Fig. 3A). This mRNA accumulated ubiquitously in leaf, apical meristem, hypocotyl, and root of carrot seedlings, although its expression level was far lower than that in cultured NC cells (Fig. 3A).

To test whether this receptor kinase is a component of the functional PSK receptor, we analyzed the effect of overexpression of this protein on PSK binding activity in membrane fractions (*9*). Transgenic carrot cells overexpressing the cDNA of this protein in sense orientation under the regulation of the cauliflower mosaic virus 35S promoter (Fig. 3A, rightmost lanes) showed accelerated growth compared with control cells in response to PSK (Fig. 3B and table S1). In contrast, expression of the antisense strand substantially inhibited callus growth. These phenotypes are consistent with the hypothesis that overexpression and antisense inhibition of this receptor kinase protein alter the response of carrot cells to PSK. We also observed a sizable increase in PSK binding activity in the membrane fractions of the sense transformants (Fig. 3, C and D). This increase in binding was due to an increase in the number of binding sites [sense transformant, B_{max} = 570 ± 18 fmol per mg of membrane protein; control B_{max} = 34 ± 2 fmol per mg of membrane protein (B_{max}, maximum number of binding sites)], with similar binding affinities (sense transformant, K_d = 4.1 ± 0.5 nM; control, K_d = 4.8 ± 1.1 nM). Photoaffinitycross-linking and immunoprecipitation analysis (*9*) of the membrane proteins derived from the sense transformants revealed the expression of the 150-kD protein in addition to the 120-kD protein, indicating that both proteins are encoded by a single gene (Figs. 3E and S4). We also characterized the specificity of the PSK binding activity by comparing the relative binding affinity for several PSK analogs (*12*) in competition binding assays (Fig. 3F). Binding of [^{3}H]PSK to the membrane fraction of sense transgenic cells was strongly inhibited by unlabeled PSK, less strongly inhibited by the less active analog [1-4]PSK, and not inhibited at all by the inactive analog [2-5]PSK. Such high specificity and affinity for PSK strongly suggest that this receptor kinase is a component of a functional PSK receptor that directly interacts with PSK.

The transgenic cells expressing high levels of sense mRNA of this receptor kinase showed accelerated proliferation, but they were unable to regenerate roots and shoots (Fig. 3G). In contrast, antisense transformants showed substantial inhibition of callus growth (Fig. 3B). One hypothesis that fits these observations is that the PSK receptor, in response to PSK binding, regulates a signaling cascade involved in cellular dedifferentiation, maintenance of the dedifferentiated state, and acquisition of competence for cellular redifferentiation in plants. PSK has been shown to stimulate tracheary element differentiation of *Zinnia* mesophyll cells (*18*) and somatic embryogenesis of carrot cells (*19*) in the presence of certain ratios and concentrations of auxin and cytokinin. Overexpression of the PSK receptor may lead to overactivation of PSK signaling in response to endogenous PSK, thereby modulating cellular competence for redifferentiation.

Plant cells retain features characteristic of totipotent stem cells; that is, they have the potential to dedifferentiate, reenter the cell cycle, proliferate, and give rise to all organs of a new plant. However, little is known about the early events that accompany cellular dedifferentiation and regeneration. Understanding how PSK and the PSK receptor are involved in these cellular events at the molecular level may have profound implications for the study of plant totipotency and also may reveal general mechanisms of ligand-receptor interactions in plants.

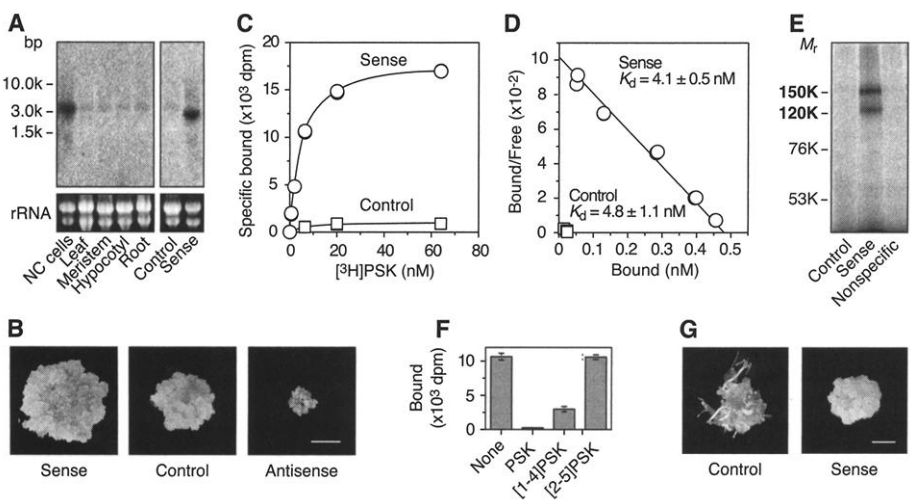

Fig. 3. **(A)** Northern blot analysis of the 120-kD receptor kinase mRNA. The total RNA was isolated from the NC cells, various parts of 2-week-old carrot seedlings, and transformed cells. rRNA, ribosomal RNA; bp, base pairs. **(B)** Callus growth of sense and antisense transformants and control cells exposed to 10 nM PSK. Transformed carrot cells and control cells were cultured for 3 weeks on B5 media containing naphthaleneacetic acid (NAA, 1.0 mg/liter), 6-benzylamino purine (6-BA, 0.5 mg/liter), and 10 nM PSK. Representative data of one of three independent experiments are shown. Scale bar, 1 cm. **(C)** Specific [^{3}H]PSK binding to the membranes of sense transformants and control cells. **(D)** Scatchard plot of the binding data in (C). **(E)** Photoaffinity labeling of the membrane proteins derived from control cells and sense transformants. Nonspecific binding for sense transformants was determined in the presence of excess unlabeled PSK. **(F)** Inhibition of [^{3}H]PSK binding to membrane fractions of sense transformants by the competitors PSK, [1-4]PSK, and [2-5]PSK. Membrane proteins were incubated in binding buffer containing 6.3 nM [^{3}H]PSK and 3.2 μM competitor. Error bars indicate ± SE from three independent experiments. **(G)** Loss of regeneration ability in sense transformants. Transformed carrot cells and control cells were cultured for 4 weeks on B5 media without plant hormones to induce plant regeneration. Scale bar, 1 cm.

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20. Single-letter abbreviations for the amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S4
Table S1
References

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