Disruption and Overexpression of Arabidopsis Phytosulfokine Receptor Gene Affects Cellular Longevity and Potential for Growth[1]

Yoshikatsu Matsubayashi*, Mari Ogawa, Hitomi Kihara, Masaaki Niwa, and Youji Sakagami

Graduate School of Bio-Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464–8601, Japan

Phytosulfokine (PSK), a 5-amino acid sulfated peptide that has been identified in conditioned medium of plant cell cultures, promotes cellular growth in vitro via binding to the membrane-localized PSK receptor. Here, we report that loss-of-function and gain-of-function mutations of the Arabidopsis (*Arabidopsis thaliana*) PSK receptor gene (*AtPSKR1*) alter cellular longevity and potential for growth without interfering with basic morphogenesis of plants. Although mutant *pskr1-1* plants exhibit morphologically normal growth until 3 weeks after germination, individual *pskr1-1* cells gradually lose their potential to form calluses as tissues mature. Shortly after a *pskr1-1* callus forms, it loses potential for growth, resulting in formation of a smaller callus than the wild type. Leaves of *pskr1-1* plants exhibit premature senescence after bolting. Leaves of *AtPSKR1ox* plants exhibit greater longevity and significantly greater potential for callus formation than leaves of wild-type plants, irrespective of their age. Calluses derived from *AtPSKR1ox* plants maintain their potential for growth longer than wild-type calluses. Combined with our finding that PSK precursor genes are more strongly expressed in mature plant parts than in immature plant parts, the available evidence indicates that PSK signaling affects cellular longevity and potential for growth and thereby exerts a pleiotropic effect on cultured tissue in response to environmental hormonal conditions.

Plants, due to their sessile nature, have developed a greater ability to adapt to dynamic environmental conditions than have animals. This plasticity allows plants to flexibly alter their developmental program and metabolism according to the environment. A particularly important adaptation of this type is the ability to form calluses from almost any plant tissue. However, studies suggest that a population of living cells is often required to support callus growth in vitro even if sufficient amounts of growth regulators and nutrients are supplied. This population dependence is alleviated by addition of conditioned medium in which cells have previously been grown, indicating that such cell-to-cell communication is mediated by chemical signal(s) produced by growing cells (Bellincampi and Morpurgo, 1987; Birnberg et al., 1988).

Phytosulfokine (PSK), a 5-amino acid sulfated peptide that has been detected in conditioned medium of plant cell cultures, is the primary signal molecule responsible for this cell-to-cell communication (Matsubayashi and Sakagami, 1996). Addition of chemically synthesized PSK to culture medium, even at nanomolar concentrations, significantly increases the rate of callus growth even when the initial cell population is below the critical density. PSK also promotes in vitro tracheary element differentiation of mesophyll cells (Matsubayashi et al., 1999b), somatic embryogenesis (Kobayashi et al., 1999; Hanai et al., 2000; Igasaki et al., 2003), adventitious root formation (Yamakawa et al., 1998), and pollen germination (Chen et al., 2000). When PSK is applied to plant seedlings at high concentrations, it retards senescence under stress conditions (Yamakawa et al., 1999).

PSK is produced from approximately 80-amino acid precursor peptides via posttranslational sulfation of Tyr residues and proteolytic processing (Yang et al., 1999, 2001). Genes encoding possible PSK precursors are redundantly distributed throughout the genome (Yang et al., 2001; Lorbiecke and Sauter, 2002) and are expressed in a variety of tissues in addition to calluses, including leaves and roots (Yang et al., 2001), suggesting that PSK plays a basic role in plant growth and development. The main difficulty in dissecting the in planta function of PSK is that PSK precursor genes are so small and redundant that the loss-of-function strategy is not applicable.

PSK binds the membrane-localized PSK receptor PSKR1, which is a Leu-rich repeat receptor kinase (LRR-RK) that has been purified from solubilized carrot (*Daucus carota*) microsomes by ligand-based affinity chromatography (hereafter referred to as DcPSKR1; Matsubayashi et al., 2002). Expression of *DcPSKR1* has been detected in the leaves, apical meristem, hypocotyl, and root of carrot seedlings, although much higher

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* Corresponding author; e-mail matsu@agr.nagoya-u.ac.jp; fax 81–52–789–4118.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Yoshikatsu Matsubayashi (matsu@agr.nagoya-u.ac.jp).

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expression has been detected in cultured carrot cells. Transgenic carrot calluses overexpressing *DcPSKR1* exhibit accelerated growth compared with control calluses.

Studies revealing the in vitro function of PSK and the molecular basis of ligand-receptor interaction in PSK signaling have paved the way for research aimed at characterization of the in vivo role of PSK and its downstream signaling pathway in plants. The carrot PSK receptor, DcPSKR1, exhibits high-percentage amino acid identity with one LRR-RK found in the Arabidopsis (*Arabidopsis thaliana*) genome. Also, a database search has revealed the presence of five paralogous PSK precursor genes in the Arabidopsis genome. In this study, we analyzed the Arabidopsis PSK receptor gene using gain-of-function and loss-of-function strategies and found that PSK signaling in plants affects their potential for growth and cellular longevity. We also examined the expression patterns of all five paralogous PSK precursor genes in the Arabidopsis genome using promoter-β-glucuronidase (GUS) analysis. In this article, we discuss possible functions of PSK signaling.

RESULTS

Five Paralogous PSK Precursor Genes in Arabidopsis

Upon analyzing the in planta role of PSK signaling, we first identified all the PSK precursor genes in Arabidopsis. In addition to two PSK precursor genes (At2g22860 and At3g49780) previously identified and confirmed to encode the functional PSK (Yang et al., 2001), we identified three paralogous genes (At1g13590, At3g44735, and At5g65870) encoding possible PSK precursors in an Arabidopsis genome database. Each predicted protein has a probable secretion signal at the N terminus and a single PSK sequence close to the C terminus. However, these proteins exhibit extreme diversity; e.g. only a small number of residues close to the C terminus are conserved (Fig. 1A). Functional expression of these novel three genes was confirmed by site-directed mutagenesis experiments in which Arabidopsis suspension cells were transformed with mutated cDNA designed to produce [Ser^4]PSK. In LC/MS analysis of culture medium derived from suspensions of transgenic cells, we detected sulfated form of 5-amino acid [Ser^4]PSK in addition to endogenous wild-type PSK (Fig. 1B). We therefore renamed these five PSK precursor genes as follows: *AtPSK1* (At1g13590), *AtPSK2* (At2g22860), *AtPSK3* (At3g44735), *AtPSK4* (At3g49780, formerly named *AtPSK3*), and *AtPSK5* (At5g65870).

Expression Patterns of PSK Precursor Genes in Arabidopsis

Northern blotting revealed that *AtPSK*s are expressed in a variety of tissues including roots, leaves, stems, flowers, siliques, and calluses, with the exception of *AtPSK1*, which was only expressed in roots (Fig. 1C). *AtPSK2*, *AtPSK4*, and *AtPSK5* were more strongly expressed in lower mature leaves than in upper young leaves (Fig. 1D), suggesting that the main in planta role of PSK is in plant homeostasis rather than morphogenesis. In addition, expression of *AtPSK4* is highly upregulated upon mechanical wounding (Fig. 1E).

To further analyze expression patterns of *AtPSK*s, we generated transgenic Arabidopsis plants harboring *AtPSK* promoter-GUS reporter gene constructs. Among the above-ground plant parts assayed, the constructs *pAtPSK2*::*GUS*, *pAtPSK3*::*GUS*, *pAtPSK4*::*GUS*, and *pAtPSK5*::*GUS* were widely expressed in cotyledons and leaves and were most abundantly expressed in vascular bundles (Fig. 1F). In roots, *pAtPSK3*::*GUS* was primarily expressed in root tips, whereas expression of *pAtPSK2*::*GUS*, *pAtPSK4*::*GUS*, and *pAtPSK5*::*GUS* was mainly detected in the more mature regions of the root. Within 12 h after leaf discs were cut, expression of *AtPSK4* had greatly increased at their outer edges, indicating that the *AtPSK4* promoter is activated by wounding (Fig. 1G).

Overexpression of PSK Precursor Gene

We generated transgenic Arabidopsis plants that overexpressed *AtPSK4*, the most prominently expressed PSK precursor gene throughout the Arabidopsis tissues, under the control of the 35S promoter, and named these plants *AtPSK4ox*. *AtPSK4ox* plants germinated normally and developed normal cotyledons and hypocotyls phenotypically indistinguishable from wild type. Growth of *AtPSK4ox* seedlings, especially root growth, was somewhat faster than wild-type growth (Fig. 1H). However, the overall growth of above-ground parts of *AtPSK4ox* plants was indistinguishable from that of wild type (data not shown). Growth of *AtPSK4ox* calluses derived from the leaves of 3-week-old plants was somewhat faster than growth of wild-type calluses (Fig. 1I).

Identification of PSK Receptor in Arabidopsis

To analyze PSK signaling in Arabidopsis, we searched for the Arabidopsis PSK receptor, based on overall amino acid similarity to DcPSKR1, and determined that At2g02220 is most likely an ortholog of *DcPSKR1*. At2g02220 encodes a 1,008-amino acid LRR-RK that has 60% amino acid sequence identity to DcPSKR1 and contains 21 tandem copies of LRR, a 36-amino acid island domain between the 17th and 18th LRR, a single transmembrane domain, and a cytoplasmic kinase domain (Fig. 2A). Amino acid sequences in the island domain are highly conserved between DcPSKR1 and At2g02220 (Fig. 2B). An island domain has also been found among the extracellular LRRs of the brassinosteroid receptor BRI1 and has been shown to be involved in ligand binding (Kinoshita et al., 2005). At2g02220 belongs to the LRR X subfamily (Shiu and Bleecker, 2001), which contains BRI1, BRL1, BRL2, BRL3 (Cano-Delgado et al., 2004; Zhou et al., 2004), and EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS required for the specialization of tapetal cells (Canales et al., 2002; Zhao et al., 2002; Fig. 2C).

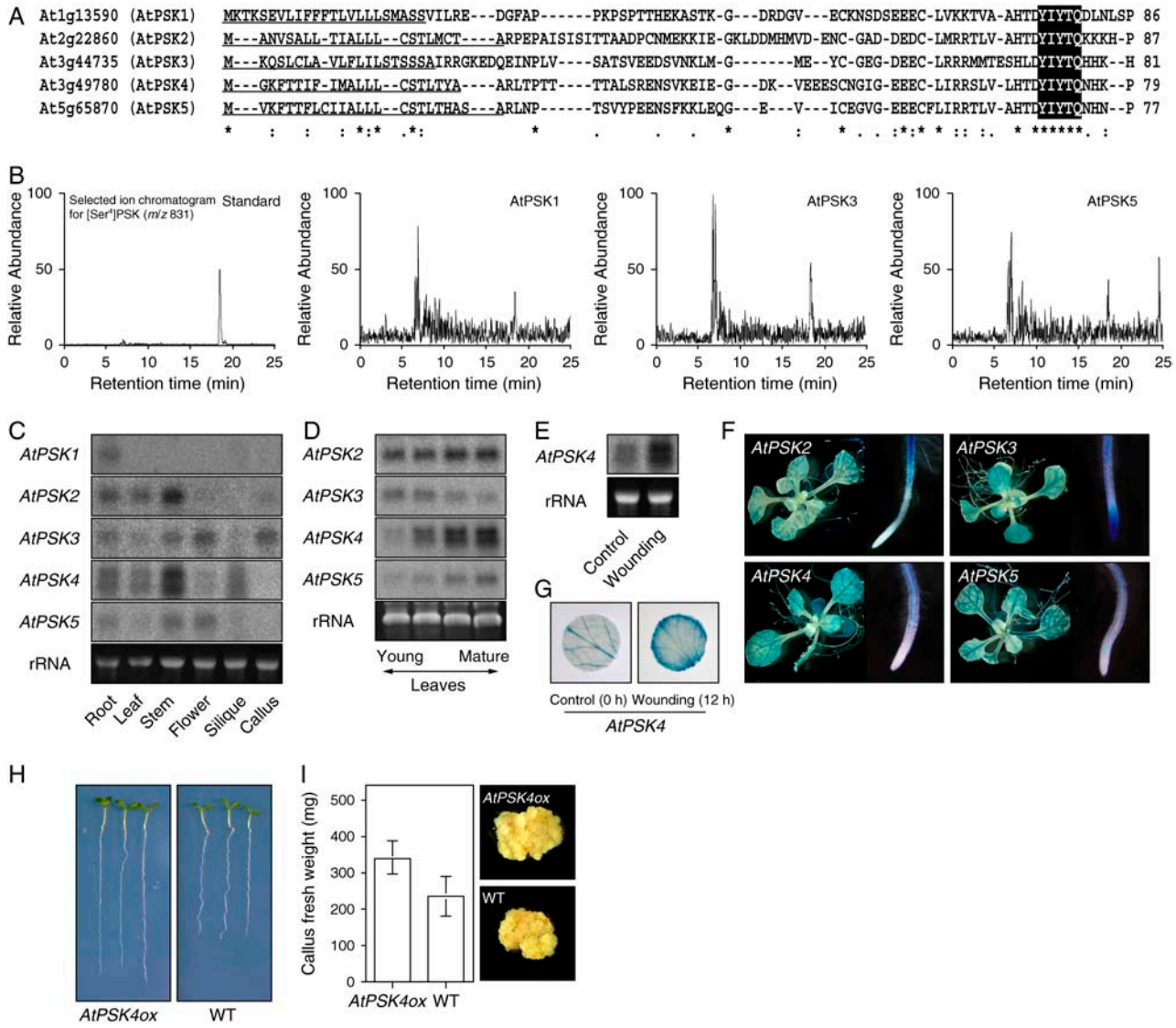

Figure 1. Five paralogous genes encoding PSK precursors in Arabidopsis. A, Sequence alignments of deduced amino acid sequences of *AtPSKs*. PSK domain is in the black box, and the predicted signal peptides are underlined. The residues conserved within all sequences are indicated by asterisks, and similar residues are indicated by dots. B, LC/MS analysis of culture medium derived from suspensions of transgenic cells expressing mutated *AtPSKs* designed to produce [Ser^4]PSK. Data are plotted as a selected ion chromatogram for mass-to-charge ratio 831 corresponding to the [M-H]$^-$ ion of [Ser^4]PSK. C, Northern-blot analysis of expression of *AtPSKs* in 3-week-old Arabidopsis plants and calluses. D, Comparison of expression of *AtPSKs* between upper young leaves and lower mature leaves of 3-week-old Arabidopsis plants. E, Wound-induced up-regulation of *AtPSK4* transcripts. F, Histochemical GUS staining of 3-week-old transgenic Arabidopsis plants expressing *pAtPSKs*::*GUS* fusions. G, Histochemical GUS staining of leaf discs derived from 3-week-old transgenic Arabidopsis plants expressing *pAtPSK4*::*GUS*. H, Growth of *AtPSK4ox* seedlings. Seedlings were grown on B5 agar plate for 8 d. I, Growth of *AtPSK4ox* and wild-type calluses. All calluses were cultured on CIM for 4 weeks. Values are means ± SD of five calluses.

To determine whether At2g02220 protein specifically interacts with PSK, we first overexpressed At2g02220 in Arabidopsis plants under the control of a constitutive 35S promoter and established suspension cell lines derived from these transgenic plants by inducing callus formation from leaf discs (overexpressor [OX1] cell line). We observed some increase in [^{3}H]PSK binding activity in the membrane fractions of the transgenic calluses, but neither the transgenic plants nor the transgenic calluses exhibited specific phenotypes (data not shown). In western blotting of microsomal fractions from cells using an anti-At2g02220 antibody, two distinct bands were recognized by the antibody: a 120-kD protein and a 150-kD protein. Compared to wild-type cells, the expression level of the 120-kD protein was increased in the transgenic cells, but the expression level of the 150-kD protein was unchanged, possibly due to posttranslational regulation (Fig. 2D).

We next overexpressed At2g02220 in Arabidopsis plants by increasing its copy number and established

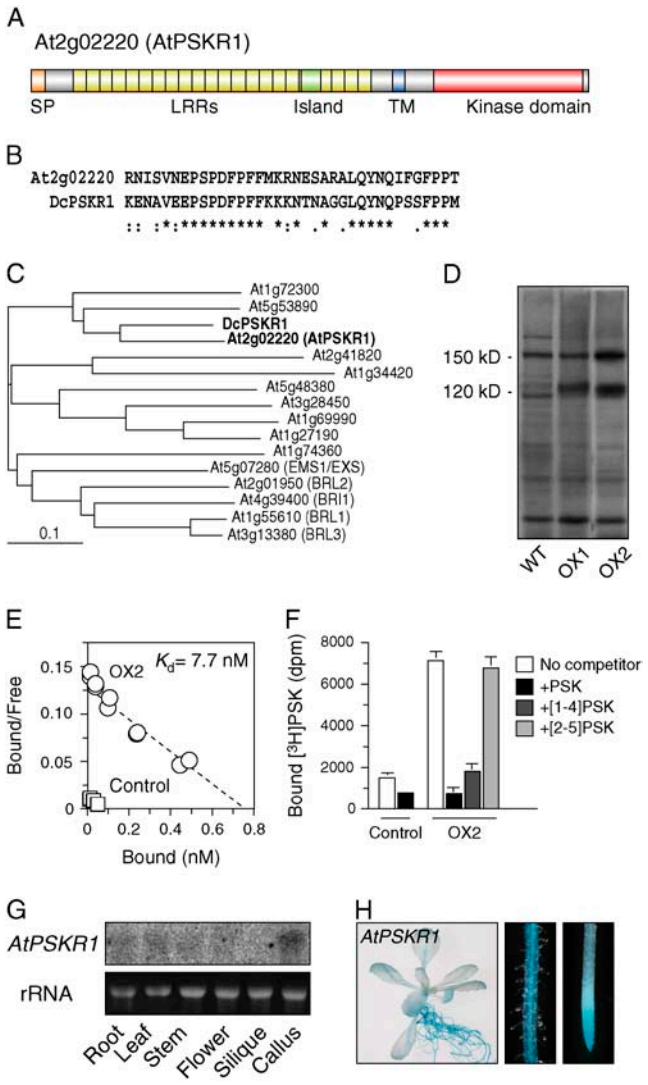

Figure 2. PSK binds At2g02220 receptor kinase (AtPSKR1). A, Schematic of At2g02220 protein. The diagram shows the signal peptide (SP), extracellular LRRs, a 36-amino acid island domain, a transmembrane domain (TM), and a cytoplasmic Ser/Thr kinase domain. B, Sequence alignments of the island domain of At2g02220 and DcPSKR1. The residues conserved within both sequences are indicated by asterisks, and similar residues are indicated by dots. C, A phylogenetic tree of Arabidopsis LRR X subfamily and DcPSKR1. Amino acid sequences of the kinase domain were aligned with ClustalW, and the graphical output was produced by TreeView. D, Immunoblot analysis of proteins in microsomal fractions of Arabidopsis callus cells overexpressing At2g02220 under control of constitutive 35S promoter (OX1, middle lane), or overexpressing At2g02220 under control of its own promoter by increasing copy number (OX2, right lane). E, Scatchard plot of the specific [^{3}H]PSK binding data for microsomal fractions of Arabidopsis cells overexpressing At2g02220 under control of its own promoter by increasing copy number (OX2). F, [^{3}H]PSK binding to microsomal fractions of OX2 cells in the presence or absence of 100-fold unlabeled PSK analogs. G, Expression patterns of *AtPSKR1* in 3-week-old Arabidopsis plants. H, Histochemical GUS staining of 3-week-old transgenic Arabidopsis plants expressing *pAtPSKR1*::*GUS* fusions.

suspension cell lines derived from these plants by inducing callus formation from leaf discs (OX2 cell line). We observed a significant increase in [^{3}H]PSK binding activity in the membrane fractions of the OX2 transgenic cells (Fig. 2E). Scatchard analysis of the binding data indicated that [^{3}H]PSK binds At2g02220 with a dissociation constant (K_d) of 7.7 $\pm$ 0.9 nM, which is similar to the K_d of DcPSKR1 (Matsubayashi et al., 2002). Western blotting of microsomal fractions from OX2 transgenic cells using an anti-At2g02220 antibody revealed significant increases in levels of both the 120- and 150-kD species (Fig. 2D). We also confirmed the specificity of binding between [^{3}H]PSK and At2g02220 by comparing relative binding affinity among several PSK analogs in competition binding assays (Fig. 2F). Binding of [^{3}H]PSK to the membrane fraction of OX2 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 affinity and specificity for PSK strongly suggest that At2g02220 is a functional Arabidopsis PSK receptor (referred to here as AtPSKR1). This transgenic Arabidopsis plant exhibited specific phenotypes of plant growth and callus proliferation (discussed below). Northern blotting and promoter analysis showed that *AtPSKR1* is weakly but widely expressed in roots, leaves, stems, and flowers of 3-week-old Arabidopsis plants as well as calluses (Fig. 2, G and H).

Identification of Loss-of-Function Mutant of *AtPSKR1*

To examine the physiological function of *AtPSKR1*, we performed a database search for loss-of-function *AtPSKR1* mutants and found a *Ds* insertion mutant (hereafter referred to as *pskr1-1*) at the *AtPSKR1* locus in transposon-tagging lines released by the Institute of Molecular Agrobiology, the National University of Singapore (Fig. 3A; Parinov et al., 1999). Sequencing of the *Ds* insertion region revealed that *Ds* was inserted into a site 1,303 bp downstream from the initiation codon, corresponding to the 15th LRR of the extracellular domain. Treatment of *pskr1-1* hypocotyl segments with auxin/cytokinin resulted in development of apparently normal calluses. Growth of wild-type calluses was significantly promoted by 10 nM PSK, whereas *pskr1-1* calluses were less sensitive to PSK (Fig. 3B). In membrane fractions derived from *pskr1-1* calluses, no AtPSKR1 protein was detected (Fig. 3C), and specific [^{3}H]PSK binding activity was significantly decreased (Fig. 3D).

Phenotypes of Loss-of-Function and Gain-of-Function Mutants of *AtPSKR1*

We compared the phenotypes of the *AtPSKR1* knockout (*pskr1-1*) and the *AtPSKR1* OX2 line (hereafter referred to as *AtPSKR1ox*) with the wild-type phenotype. *AtPSKR1ox* and *pskr1-1* seedlings grown on B5 agar plate germinated normally and developed normal cotyledons and hypocotyls pheno-

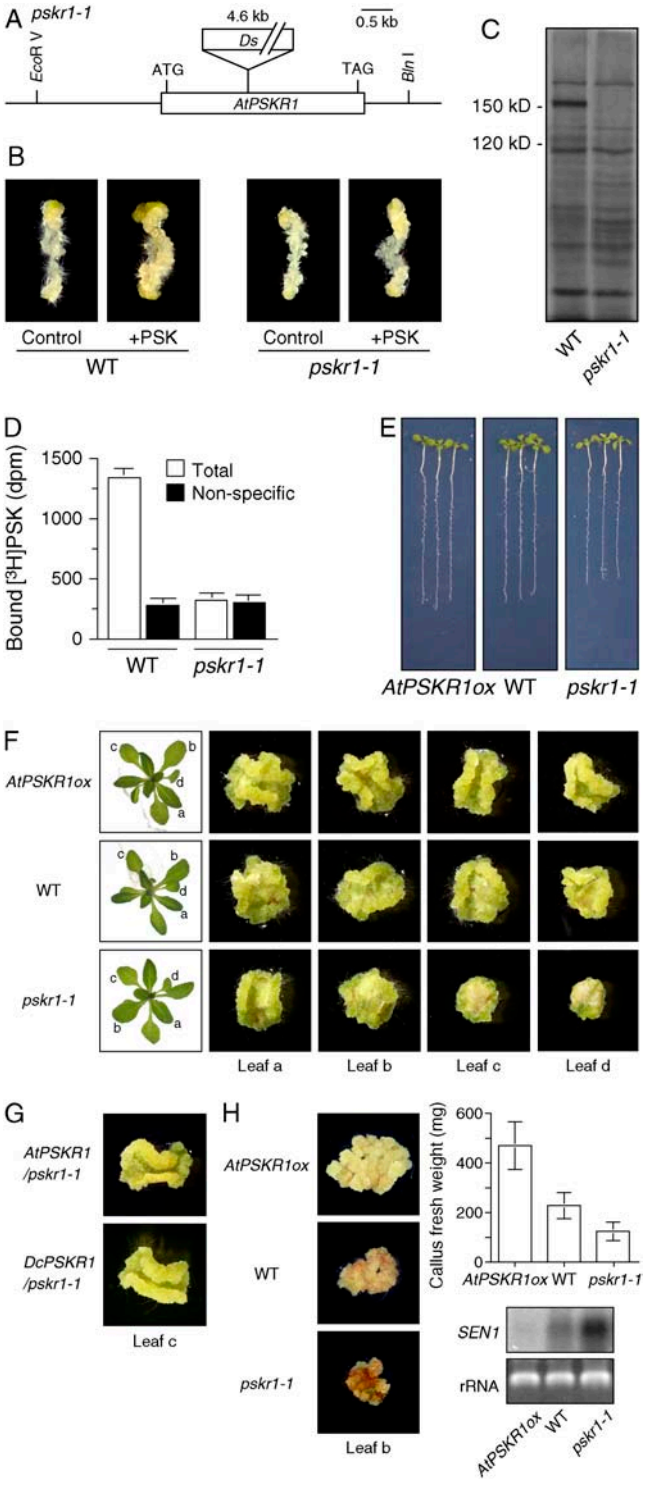

Figure 3. Phenotypes of *pskr1-1* and *AtPSKR1ox*. A, Schematic map of the *Ds* insertion site of *pskr1-1*. B, Response of wild-type (WT) and *pskr1-1* calluses to PSK. Hypocotyls of 7-d-old seedlings were cut into segments about 1 cm long, which were cultured in CIM for 10 d in the presence or absence of 30 nM PSK. C, Absence of AtPSKR1 protein in membrane fractions derived from *pskr1-1* calluses. D, Absence of PSK binding activity in membrane fractions derived from *pskr1-1* calluses. E, Growth of *pskr1-1*, *AtPSKR1ox*, and wild-type seedlings. Seedlings were grown on B5 agar plate for 10 d. F, Callus formation from leaf discs of *pskr1-1*, *AtPSKR1ox*, and wild-type plants. Leaf discs used for callus induction were cut from leaves a, b, c, and d of the 3-week-old plants, shown in the leftmost sections. All leaf discs were incubated on CIM for 2 weeks. G, Complementation of *pskr1-1* homozygous plants with a wild-type 6.5-kb *Eco*RV/*Bln*I genomic fragment of *AtPSKR1* or *pAtPSKR1::DcPSKR1* construct restored their callus-forming potential. H, Growth of *pskr1-1*, *AtPSKR1ox*, and wild-type calluses. All calluses were cultured on CIM for 6 weeks. Values are means ± SD of five calluses. Expression of *SEN1* is indicative of senescence.

typically indistinguishable from wild type. Root growth of *pskr1-1* seedlings was slightly reduced, whereas root growth of *AtPSKR1ox* was comparable to that of wild type (Fig. 3E). In the first 3 weeks of culture on B5 agar, there were no morphological differences in overall growth of above-ground plant parts between *pskr1-1*, *AtPSKR1ox*, and wild type (Fig. 3F, leftmost section).

The most striking phenotype of the *pskr1-1* plants was that individual cells gradually lost their potential to form calluses as the tissues matured (Fig. 3F, bottom). Leaf discs from the fully expanded leaves of *pskr1-1* plants exhibited severe defects in hormone-induced callus formation. Young immature *pskr1-1* leaves close to the meristem retained full callus-forming potential. Leaf discs of wild-type plants exhibited high potential for callus formation irrespective of the age of the leaves. Complementation of homozygous *pskr1-1* plants with a wild-type 6.5-kb *Eco*RV/*Bln*I genomic fragment of *AtPSKR1* restored their callus-forming potential, confirming that the phenotypes of homozygous *pskr1-1* plants were caused by the disruption of *AtPSKR1* (Fig. 3G, top). The *pskr1-1* mutant can also be functionally complemented by expressing the cDNA of the carrot *DcPSKR1* gene under the control of the Arabidopsis *AtPSKR1* promoter, suggesting that *AtPSKR1* and *DcPSKR1* function in the same pathway (Fig. 3G, bottom). In contrast, leaf discs of *AtPSKR1ox* plants exhibited a significantly greater callus-forming potential than wild type, irrespective of the age of the leaves (Fig. 3F, top).

There were also significant differences in relative growth rate of calluses between *pskr1-1*, *AtPSKR1ox*, and wild type. The *pskr1-1* calluses exhibited premature senescence with browning within 3 weeks of culture, resulting in formation of a smaller callus than the wild type after 6 weeks (Fig. 3H, left). The senescence marker *SEN1* transcript (Oh et al., 1996) was significantly increased in *pskr1-1* calluses (Fig. 3H, bottom right). Wild-type calluses did not exhibit senescence in the first 3 weeks of culture but gradually senesced thereafter. In contrast, *AtPSKR1ox* calluses vigorously proliferated and did not exhibit symptoms of senescence, even after 6 weeks of culture. At 6 weeks of culture, *AtPSKR1ox* calluses were almost twice as large as wild-type calluses (Fig. 3H, top right).

The *pskr1-1* seedlings grew at almost the same rate as wild-type seedlings, flowered normally, and completed the normal life cycle, but their leaves exhibited premature senescence phenotypes 4 weeks

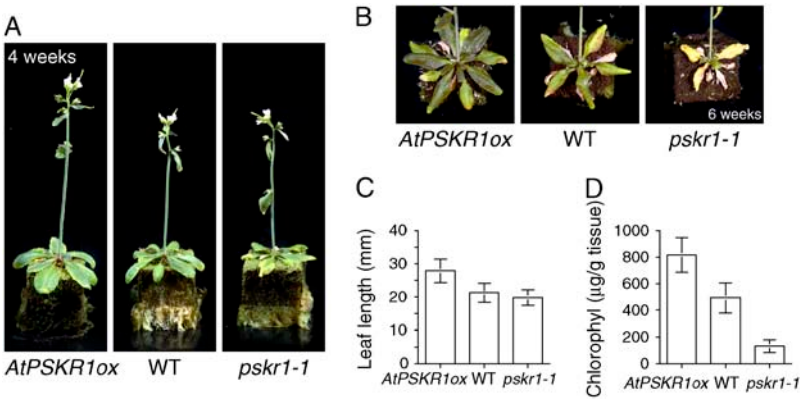

Figure 4. Phenotypes of *pskr1-1* and *AtPSKR1ox* plants after bolting. A, Plants grown on rockwool for 4 weeks. B, Leaves of plants grown on rockwool for 6 weeks. C, Comparison of leaf size measured as the length of the longest axis on the fully expanded leaves (seventh to eighth leaves) of 4-week-old plants. D, Chlorophyll content of the leaves (third to eighth leaves) of 6-week-old plants.

after germination (Fig. 4A). All the leaves of *pskr1-1* plants were senesced 6 weeks after germination (Fig. 4, B and D). The *AtPSKR1ox* seedlings also grew at almost the same rate as wild-type seedlings and completed the normal life cycle, but they developed larger leaves than the wild type (Fig. 4C) and exhibited delayed senescence (Fig. 4, B and D). This enlargement of leaves was due to an increase in cell number but not an increase in cell size (data not shown).

Expression patterns of *AtPSKs* are consistent with the greater severity of *pskr1-1* phenotypes in the lower mature leaves where *AtPSKs* are more strongly expressed. All these results strongly suggest that PSK signaling in plants affects their potential for growth and cellular longevity.

DISCUSSION

Our findings indicate that the At2g02220 gene encodes a functional PSK receptor (AtPSKR1) in Arabidopsis. AtPSKR1 is a member of the LRR-RK family and is characterized by 21 tandem copies of extracellular LRR, a 36-amino acid island domain between the 17th and 18th LRR, a single transmembrane domain, and a cytoplasmic kinase domain. AtPSKR1 interacts with [^{3}H]PSK with a K_d of 7.7 $\pm$ 0.9 nM, which is similar to the K_d of DcPSKR1 (4.2 $\pm$ 0.4 nM).

PSK receptors are often detected on SDS-PAGE as two distinct bands with different molecular size (Matsubayashi and Sakagami, 2000; Matsubayashi et al., 2002). In this study, when *AtPSKR1* was overexpressed under the control of the 35S promoter, we detected an increase in levels of the 120-kD protein but not of the 150-kD protein. In contrast, when *AtPSKR1* was overexpressed by increasing its copy number, we detected significant increase in levels of both the 120- and 150-kD proteins. Although previous photoaffinity label experiments have revealed high-affinity interaction between PSK and both the 120- and 150-kD proteins (Matsubayashi and Sakagami, 2000), this analysis indicates that phenotypic alteration occurs only when the 150-kD protein is overexpressed in Arabidopsis. This finding suggests that the 150-kD protein is the functional PSK receptor, which can activate intercellular signaling upon PSK binding, and that the 120-kD protein is a partially truncated dysfunctional form of AtPSKR1. Expression of exceptionally high levels of *AtPSKR1* mRNA and/or the corresponding membrane receptor protein may trigger a regulatory system by which the relative amount of functional receptor is maintained at a constant level. It has been reported that protein expression levels of LRR-RKs do not always correlate with their mRNA levels, due to posttranslational or posttranscriptional regulation (Jeong et al., 1999).

In this study, although seedlings of *pskr1-1* exhibited normal growth and developed rosette leaves phenotypically indistinguishable from wild type for the first 3 weeks after germination, individual *pskr1-1* cells gradually lost their potential to form calluses as tissues matured. The *pskr1-1* calluses derived from immature tissues also exhibited premature senescence accompanied by browning within 3 weeks of culture, resulting in formation of a smaller callus than the wild type. Premature senescence phenotypes were also observed in leaves of *pskr1-1* plants at the late bolting stage. In contrast, *AtPSKR1ox* calluses vigorously proliferated and did not exhibit symptoms of senescence even after 6 weeks of culture, resulting in formation of calluses almost twice the size of wild type. *AtPSKR1ox* plants exhibited delayed senescence, and its leaves underwent prolonged expanding growth, resulting in formation of bigger leaves than the wild type. This enlarged leaf phenotype was not obvious until the bolting stage, suggesting that it is due to continuous proliferation rather than accelerated cell division. These results strongly suggest that PSK represents a new class of hormones that affect the potential for cellular growth and longevity of individual cells but are not simple mitogens or differentiation factors. Overactivation or disruption of PSK signaling did not interfere with meristem organization or subsequent plant morphogenesis, except for the changes in cellular longevity and slight differences in root and leaf growth, which is consistent with our previous observation that treatment of dispersed mesophyll cells with PSK peptide alone does not directly induce any morphological

changes (Matsubayashi et al., 1999a). We speculate that PSK reactivates (or maintains) the cellular potential to proliferate and differentiate in response to endogenous or external stimuli, which gradually decreases during cellular aging, and thereby exerts a pleiotropic effect on cultured tissue in response to environmental hormonal conditions.

Our biochemical experiments confirm that Arabidopsis has five paralogous genes encoding PSK precursors (*AtPSKs*) that are functionally secreted after posttranslational sulfation and processing. Multiple alignment of the deduced amino acid sequences revealed that, in addition to the 5-amino acid PSK domain, certain residues between amino acid positions −25 and −1 are highly conserved among the PSK precursor peptides, including multiple acidic residues, one Cys pair, several hydrophobic residues, consecutive basic residues, and one His residue. We speculate that some of these residues synergistically determine Tyr sulfation efficiency and others are involved in proteolytic processing.

Expression of *AtPSKs* was detected in almost all plant tissues, including fully developed mature leaves. In addition, *AtPSK2*, *AtPSK4*, and *AtPSK5* were more strongly expressed in the lower mature leaves than in upper young leaves. These expression patterns are consistent with our conclusion that PSK regulates potential for cellular growth and longevity of individual cells rather than directly inducing cell division or differentiation. One of the PSK precursor genes is also highly up-regulated upon wounding, suggesting that PSK is involved in wound response signaling.

In contrast to the relatively obvious phenotypes of *AtPSKR1ox* plants, the only phenotypic alterations that we observed in Arabidopsis plants overexpressing PSK precursor gene were a slight increase in root growth at the seedling stage and a slight increase in callus proliferation in vitro. One possibility is that basal expression of endogenous *AtPSKs* is at a sufficiently high level to activate PSK signaling, and exogenous transgene-encoded PSK therefore does not cause any additional effects. Indeed, PSK shows a dose-response curve that reaches a plateau at PSK levels of around 10 nM (Matsubayashi and Sakagami, 1996). Another possibility is that posttranslational modification such as Tyr sulfation and processing acts as a rate-limiting step for production of mature 5-amino acid PSK peptide.

How might PSK activate the potential for cellular growth and longevity of individual cells? One may speculate that PSK modulates sensitivity to endogenous plant hormones such as auxin and cytokinin, thereby indirectly causing growth alteration. However, that hypothesis is not consistent with our finding that *pskr1-1* hypocotyls can respond to auxin/cytokinin and form calluses at normal auxin/cytokinin concentrations. Moreover, in the root elongation assay, *pskr1-1* seedlings exhibited the same cytokinin response as wild-type seedlings (data not shown). In animals, there is much evidence indicating that cellular growth and longevity are tightly coupled to protein translation and protein turnover via TOR (target of rapamycin) protein kinase, which is an integrator molecule of nutrient availability, growth factors, and the energy status of the cells (Martin and Hall, 2005). Several recent studies of plants suggest that regulation of protein synthesis and turnover is an important determinant of cellular proliferation and senescence (Doelling et al., 2002; Hanaoka et al., 2002; Thompson et al., 2004). This possibility is indirectly supported by the finding that cellular response to PSK is affected by the nutrient status of the medium (Matsubayashi and Sakagami, 1998). The presence of ammonium ion, a ready nitrogen source, disturbs PSK-dependent promotion of cellular proliferation of dispersed asparagus (*Asparagus officinalis*) mesophyll cells.

In contrast to the relatively clear understanding of the mechanism of maintenance of cellular homeostasis in the shoot apical meristem of plants, little is known about the molecular basis for the cellular plasticity that allows plants to flexibly alter their developmental program according to the environment. We are currently researching the downstream target of PSK signaling, using *AtPSKR1ox* and *pskr1-1* plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*, Landsberg *erecta* [L*er*]) plants were grown at 22°C under continuous light, on rockwool or on B5 medium containing 1.0% Suc solidified with 0.7% agar. For callus induction, leaf discs or hypocotyl segments were excised from donor plants grown on B5 agar plate and were cultured on callus induction medium (CIM) containing B5 medium with 0.5 mg/L 2,4-dichlorophenoxyacetic acid, 0.1 mg/L kinetin, 20 g/L Glc, 0.5 g/L MES, and 2.5 g/L gellan gum, at 22°C under continuous light. Arabidopsis (Columbia) T87 cells were maintained in B5 medium containing 1.0 μM naphthylacetic acid and 1.5% Suc by gentle agitation at 120 rpm under continuous light at 22°C.

The loss-of-function mutant of *AtPSKR1* (SGT5281) was found in the searchable database of *Ds* insertion sequences (L*er* background) released by the Institute of Molecular Agrobiology, the National University of Singapore (Parinov et al., 1999). The DNA sequence flanking the left and right border of the *Ds* mutation was confirmed by PCR. Leaf size was measured as the length of the longest axis on the fully expanded leaves (seventh to eighth leaves) of 4-week-old plants. Chlorophyll content of leaves (third to eighth leaves) of 6-week-old plants was determined using dimethylformamide as the extraction (Moran, 1982).

Plasmid Constructs and Plant Transformation

cDNA clones coding for *AtPSK*s were obtained by RT-PCR from total RNA of Arabidopsis plants. Site-directed mutagenesis of *AtPSK1*, *AtPSK3*, and *AtPSK5* cDNA was performed by PCR using the overlap extension technique. The resulting cDNAs were ligated into the binary vector pBI121 by replacing the GUS coding sequence downstream of the cauliflower mosaic virus (CaMV) 35S promoter. Arabidopsis T87 suspension cells were coincubated with *Agrobacterium tumefaciens* (C58C1) harboring these constructs for 2 d and further incubated on CIM plate containing 50 mg/L kanamycin and 200 mg/L carbenicillin for 4 weeks. The selected calluses were then transferred to suspension culture.

The *AtPSKR1* (At2g02220) full-length cDNA was obtained from the RIKEN BioResource Center (Tsukuba, Japan; Seki et al., 1998, 2002). For the overexpression of *AtPSKR1* under the control of the CaMV 35S promoter, a 3.1-kb entire coding sequence of the *AtPSKR1* cDNA was amplified by PCR and cloned in the binary vector pBI121 by replacing the GUS coding sequence downstream of the CaMV 35S promoter with the *AtPSKR1* fragment. For the

expression of *AtPSKR1* under its own promoter, a 9.4-kb genomic fragment containing the *AtPSKR1* gene was cloned from an Arabidopsis L*er* genomic DNA library. Then, an *Eco*RV/*Bln*I fragment (6.2 kb) containing the complete *AtPSKR1* gene and 2 kb of the 5′ upstream region was cloned in the binary vector pBI101-Hm (kindly provided by Dr. K. Nakamura, Nagoya University, Nagoya, Japan), which is a derivative of pBI101 and carries the hygromycin phosphotransferase gene in addition to the neomycin phosphotransferase gene as a selective marker gene. For the expression of *DcPSKR1* under the control of the *AtPSKR1* promoter, a 3.1-kb entire coding sequence of the *DcPSKR1* cDNA and 2.0 kb of the 5′ upstream region of *AtPSKR1* were amplified by PCR and cloned in the binary vector pBI101-Hm. For the overexpression of *AtPSK4* under the control of the CaMV 35S promoter, a 0.3-kb entire coding sequence of the *AtPSK4* cDNA was amplified by PCR and cloned in the binary vector pBI121 by replacing the GUS coding sequence downstream of the CaMV 35S promoter with the *AtPSK4* fragment. Arabidopsis (L*er*) was transformed with these constructs via Agrobacterium (C58C1) using the floral dip method (Clough and Bent, 1998).

To study expression patterns of *AtPSKR1*, *AtPSK2*, *AtPSK3*, *AtPSK4*, and *AtPSK5* in detail, we amplified the upstream 2.0-kb promoter regions of *AtPSKR1* or the other *AtPSK*s by genomic PCR and cloned them by translational fusion in frame with the GUS coding sequence in the binary vector pBI101. Histochemical analysis of GUS gene expression in the transformed plants was performed as described elsewhere (Kosugi et al., 1990).

LC/MS Analysis of [Ser^4]PSK

Culture medium (20 mL) derived from suspensions of transgenic T-87 cells expressing mutated *AtPSKs* were loaded onto a DEAE Sephadex A-25 column (5.0 × 30 mm, packed in an Econo-column [Bio-Rad]) equilibrated with 20 mM Tris-HCl, pH 7.5. The column was washed with 3.0 mL of the same buffer containing 500 mM KCl, and PSKs were eluted with 1.0 mL of the same buffer containing 2,000 mM KCl. Eluate from the DEAE Sephadex A-25 column was acidified by adding formic acid at a final concentration of 1.0%.

LC/MS analysis was performed using a JASCO semimicro HPLC pump system (model PU-2085) equipped with a column switching-valve unit (model HV-2080-01). Sample (200 μL) was loaded to the first reverse-phase column (Develosil ODS-HG-5 column, 2.0 × 50 mm) using 5% acetonitrile (containing 0.1% formic acid) with a flow rate of 200 μL/min. After this cleanup and enrichment step (5.0 min), the first column was connected to the second reverse-phase analytical column (Develosil ODS-HG-5 column, 2.0 × 150 mm) in back-flush mode by means of the time-controlled switching valve, and eluted with a gradient of 5%/20%/20% acetonitrile (containing 0.1% formic acid) in 0/15/20 min at a flow rate of 200 μL/min. The HPLC eluate was introduced into an electrospray ionization ion-trap mass spectrometer (LCQ Deca XP-plus, Thermo Electron) via an electrospray ionization interface at a spray voltage of 5.0 kV. The mass spectrometer was operated in negative ion mode with a capillary temperature of 220°C, a capillary voltage of −38 V, and the tube lens offset at 30 V. The mass spectra were obtained by scanning the mass range from mass-to-charge ratio 700 to 900.

Anti-AtPSKR1 Antibodies

An N-terminal 100-amino acid region of AtPSKR1 (excluding the signal peptide) was expressed in *Escherichia coli* using the pET-24b expression vector and purified as a His_6 fusion. This recombinant protein was used as the antigen to generate the antibodies in rabbits and was used for affinity purification of the antibodies using Hi-Trap NHS activated Sepharose.

[^{3}H]PSK Binding Assays

Tritium-labeled PSK was prepared by tritium reduction of a PSK analog containing tetradehydro-Ile (Matsubayashi and Sakagami, 1999). The specific radioactivity of [^{3}H]PSK was estimated to be 67 Ci/mmol. Nonlabeled PSK was prepared as described elsewhere (Matsubayashi et al., 1996). Plant microsomal fractions were prepared from Arabidopsis callus cells using a protocol described elsewhere (Matsubayashi and Sakagami, 1999). Membrane pellets were resuspended in binding buffer (20 mM HEPES-KOH, pH 7.5, 100 mM Suc) and stored at −80°C until use. Each binding assay mixture contained 500 μg of membrane proteins and 0.6 to 62.5 nM [^{3}H]PSK with or without 32 μM unlabeled PSK, in a total volume of 250 μL. The binding reactions were performed by incubating the mixture for 30 min on ice. The bound and free [^{3}H]PSK were separated by layering the reaction mixture onto 900 μL of wash buffer (20 mM HEPES-KOH, pH 7.5, 500 mM Suc) and centrifuging for 10 min at 100,000*g* at 4°C. The radioactivity in each pellet was measured using a liquid-scintillation counter at approximately 40% counting efficiency.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_126282 (*AtPSKR1*), NM_101229 (*AtPSK1*), NM_127851 (*AtPSK2*), NM_114342 (*AtPSK3*), NM_114838 (*AtPSK4*), and NM_125984 (*AtPSK5*).

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LITERATURE CITED

Bellincampi D, Morpurgo G (1987) Conditioning factor affecting growth in plant cells in culture. Plant Sci **51:** 83–91

Birnberg PR, Somers DA, Brenner ML (1988) Characterization of conditioning factors that increase colony formation from Black Mexican sweet corn protoplasts. J Plant Physiol **132:** 316–321

Canales C, Bhatt AM, Scott R, Dickinson H (2002) EXS, a putative LRR receptor kinase, regulates male germline cell number and tapetal identity and promotes seed development in *Arabidopsis*. Curr Biol **12:** 1718–1727

Cano-Delgado A, Yin Y, Yu C, Vafeados D, Mora-Garcia S, Cheng JC, Nam KH, Li J, Chory J (2004) BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*. Development **131:** 5341–5351

Chen YF, Matsubayashi Y, Sakagami Y (2000) Peptide growth factor phytosulfokine-α contributes to the pollen population effect. Planta **211:** 752–755

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Doelling JH, Walker JM, Friedman EM, Thompson AR, Vierstra RD (2002) The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in *Arabidopsis thaliana*. J Biol Chem **277:** 33105–33114

Hanai H, Matsuno T, Yamamoto M, Matsubayashi Y, Kobayashi T, Kamada H, Sakagami Y (2000) A secreted peptide growth factor, phytosulfokine, acting as a stimulatory factor of carrot somatic embryo formation. Plant Cell Physiol **41:** 27–32

Hanaoka H, Noda T, Shirano Y, Kato T, Hayashi H, Shibata D, Tabata S, Ohsumi Y (2002) Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an Arabidopsis autophagy gene. Plant Physiol **129:** 1181–1193

Igasaki T, Akashi N, Ujino-Ihara T, Matsubayashi Y, Sakagami Y, Shinohara K (2003) Phytosulfokine stimulates somatic embryogenesis in *Cryptomeria japonica*. Plant Cell Physiol **44:** 1412–1416

Jeong S, Trotochaud AE, Clark SE (1999) The Arabidopsis *CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. Plant Cell **11:** 1925–1934

Kinoshita T, Cano-Delgado A, Seto H, Hiranuma S, Fujioka S, Yoshida S, Chory J (2005) Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. Nature **433:** 167–171

Kobayashi T, Eun C, Hanai H, Matsubayashi Y, Sakagami Y, Kamada H (1999) Phytosulphokine-α, a peptidyl plant growth factor, stimulates somatic embryogenesis in carrot. J Exp Bot **50:** 1123–1128

Kosugi S, Arai Y, Nakajima K, Ohashi Y (1990) An improved assay for β-glucuronidase (GUS) in transformed cells: methanol almost suppresses a putative endogenous GUS activity. Plant Sci **70:** 133–140

Lorbiecke R, Sauter M (2002) Comparative analysis of PSK peptide growth factor precursor homologs. Plant Sci **163:** 321–332

Martin DE, Hall MN (2005) The expanding TOR signaling network. Curr Opin Cell Biol **17:** 158–166

Matsubayashi Y, Hanai H, Hara O, Sakagami Y (1996) Active fragments and analogs of the plant growth factor, phytosulfokine: structure-activity relationships. Biochem Biophys Res Commun **225:** 209–214

Matsubayashi Y, Morita A, Matsunaga E, Furuya A, Hanai N, Sakagami Y (1999a) Physiological relationships between auxin, cytokinin, and a peptide growth factor, phytosulfokine-α, in stimulation of asparagus cell proliferation. Planta **207:** 559–565

Matsubayashi Y, Ogawa M, Morita A, Sakagami Y (2002) An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine. Science **296:** 1470–1472

Matsubayashi Y, Sakagami Y (1996) Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. Proc Natl Acad Sci USA **93:** 7623–7627

Matsubayashi Y, Sakagami Y (1998) Effects of the medium ammonium-nitrate ratio on competence for asparagus cell division induced by phytosulfokine-α. Plant Cell Rep **17:** 368–372

Matsubayashi Y, Sakagami Y (1999) Characterization of specific binding sites for a mitogenic sulfated peptide, phytosulfokine-α, in the plasma-membrane fraction derived from *Oryza sativa* L. Eur J Biochem **262:** 666–671

Matsubayashi Y, Sakagami Y (2000) 120- and 160-kDa receptors for endogenous mitogenic peptide, phytosulfokine-α, in rice plasma membranes. J Biol Chem **275:** 15520–15525

Matsubayashi Y, Takagi L, Omura N, Morita A, Sakagami Y (1999b) The endogenous sulfated pentapeptide phytosulfokine-α stimulates tracheary element differentiation of isolated mesophyll cells of zinnia. Plant Physiol **120:** 1043–1048

Moran R (1982) Formulae for determination of chlorophyllous pigments extracted with *N,N*-dimethylformamide. Plant Physiol **69:** 1376–1381

Oh SA, Lee SY, Chung IK, Lee CH, Nam HG (1996) A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. Plant Mol Biol **30:** 739–754

Parinov S, Sevugan M, Ye D, Yang WC, Kumaran M, Sundaresan V (1999) Analysis of flanking sequences from dissociation insertion lines: a database for reverse genetics in Arabidopsis. Plant Cell **11:** 2263–2270

Seki M, Carninci P, Nishiyama Y, Hayashizaki Y, Shinozaki K (1998) High-efficiency cloning of *Arabidopsis* full-length cDNA by biotinylated CAP trapper. Plant J **15:** 707–720

Seki M, Narusaka M, Kamiya A, Ishida J, Satou M, Sakurai T, Nakajima M, Enju A, Akiyama K, Oono Y, et al (2002) Functional annotation of a full-length *Arabidopsis* cDNA collection. Science **296:** 141–145

Shiu SH, Bleecker AB (2001) Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA **98:** 10763–10768

Thompson JE, Hopkins MT, Taylor C, Wang TW (2004) Regulation of senescence by eukaryotic translation initiation factor 5A: implications for plant growth and development. Trends Plant Sci **9:** 174–179

Yamakawa S, Matsubayashi Y, Sakagami Y, Kamada H, Satoh S (1999) Promotive effects of the peptidyl plant growth factor, phytosulfokine-α, on the growth and chlorophyll content of *Arabidopsis* seedlings under high night-time temperature conditions. Biosci Biotechnol Biochem **63:** 2240–2243

Yamakawa S, Sakuta C, Matsubayashi Y, Sakagami Y, Kamada H, Satoh S (1998) The promotive effects of a peptidyl plant growth factor, phytosulfokine-α, on the formation of adventitious roots and expression of a gene for a root-specific cystatin in cucumber hypocotyls. J Plant Res **111:** 453–458

Yang H, Matsubayashi Y, Nakamura K, Sakagami Y (1999) *Oryza sativa* PSK gene encodes a precursor of phytosulfokine-α, a sulfated peptide growth factor found in plants. Proc Natl Acad Sci USA **96:** 13560–13565

Yang H, Matsubayashi Y, Nakamura K, Sakagami Y (2001) Diversity of Arabidopsis genes encoding precursors for phytosulfokine, a peptide growth factor. Plant Physiol **127:** 842–851

Zhao DZ, Wang GF, Speal B, Ma H (2002) The *excess microsporocytes1* gene encodes a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in the *Arabidopsis* anther. Genes Dev **16:** 2021–2031

Zhou A, Wang H, Walker JC, Li J (2004) BRL1, a leucine-rich repeat receptor-like protein kinase, is functionally redundant with BRI1 in regulating *Arabidopsis* brassinosteroid signaling. Plant J **40:** 399–409