The Phytosulfokine (PSK) Receptor Is Capable of Guanylate Cyclase Activity and Enabling Cyclic GMP-dependent Signaling in Plants**§

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Phytosulfokines (PSKs) are sulfated pentapeptides that stimulate plant growth and differentiation mediated by the PSK receptor (PSKR1), which is a leucine-rich repeat receptor-like kinase. We identified a putative guanylate cyclase (GC) catalytic center in PSKR1 that is embedded within the kinase domain and hypothesized that the GC works in conjunction with the kinase in downstream PSK signaling. We expressed the recombinant complete kinase (cytoplasmic) domain of AtPSKR1 and show that it has serine/threonine kinase activity using the Ser/Thr mutant protein also has GC activity in vitro that is dependent on the presence of either Mg2+ or Mn2+. Overexpression of the full-length AtPSKR1 receptor in Arabidopsis leaf protoplasts raised the endogenous basal cGMP levels over 20-fold, indicating that the receptor has GC activity in vivo. In addition, PSK-α itself, but not the non-sulfated backbone, induces rapid increases in cGMP levels in protoplasts. Together these results indicate that the PSKR1 contains dual GC and kinase catalytic activities that operate in vivo and that this receptor constitutes a novel class of enzymes with overlapping catalytic domains.

Guanylate cyclases (GCs)3 (EC 4.6.1.2) are the enzymes that generate production of cyclic GMP (cGMP), which is a key signaling intermediate in eukaryotes. In higher plants, the Ser/GMP has been identified as an important molecule involved in regulating a wide variety of physiological effects ranging from chloroplast development and plant hormone-dependent responses to the induction of plant defense responses (for reviews, see Refs. 1–3). Nitric oxide signaling is thought to stimulate cGMP production in defense and stress responses, but how it generates cGMP is still uncertain (4, 5). Cyclic GMP acts on cyclic nucleotide-gated ion channels (6), various other downstream molecules, and the transcriptome (1, 7) to modulate plant responses. As such a diverse group of plant responses has shown direct or indirect dependence on cGMP, we hypothesized that several different functional GCs exist in higher plants and have undertaken to search for them. The strategy we used was based on the assumption that the catalytic center of known GCs was at least in part conserved across the different kingdoms. Consequently, we designed and tested a search motif based on several functionally assigned amino acids in the catalytic domain of known GCs from lower eukaryotes and animals (see Fig. 1A and identified and experimentally confirmed the first three molecules with GC activity in higher plants (8–10). AtGC1 is a soluble protein with the GC domain toward the N terminus but does not contain a heme binding motif essential for nitric oxide binding (9), indicating that other GCs are likely to be present. Recently, a GC1 homologue has been characterized in Pharbitis nil (morning glory), and the expression of this molecule is regulated by light (11). The second functional GC was identified by relaxing the initial search motif and surprisingly is the brassinosteroid receptor AtBRI1. The GC domain is found within the intracellular kinase domain, and it is this region that has been shown to have functional activity in vitro (8). AtBRI1 is a leucine-rich repeat receptor-like kinase (LRR RLK) with both serine/threonine and tyrosine kinase activity (EC 2.7.12.1) (12) and was one of several LRR RLKs identified in the screen that all shared a similar kinase–GC domain structure (8). In addition, another membrane-associated kinase molecule, the wall-associated kinase-like 10 (WAKL10), has also recently been reported to have GC activity in vitro as well as being transcriptionally up-regulated in response to biotic stress (10).

Among the other LRR RLKs detected in this screen for novel GCs, we identified the phytosulfokine (PSK) receptor AtPSKR1, and its domain organization is shown in Fig. 1B. AtPSKR1 is a typical LRR RLK with an island that binds PSK in its extracellular leucine-rich repeat domain, a single transmembrane-spanning domain, and an intracellular kinase domain.
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(13, 14). PSKR has been identified in several other species where the GC domain is also present (see Fig. 1C), indicating that it is of widespread occurrence. Mutant studies indicate that AtPSKR1 is involved in regulating root elongation (13). PSK-α is the natural ligand for AtPSKR1, and it is a pentapeptide that is sulfated on its two tyrosine residues (Y(SO3H)IY(SO3H)TQ). PSK-α was first discovered as a cell proliferation agent essential for low density cell cultures (15). Several precursor proteins for PSK have been identified in different species that contain an N-terminal secretory signal sequence and a PSK sequence near the C terminus (16–18). Cellular activity depends on sulfation of the tyrosine residues (15), and it is likely that PSK precursor protein is sulfated by tyrosylprotein sulfotransferase as the protein is processed through the Golgi network (19) before secretion. Specific subtilisin serine proteases in the apoplast cleave PSK precursor protein at dibasic residues upstream from the PSK sequence (20) before further processing to the active pentapeptide. Like other secreted peptide signaling molecules in plants, PSK acts in a paracrine or autocrine fashion on nearby cells expressing the receptor (21). This is most clearly shown in roots, where PSK enhances root elongation by controlling cell size (13, 22). PSKs also have a role in attenuating expression of stress response genes during differentiation of tracheary elements (23).

The signaling processes activated by PSK-α binding to PSKR have not been resolved, although it is speculated that the receptor kinase is activated to stimulate a phosphorylation cascade. Because we had identified a putative GC catalytic center in PSKR, we hypothesized that this worked in conjunction with the kinase to signal downstream events. Here we show that the kinase domain of AtPSKR1 has kinase and also GC activity in vitro. In addition, we demonstrate that PSK-α itself induces small and rapid increases in cGMP levels and that overexpression of AtPSKR1 in protoplasts raises endogenous basal cGMP levels. Together these results indicate that AtPSKR1 contains dual functioning GC and kinase catalytic activity that operates in vivo and represents a novel class of kinases with overlapping catalytic domains.

EXPERIMENTAL PROCEDURES

Gene Constructs—The AtPSKR1-KD1 encoding the cytoplasmic domain of AtPSKR1 (AT2G02220) was prepared using cDNA synthesized from RNA freshly isolated from Arabidopsis thaliana (Col) as a template with the gene-specific primers AtPskR1-F and AtPskR1-R (supplemental Table 1). The PCR product was cloned into pTOPO and confirmed by sequencing. The AtPSKR1-KD3 (wild type and mutant) constructs were prepared from AtPSKR1-KD1 and cloned into pET SUMO. The mutant (glycine to lysine) was generated using the Expand long template PCR system (Roche Applied Science), with amplimers A and B: were generated to contain the mutation at the catalytic site using the primers AtPskR1-F and amplimer A-rev or amplimer B-for and AtPskR1-Rns (supplemental Table 1), respectively. Amplimer A and B were used as the template in another PCR reaction that contained AtPskR1-F and AtPskR1-Rns as primers to create the mutant construct cloned into pET SUMO. Gateway cloning was used to prepare full-length receptor and cytoplasmic domain (AtPSKR1-KD2) constructs of the AtPSKR1 (AT2G02220) gene. Because AtPSKR1 is intronless, genomic DNA was used as the source material and amplified with KAPA HiFi DNA polymerase (Kapa Biosystems). Primers AtPSKR1-f-wd and AtPSKR1-rev (supplemental Table 1) were used to amplify the gene, and then nested gateway primers (AtPSKR1-f-wd-gw or AtPSKR1-cd-fwd-gw with AtPSKR1-rev-gw-stop) were used to amplify AtPSKR1 full-length and cytoplasmic domain fragments and constructs cloned into pDONR207. The cytoplasmic domain of AtPSKR1 was recombined into pDEST17 (Invitrogen) and used to prepare recombinant protein (pDEST-PSKR1-KD2). The full-length receptor was cloned into the high copy number destination vector p2GW7.0 (24) and used in transient transfection studies (p2GW7.0-PSKR1-fl-s).

Expression, Purification, and Refolding of Recombinant Proteins—For expression of the recombinant AtPSKR1-KD1 and AtPSKR1-KD3 proteins (wild type or mutant), the appropriate constructs were transformed into BL21 (DE3) Escherichia coli cells (Invitrogen) and induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) and grown for a further 3 h at 37 °C. The recombinant protein(s) was purified by preparing a cleared cell lysate under denaturing conditions essentially as described in Protocols 10 and 17 of the Qiagen expressionist manual (Qiagen) but with some slight modifications described in the supplemental methods. The eluted protein was subsequently desalted and concentrated using Centrimentos filtration devices (Millipore Corp., Bedford, MA) with a molecular mass cut-off point of 3000 Da. Protein concentration was determined by the Bradford method (25) before the recombinant protein was stored at −20 °C. The AtPSKR1-KD2 (pDEST-PSKR1-KD2) was transformed into BL21-A1 cells (Invitrogen), and AtPSKR1-KD2 protein expression was induced by 0.2% arabinose for 3 h or with both 0.2% arabinose and 100 μM isopropyl-β-D-thiogalactopyranoside as the vector contains an isopropyl-β-D-thiogalactopyranoside-inducible promoter and the host cells contain an arabinose-inducible promoter. Soluble proteins were purified under native conditions on a nickel column following Protocol 12 in the Qiagen expressionist manual (Qiagen) in the presence of 1 mM PMSF (phenylmethylsulfonyl fluoride) and 45 mM imidazole. Eluted protein was concentrated using Centriplus filtration columns (3000 molecular mass cut-off point) by centrifugation (2 h at 4300 × g at 5 °C) and desalted with washing buffer (20 mM Tris, pH 8.0, 1 mM PMSF) at 4300 × g for 60 min at 5 °C. Protein concentration was determined using a Quant-iT™ protein assay kit (Invitrogen) in a Qubit® fluorometer (Invitrogen).

RNA Extraction and RT-PCR—Total RNA was extracted from tissue samples using the RNeasy plant mini kit (Qiagen), and RNA was digested with DNase I (Ambion) for 1 h at 37 °C. Total RNA (1 μg) was reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen) using oligo(dT)15 primers according to the manufacturer’s specifications in a total volume of 20 μl. PCR reaction mixtures contained 1 μl of cDNA (or negative RT reaction), 1× PCR coral load buffer (Invitrogen), 0.1 μM primers, 200 μM dNTPs, and 0.2 units of Taq DNA polymerase (Qiagen) per 20-μl reaction. The primers used are described in supplemental Table 1, and the product from the amplification for AtPSKR1 is expected to be 220 bp. All tubes
were denatured at 94 °C for 3 min, and then 35 cycles of amplification were performed (60 s of denaturation at 94 °C, 60 s of annealing at 52 °C, and 60 s of extension at 72 °C) with a final extension at 72 °C for 8 min in a MyCycler thermal cycler (Bio-Rad Laboratories). The quality of cDNA produced was assessed by amplifying cDNA for the LIBQ-10 gene (expected product: 122 bp), which is a suitable reference gene as it is expressed relatively stably (26). After PCR amplification, equal volumes of PCR products were separated in 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

**Cyclic Nucleotide Assays**—The GC activity of AtPSKR1-KD (1–3) was measured *in vitro* by incubating 2–10 μg of protein in 50 mM Tris-HCl (pH 8.0), 2 mM isobutylmethylxanthine, 5 mM MgCl₂, or 5 mM MnCl₂, and 1 mM GTP in a final volume of 100 μl (27). Residual cGMP levels resulting from non-GC activity were measured in tubes that contained the incubation medium, but no protein was added. Incubations were performed for 5, 15, and 20 min at room temperature (~24 °C) and terminated by the addition of 10 mM EDTA. Tubes were then boiled for 3 min, cooled on ice for 2 min, and centrifuged at 2300 × g for 3 min, and the supernatant was kept to assay cGMP content. Cyclic GMP was also measured in protoplasts isolated from *A. thaliana* leaves and treated with PSK-α or non-sulfated PSK (see below). In other cases, protoplasts were transiently transfected as described (28) with empty vector (p2GW7.0) or full-length AtPSKR1 (p2GW7.0-PSKR1-fl-s) and incubated for at least 2 h to express protein. Immediately following treatments, protoplasts were placed in liquid nitrogen to stop any further reactions and stored at ~80 °C. The cGMP content from recombinant protein and protoplast assays was analyzed using the Amersham Biosciences cGMP enzyme immunoassay Biotrak system following the standard (Protocol 2) or the total cellular cGMP (Protocol 4) acetylation protocols as described in the supplier’s manual (GE Healthcare, code RPN226). The optical density was read at 450 nm using an Envision 2101 plate reader (PerkinElmer Life Sciences). Cyclic GMP levels were then calculated as fmol/μg of protein or fmol/10⁵ protoplasts. The anti-cGMP antibody is highly specific for cGMP and has ~10⁶ times lower affinity for cAMP.

**RESULTS**

**In Vitro GC Activity of AtPSKR1**—The domain organization of PSKR1 is typical of LRR RLKs with a highly conserved leucine-rich repeat domain in the extracellular space, a transmembrane domain, and a kinase domain that forms the majority of the intracellular protein. Unusually, a putative GC catalytic center is also found in the latter part of the kinase domain just downstream of the key kinase catalytic residues and the predicted activation loop (Fig. 1D). To test whether the putative GC domain was functional, we first prepared a construct that contained the complete cytoplasmic domain of PSKR amino acids 683–1008 in pTOPO, which we called AtPSKR1-KD1. We expressed AtPSKR1-KD1 in BL21 (DE3) cells and purified the recombinant protein. We assessed the ability of AtPSKR1-KD1 to generate cGMP using mass spectrometry as we had previously shown that Q-TOF mass spectrometry detected fmol amounts of cGMP (8, 10). Recombinant AtPSKR1-KD1 catalyzed the production of cGMP (Fig. 2A). Moreover, we undertook a series of tests to determine whether AtPSKR1 also functioned as an adenylate cyclase. No cAMP production was
detected (supplemental Fig. 1), indicating that AtPSKR1-KD1 acts as a GC only.

**In Vitro Dual Kinase and GC Activity of AtPSKR1**—To test whether the kinase and GC domain were both functional, we prepared another construct that contained the cytoplasmic part of AtPSKR1 in pDEST17 named AtPSKR1-KD2, which included the kinase domain but was 3 amino acids shorter than AtPSKR1-KD1 at the N terminus of the kinase domain (Fig. 1D). The recombinant protein was expressed in BL21-AI cells as an N-terminal His tag protein with a predicted molecular mass of 38.9 kDa and used in both GC and phosphorylation *in vitro* studies. Firstly, we demonstrated that AtPSKR1-KD2 also exhibited GC activity that showed no preference in metal ion selectivity between Mn²⁺/H₁₁₀₀₁ or Mg²⁺/H₁₁₀₀₁ ions; however, the presence of one or the other metal ions was essential to observe activity (Fig. 2B). In a second set of experiments, we used a separately prepared construct in pET SUMO that was 3 amino acids longer than AtPSKR1-KD2 with a kinase domain identical to AtP-
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SKR2-KD1 and termed At-PSKR1-KD3. The recombinant protein was expressed as a His-tagged SUMO fusion protein in BL21 (DE3) E. coli cells and has a predicted molecular mass of 50.3 kDa. To test whether the GC search motif present was essential for the observed GC activity, we mutated the glycine residue in position 3 of the search motif to a lysine residue (Fig. 1D) as the glycine is predicted to face the purine and determine substrate specificity for GTP rather than ATP (33–35). The recombinant AtPSKR1-KD3 mutant protein exhibited reduced ability to generate cGMP (Fig. 2C), indicating that the glycine residue is necessary for this function.

Using the Omnia kinase assay, we also showed that AtPSKR1-KD2 has serine/threonine kinase activity using the Ser/Thr peptide 1 as a substrate. Under these conditions, AtPSKR1-KD2 has an approximate \( K_m \) of \( \sim 7.5 \) \( \mu M \) and a \( V_{\text{max}} \) of \( \sim 1800 \) nmol min\(^{-1}\) mg\(^{-1}\) protein (supplemental Fig. 2 and Fig. 3A). To our knowledge, this is the first reported kinetic value for AtPSKR1 kinase activity, and it is similar to the \( K_m \) values reported for WAKL10-KD using the same substrate (10). In addition, AtPSKR1-KD2 can use GTP as an alternative substrate to ATP to phosphorylate Ser/Thr peptide 1 (Fig. 3B). Because the AtPSKR1-KD2 also produced cGMP (Fig. 2B), we tested the effects of cGMP on its kinase activity and show that cGMP suppresses kinase activity (Fig. 3C).

Overexpression of AtPSKR1 in Protoplasts Raises Basal cGMP Levels—If the GC activity observed \( \text{in vitro} \) for AtPSKR1 has a biological role in general and a signaling function in particular, then we might expect to observe it \( \text{in vivo} \). To test this possibility, we isolated protoplasts from Arabidopsis leaves, transfected them, and transiently expressed the full-length AtPSKR1 under the control of the cauliflower mosaic virus promoter to overexpress the receptor. The protoplasts were then incubated for at least 2 h to allow expression of the protein before they were harvested. The protoplasts expressing the full-length receptor contained markedly higher basal levels of cGMP than protoplasts transfected with the empty vector (Fig. 4A).

PSK-\( \alpha \) Stimulates cGMP Production—Because strong overexpression of AtPSKR1 resulted in increased cGMP levels, it suggested that PSK-\( \alpha \) may stimulate AtPSKR1 GC activity as part of its signaling cascade. Protoplasts have been suggested as an ideal tissue to carry out investigations into the roles of cyclic nucleotide signaling as they can respond rapidly to extracellular signals (36). Firstly, we determined that young Arabidopsis leaves and our protoplast preparations were expressing AtPSKR1 RNA (Fig. 4B) and so could conceivably respond to PSK-\( \alpha \) treatment by inducing cGMP production. We prepared PSK-\( \alpha \) and the relatively inactive nPSK to test whether these peptides could induce cGMP production in protoplasts pre-
cascade. indicating that cGMP production is part of the PSK signaling
pathway. To further explore this possibility, we undertook to
investigate both the GC and the kinase activity of PSKR1 as it is
posed from Arabidopsis leaves. In the presence of PSK-α but
not nPSK, cGMP levels were raised in protoplasts. This increase
in cGMP levels was observed in the absence of any phospho-
diesterase inhibitors (Fig. 4C). The inhibitor dipyridamole has
been used successfully in plants to decrease basal phosphodi-
erase activity (37), and when it was present, we observed a
3-fold increase in cGMP levels over control and nPSK treat-
ment (Fig. 4D). The levels of cGMP detected in the protoplasts
are in the normal range reported for plants (e.g. (31, 37–41)).
Importantly, cGMP levels are observed to rise in response to
PSK-α but not the non-sulfated PSK backbone (Fig. 4, C and D),
indicating that cGMP production is part of the PSK signaling
cascade.

discussion
In a previous study, we demonstrated that the BRI1 receptor
contained a functional GC domain within its kinase domain (8),
which raised the intriguing possibility that this GC domain also
contributed to downstream signaling. Moreover, we had also
observed that WAKL10 and several other LRR RLKs contained
putative GC centers embedded within kinase domains, suggest-
ing that this may be a widespread and novel molecular archi-
tecture. To further explore this possibility, we undertook to
investigate both the GC and the kinase activity of PSKR1 as it is
an LRR RLK with a known ligand. We demonstrate that the
kinase domain of the PSKR1 has functional kinase and also GC
activity in vitro and that mutating a key residue in the GC cat-
alytic center reduces GC activity. Most importantly, we also
show that PSK-α, the natural ligand of PSKR1, does induce
cGMP production in vivo, indicating that modulating GC activ-
ity is a likely component of the signaling cascade initiated by
PSK-α.

In animals, all characterized GCs contain sequence similarity
to adenylate cyclase class III cyclase and are predicted to func-
tion as dimers (33). Changes in two residues (Asp and Lys to Glu
replaced by Ala) in an extracellular domain of a mammalian
membrane-bound GC, GUCY2D (retina-specific membrane guanylate cyclase 2D, human retGC-1), to an adenylate cyclase (35). The mammalian
receptor GCs are well characterized and include receptors for
natriuretic peptides (42) and the guanylin peptide family
(receptor guanylyl cyclase C) (43) as well as GUCY2D (44). These receptor GCs contain an extracellular domain where ligand binding occurs, a single transmembrane domain, a
kinase homology domain, a linker domain, and a C-ter-
minal GC domain and form homodimers (33). In a recent study (45),
the relationship between the receptor GC catalytic domains
and kinase homology domains was analyzed, and it was sug-
gested that these domains coevolved. The kinase domain is sepa-
rated from the GC domain by a linker domain whose length
appears to have been evolutionary conserved (45). Animal
receptor GC domain topology is thus distinctly different from
that observed in the plant receptor GCs, where the kinase
domain encapsulates the GC catalytic center (Fig. 1). Interest-

FIGURE 3. Demonstration of kinase activity of PSKR1-KD. A, Hanes-Woolf
plot of the kinetic determinants of serine-threonine kinase activity of the
recombinant PSKR1-KD2 using the Ser/Thr peptide 1 as a substrate and mea-
suring activity with the Omnia kinase assay. B, effect of substituting GTP for
ATP on the kinase activity of PSKR1-KD2. C, effect of cGMP on the kinase
activity of PSKR1-KD2. All experiments were performed in triplicate, and error
bars represent S.E. of a representative experiment from two independent
assays.

FIGURE 4. PSK-α stimulates cGMP production in freshly isolated leaf mes-
ophyll protoplasts. A, overexpression of full-length AtPSKR1 in protoplasts
stimulates cGMP production. Freshly isolated protoplasts were transiently
transfected with full-length AtPSKR1 or the empty vector control, and after
2–3 h, the protoplasts were harvested for cGMP assays. B, AtPSKR1 is ex-
pressed in leaves of 4-week-old soil grown Arabidopsis plants and also in
the protoplasts derived from mesophyll cells in leaves of 5–6-week-old plants
(cDNA (+), minus RT (−), non-template control (N)). C, cyclic GMP produc-
tion following treatment with PSK-α (0.1 μM) or nPSK (0.1 μM) in protoplasts
over 15 min in the absence of any phosphodiesterase inhibitor of a represent-
ative experiment in triplicate repeated at least three times. Asterisks indicate
significant differences (p < 0.05: two-way analysis of variance, Bonferroni post
test). D, cyclic GMP production following treatment with PSK-α (0.1 μM) or
nPSK (0.1 μM) for 15 min in protoplasts (10^6) after 45 min of pretreatment
with the phosphodiesterase inhibitor dipyridamole (100 μM) of a representa-
tive experiment performed in duplicate and repeated twice.
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ingly, GUCY2D appears to be the only animal receptor GC to contain an active kinase domain that autophosphorylates (44) as the kinase homology domains in other receptor GCs are predicted to be inactive (45). Membrane-bound GCs also occur in lower eukaryotes and have a topology more similar to mammalian adenylyl cyclases of two cassettes of six transmembrane spans that fold together to form the functional GC (46). Unicellular GCs generally do not contain kinase homology domains (45). Hence, the plant receptor GCs (AtPSKR1 (this study), AtBRI1 (8), and WAKL10 (10)) appear to be unique molecules as they contain overlapping functional kinase and GC catalytic domains.

To the best of our knowledge, this is the first report of kinase activity determined for the PSKR1 receptor. The recombinant kinase domain has a $K_m$ value of $\sim$7.5 μM obtained using a standard commercial substrate developed for serine/threonine kinases (Fig. 3A). This value is comparable with the values obtained for WAKL10-KD of 2.7 μM (10) and for the mouse cAMP-dependent kinase (PKA) catalytic subunit of 1.8 μM (32) using the same substrate. However, it is lower than the $K_m$ values of 71 or 82 μM reported for the related LRR RLK kinase domain BR11-KD using the BR112 peptide substrate (47, 48). Hence, the kinase domain of PSKR1 has a typical functioning catalytic activity at least in vitro. To date, there are no reports on the natural downstream substrates of PSKR1 kinase activity. One possibility might be the BR11-associated kinase 1 (BAK1), which partners other LRR RLKs such as BRI1 and flagellin sensing 2 (FLS2) (49–52). BAK1 is a promiscuous LRR RLK that interacts with BRI1 or FLS2 and other receptors to form hetero- and homo-dimers that initiate trans- or cross-phosphorylation of the intracellular domains of the receptor, and this in turn generates a downstream signaling cascade (49).

It is also noteworthy that GTP can also substitute for ATP in the kinase reaction (Fig. 3B). This ability of the kinase to use either purine nucleotide as a substrate possibly is an essential feature of the overlapping dual enzymatic activity as the GC catalytic center is located near the activation loop (Fig. 1D) that positions the purine in place for kinase activity. This ability may encourage GC activity in the presence of GTP as ATP cannot act as a substrate for adenylyl cyclase activity of the molecule (supplemental Fig. 1), presumably because of the GC-favoring amino acids present in the GC catalytic domain. Interestingly, we also observe that the product of GC activity, cGMP, itself suppresses kinase activity of PSKR1 in vitro (Fig. 3C), and this result raises the possibility that cGMP can directly feed back to modulate the receptor. The same recombinant protein preparations of PSKR1-KD were used to demonstrate both kinase and GC activity. Initial experiments revealed a relatively low GC activity in vitro with no preference for either Mn$^{2+}$ or Mg$^{2+}$, although their presence was critical for activity (Fig. 2). We have previously argued that such low activity was possibly due to lack of other essential co-factors in the in vitro assay buffer (8) and could also reflect suboptimal folding of the recombinant protein. Furthermore, plant GC activities as measured by cGMP levels are also relatively low particularly when compared with the levels observed with some animal soluble GCs (3). Significantly, when the full-length receptor was transiently expressed in plant cells, we observed about a 20-fold increase in cGMP levels when compared with vector-transfected control cells (Fig. 4A). This indicates that PSKR1 can produce cGMP in vivo and also that proper function of plant GCs probably requires additional co-factors that are critical for optimal activity. The level of cGMP production in transiently expressing cells is similar to that reported for Arabidopsis plants expressing α and β subunits of mammalian soluble GC (see unpublished observations in Ref. 4). Residues at position 3 in the catalytic center of the GC that are predicted to enable specificity for GTP in the active site include Gly, Cys, Thr, His, and Ser (33–35, 45, 46, 53, 54). PSKR1 contains a Gly at this position in the GC catalytic center, and when it was mutated to a Lys, GC activity was significantly reduced (Fig. 2C). This is a relevant finding as it shows that GC activity is dependent upon the predicted catalytic center and the presence of specific amino acids predicted to directly interact with the guanine moiety.

The effects of PSK-α signaling include cell proliferation, cell elongation, and root elongation mainly due to cell extension (13, 22, 30). As mentioned earlier, the actual proteins and other components relaying the downstream signaling cascade following binding of PSK-α to PSKR1 are not known. Our results implicate cGMP in the early events and so raise the possibility that cGMP-dependent proteins as well as kinase substrates form part of the relay network. Increases in cGMP levels were observed in protoplasts derived from leaves within minutes following application of PSK-α but not the non-sulfated nPSK (Fig. 4). The increases in cGMP are of similar orders of magnitude to those reported previously in response to extracellular signals such as gibberellic acid (40) or plant natriuretic peptide (31, 41, 55), where they have proven sufficient and critical for a downstream response. Furthermore, the increases in cGMP occur in a time frame that is consistent with activating the GC catalytic function of the PSKR1 receptor. A similar time frame would be expected to result in protein phosphorylation from the kinase as well. These early effects are unlikely to have been previously observed as the initial assays used to identify and characterize PSK activity were over considerably longer periods of 3–6 days, in which cell growth was assessed (30). This in turn raises the question of the role of cGMP in PSK signaling. Cyclic GMP modulates various cyclic nucleotide-gated ion channels and the transcriptome, where among other roles it regulates chloroplast development (1, 7, 56). In addition, cGMP has been implicated as a signaling molecule in cell expansion (31, 57), which is one of the described functional responses to PSK-α (13, 22). It is also conceivable that cGMP has a role in regulating the receptor, possibly by allosteric modulation.

Finally, we have developed a speculative model (Fig. 5) that takes into account the novel finding that PSKR1 has overlapping dual enzymatic activity. The model proposes that in its inactive form, PSKR1 can be either a monomer or a dimer. Upon binding of PSK-α, PSKR1 becomes catalytically active. Whether the activated PSKR1 acts as a kinase or GC first is unclear at this stage. However, it is likely that cyclic nucleotide activity may require the homodimer to correctly form the GC catalytic site. Thus it is conceivable that cross-phosphorylation of PSKR1 may enhance dimerization, which in turn may allow formation of cGMP. In support of this notion, the GC activity of
AtPSKR1-KD2 is increased in the presence of ATP. Production of cGMP (whether from AtPSK1 or other GCs) will modulate cGMP-dependent proteins such as various cyclic nucleotide-dependent ion channels (1, 6) and could also lead to formation of cGMP-PSKR1 complexes that suppress the kinase activity. It is also conceivable that heterocomplexes of PSKR1 and receptor-associated kinases such as BAK1, and this possibly activates the delayed adaptive responses to PSK-α (e.g. activation of cGMP-dependent transcriptome).

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