Phytosulfokine Regulates Growth in Arabidopsis through a Response Module at the Plasma Membrane That Includes CYCLIC NUCLEOTIDE-GATED CHANNEL17, H+-ATPase, and BAK1

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Phytosulfokine (PSK) is perceived by the leucine-rich repeat receptor kinase PSKR1 and promotes growth in Arabidopsis thaliana. PSKR1 is coexpressed with the CYCLIC NUCLEOTIDE-GATED CHANNEL gene CNGC17. PSK promotes protoplast expansion in the wild type but not in cngc17. Protoplast expansion is likewise promoted by cGMP in a CNGC17-dependent manner. Furthermore, PSKR1-deficient protoplasts do not expand in response to PSK but are still responsive to cGMP, suggesting that cGMP acts downstream of PSKR1. Mutating the guanylate cyclase center of PSKR1 impairs seedling growth, supporting a role for PSKR1 signaling via cGMP in planta. While PSKR1 does not interact directly with CNGC17, it interacts with the plasma membrane-localized H+-ATPases AHA1 and AHA2 and with the BRI1-associated receptor kinase 1 (BAK1). CNGC17 likewise interacts with AHA1, AHA2, and BAK1, suggesting that PSKR1, BAK1, CNGC17, and AHA assemble in a functional complex. Roots of deetiolated bak1-3 and bak1-4 seedlings were unresponsive to PSK, and bak1-3 and bak1-4 protoplasts expanded less in response to PSK but were fully responsive to cGMP, indicating that BAK1 acts in the PSK signal pathway upstream of cGMP. We hypothesize that CNGC17 and AHA form a functional cation-translocating unit that is activated by PSKR1/BAK1 and possibly other BAK1/RLK complexes.

INTRODUCTION

Peptide signaling has been recognized as a central component of cell-cell communication in plants (Murphy et al., 2012). Phytosulfokine (PSK) is a pentapeptide of the sequence Tyr-Ile-Tyr-Thr-Gln with a sulfate group attached to each of the two tyrosine residues (Matsubayashi and Sakagami, 1996; Sauter, 2015). Sulfation occurs in the trans-Golgi and is catalyzed by a tyrosylprotein sulfotransferase during passage of the proprotein through the secretory pathway (Komori et al., 2009). Partial proteolytic processing of the Arabidopsis thaliana PSK4 proprotein but not of other proproteins was shown to occur in the apoplasm by the subtilase SBT1.1 (Srivastava et al., 2008). PSK is perceived by membrane-bound leucine-rich repeat (LRR) receptor kinases (Matsubayashi et al., 2006) that are encoded by two genes, PSKR1 and PSKR2, in Arabidopsis.

Within the large family of LRR receptor kinases, the PSK receptors PSKR1 and PSKR2 are closely related to the brassinosteroid receptor BRASSINOLIDE INSENSITIVE1 (BRI1). The leucine-rich repeats of PSKR1, PSKR2, and BRI1 contain an island domain that was shown to bind the respective ligands, i.e., PSK and brassinolide (Kimishita et al., 2005; Shinohara et al., 2007; Clouse, 2011). A single transmembrane domain links the LRR region to a cytoplasmic kinase domain. The intracellular PSKR1 protein part was shown to possess serine/threonine kinase activity (Kwezi et al., 2011; Hartmann et al., 2014). The kinase domain of PSKR1 overlaps with a canonical guanylate cyclase (GC) core and was shown to also have, albeit weak, GC activity in vitro. Overexpression of PSKR1 in protoplasts raised the cGMP level 20-fold (Kwezi et al., 2011). cGMP had an inhibitory effect on PSKR1 kinase activity, suggestive of an autoregulatory signaling loop. A GC activity was also predicted for BRI1 (Kwezi et al., 2007; Wong and Gehring, 2013), but this was recently disputed based on crystal structure analysis of BRI1 and on enzyme assays (Bojar et al., 2014) such that the question of whether or not BRI1 produces cGMP remains a matter of debate.

PSK signaling through PSKR1 was shown to regulate root and hypocotyl elongation of Arabidopsis seedlings, while signaling via PSKR2 contributes to root but not hypocotyl elongation (Matsubayashi et al., 2006; Kutschmar et al., 2009; Stührwohldt et al., 2011). PSK-dependent growth was attributed mainly to enhanced cell elongation rather than increased cell proliferation rates (Kutschmar et al., 2009; Stührwohldt et al., 2011). Downstream of perception, the PSK signaling pathway is not understood. Recent data describe binding of PSKR1 to calmodulin (CaM) (Hartmann et al., 2014). When a conserved tryptophan at the CaM binding site of PSKR1 was mutated, the receptor variant was no longer able to bind CaM. Expression of this receptor variant in the receptor null background led to the development of roots that were shorter than those of receptor-less seedlings, indicating that Ca²⁺/CaM binding to PSKR1 is required for receptor activity.

The brassinosteroid receptor BRI1 is known to form a complex with a coreceptor, the BRI1-associated receptor kinase 1 (BAK1) (Chinchilla et al., 2009; Bücherl et al., 2013). BAK1 is
a promiscuous kinase that associates not only with BRI1 but also with receptors implicated in plant immunity, such as the flagellin receptor FLS2 (Postel et al., 2010), and BAK1 is also a target of the bacterial effector proteins AvrPto and AvrPtoB (Shan et al., 2008). Based on the close relationship of PSK receptors with BRI1 and on the promiscuity of BAK1, we hypothesized that PSKR1 may interact with BAK1.

Among the few known targets of cGMP regulation in plant cells are cyclic nucleotide-gated channels (CNGCs; Talke et al., 2003). The Arabidopsis genome encodes 20 members of the CNGC family, which fall into five subgroups in the monocot rice (Oryza sativa) as well as in the dicot species Arabidopsis (Talke et al., 2003; Ma et al., 2009). CNGC proteins possess six transmembrane helices with a P loop between helices 5 and 6 that is responsible for ion selectivity and ion passage. The C terminus is characterized by a CaM binding domain that partially overlaps with a cyclic nucleotide binding domain (Talke et al., 2003). A recent study revealed an additional CaM binding site characterized by an IQ motif that lies C-terminal of the previously described CaM binding domain and that does not overlap with the cyclic nucleotide binding site (Fischer et al., 2013). CNGCs are opened upon binding of cAMP or cGMP and closed upon binding of Ca²⁺/CaM, which was explained by the competitive binding of cyclic nucleotides and CaM to overlapping binding sites at the C terminus of the channel protein (Kaplan et al., 2007). Binding of CaM to a nonoverlapping site (Fischer et al., 2013) may allow for other modes of regulation. CNGCs are considered to be nonselective cation channels, but the selectivity and properties of most CNG channel proteins are not well characterized (Ward et al., 2009). Some were shown to facilitate K⁺ or monovalent cation fluxes, but permeability to Ca²⁺ has also been described (White et al., 2002; Ma et al., 2009). Arabidopsis CNGC1 and CNGC2 partially restored K⁺ permeability in an uptake-deficient yeast mutant (Köhler et al., 1999). Expression of Arabidopsis CNGC2 in a yeast mutant lacking low-affinity K⁺ uptake complemented growth inhibition in the presence of cyclic nucleotides. Furthermore, Xenopus laevis oocytes showed cyclic nucleotide-dependent, inward-rectifying K⁺ currents upon expression of CNGC2, whereas human cells expressing CNGC2 displayed increased Ca²⁺ uptake when treated with membrane-permeable cAMP or cGMP (Leng et al., 1999).

Substrate specificity and far-reaching implications for CNGC function in, e.g., osmotic adjustment or Ca²⁺ signaling. Functions have been suggested for only a few members to date. Knockout of CNGC2 in the mutant defense, no death 1 resulted in reduced local hypersensitive cell death and in elevated systemic defense (Clough et al., 2000). Loss of Arabidopsis CNGC18 function abolished normal pollen tube growth, resulting in male sterility (Frietsch et al., 2007). CNGC18 appeared to be distributed asymmetrically in the cell membrane with predominant localization at the flanks of the pollen tube tip (Frietsch et al., 2007). CNGC18 is one of five members of subgroup III of the CNGC family along with CNGC14, CNGC15, CNGC16, and CNGC17 (Talke et al., 2003). Overall, cGMP signaling in plants and the biochemical features, interacting proteins, and physiological functions of most CNGCs are not well understood.

In a screen for genes that are coexpressed with PSK receptors, we identified CNGC17. A report on GC activity of PSKR1 (Kwezi et al., 2011) prompted us to further study a possible role of CNGC17 in PSK-induced cell growth. Results described in this study unveiled interactions between PSKR1, CNGC17, AHA1, and AHA2, two isoforms of the plasma membrane-localized H⁺-ATPase, and BAK1, and we provide evidence that cGMP, CNGC17, and BAK1 participate in PSK-induced cell expansion.

RESULTS

The Cyclic Nucleotide-Gated Channel Gene CNGC17 Is Coexpressed with PSKR1 and Contributes to Seedling Growth

We performed a screen using the coexpression tool CressExpress (Srinivasasainagendra et al., 2008) to identify genes that are coexpressed with the PSK receptor PSKR1 in Arabidopsis. As a result, the CYCLIC NUCLEOTIDE-GATED CHANNEL17 (CNGC17) gene was identified. CNGC17 belongs to subgroup III of the CNGC family that further includes CNGC14, CNGC15, CNGC16, and CNGC18. CNGC17 has five introns (Figure 1). A T-DNA insertion is present in the fifth exon of line N541923 (SALK_041923) that was employed to study CNGC17 function (Figure 1A). CNGC17 transcript levels of homozygous cngc17 plants were strongly reduced compared with the wild type (Figure 1B; Supplemental Figure 1). To test if downregulation of CNGC17 induces expression of the related subgroup III genes of the CNGC family, which might compensate for CNGC17 knockdown, mRNA levels of CNGC14, CNGC15, and CNGC18 were compared in roots and shoots of wild-type and cngc17 seedlings (Figure 1B; Supplemental Figure 1A). Analysis of CNGC16 expression by quantitative real-time PCR (qPCR) was not successful using various sets of primers and yielded an additional band in RT-PCR analysis (Supplemental Figure 1A). CNGC14 transcript levels were higher in roots than in shoots, but expression did not differ significantly in cngc17 compared with the wild type. Likewise, CNGC15 and CNGC18 mRNA levels were not significantly altered in cngc17, supporting the idea that seedlings did not upregulate related CNGC genes to compensate for reduced CNGC expression.

Hypocotyls of etiolated cngc17 seedlings had wild-type lengths, whereas roots were significantly shorter and were less responsive to 1 μM PSK with a 13% increase in root length compared with a 22% increase in the wild type (Figures 1D and 1E). CNGC17 expression under the strong constitutive 35S promoter in the background rescued the short-root phenotype of cngc17 seedlings both in the absence of PSK and after treatment with 1 μM PSK, pointing to a role of CNGC17 in seedling growth (Figure 1E; Supplemental Figure 1B).

CNGC17 Is Required for PSK-Induced Protoplast Expansion

We showed previously that protoplasts expand rapidly in response to PSK but not in response to unsulfated PSK in a PSKR1-dependent manner (Stühwohldt et al., 2011; Figure 2A). Since PSKR1 exhibits GC activity in vitro and in vivo (Kwezi et al., 2011), we hypothesized that CNGC17 might be a target of PSKR1 signaling with cGMP as a second messenger (Figure 2). Protoplasts isolated from cngc17 seedlings showed delayed and reduced

CNGC17 Mediates PSK-Induced Cell Expansion
swelling in response to treatment with 1 nM PSK compared with the wild type, supporting this hypothesis (Figure 2A). Reduced PSK responsiveness in cngc17 was not overcome by applying elevated levels of PSK up to 100 nM (Figure 2B). However, the swelling response was restored when CNGC17 was expressed under the 35S promoter in the cngc17 background. Protoplasts from cngc17 35S:CNGC17-3 and cngc17 35S:CNGC17-7 lines showed wild-type swelling in response to 1 nM PSK, supporting the view that CNGC17 was a mediator of PSK-induced protoplast swelling (Figures 2B and 2C).

Swelling of tobacco (Nicotiana tabacum) protoplasts is induced by cGMP (Volotovski et al., 1998). Similarly, in this study, 50 μM of the membrane-permeable active cGMP variant 8-Br cGMP induced swelling of protoplasts from wild-type Arabidopsis seedlings. The effect of 8-Br cGMP after 30 min was comparable to that of PSK (Figures 2A and 2D). By contrast, cngc17 protoplasts showed delayed swelling and an overall smaller increase in volume than wild-type protoplasts, indicating that cGMP acts specifically through CNGC17 (Figure 2D).

CNGC17 and PSKR1 Interact with AHA1, AHA2, and BAK1 in Vivo at the Plasma Membrane

Protoplast swelling is a rapid response that occurs in response to PSK and is independent of protein synthesis (Stührwohldt et al., 2011). We hypothesized that it may rely on interaction of the PSK receptor with channel proteins in the plasma membrane. To test this hypothesis, we performed a split-ubiquitin assay (Figure 3; Supplemental Figure 2; Grefen et al., 2009). Full-length PSKR1 and CNGC17 proteins were expressed as Nub (Figure 3) or Cub (Supplemental Figure 2) fusions in yeast. After mating, the presence of the plasmids was verified by growth of yeast cells on synthetic complete medium containing adenine and histidine (SC-AH), whereas the putative interactions of the
fusion proteins were assayed by growth on SC medium and SC medium containing methionine (SC+met), which enhances the stringency of the interaction test. PSKR1 and CNGC17 alone did not show complementation as either Cub or Nub fusions (Figures 3A and 3B; Supplemental Figure 2).

Cell expansion during acid growth and in reversible stomata opening involves activity of the plasma membrane (PM)-localized H⁺-ATPase. To explore the possibility that PSK signaling of protoplast expansion may involve PM H⁺-ATPase, we tested for interactions of PSKR1 and CNGC17 with AHA1 and AHA2, the two most highly expressed and crucial isoforms of PM H⁺-ATPase (Haruta et al., 2010). Strong interactions of PSKR1-Nub and CNGC17-Nub with AHA1-Cub and AHA2-Cub were observed (Figures 3A and 3B). As AHA1 and AHA2 were previously shown to interact with the brassinosteroid receptor BRI1 (Caesar et al., 2011), which in turn forms heterodimers with the promiscuous kinase BAK1 (Chinchilla et al., 2009), we next explored possible interactions of PSKR1 and CNGC17 with BRI1 and BAK1. Neither PSKR1 nor CNGC17 interacted with BRI1, but both interacted with BAK1 (Figures 3A and 3B). The interactions of CNGC17 with AHA1, AHA2, and BAK1 were confirmed in reverse fusions in which CNGC17-Cub interacted with AHA1-Nub, AHA2-Nub, and BAK1-Nub, while the PSKR1-Cub protein did not interact with Nub fusions at all (Supplemental Figure 2). Taken together, the yeast mbSUS assay suggests that PSKR1 and CNGC17 do not interact directly but share the common binding partners AHA1, AHA2, and BAK1.

To verify the results of the mbSUS study, we performed a Förster resonance energy transfer (FRET)-fluorescence lifetime imaging microscopy (FLIM) analysis. PSKR1-GFP or BRI1-GFP were transiently coexpressed in tobacco leaf epidermal cells with RFP fusions of PSKR1, CNGC17, BAK1, AHA1, and AHA2 (Figures 4 and 5). To determine the background FLIM values, C-terminal GFP fusion constructs of PSKR1 and BRI1 were expressed alone (Figures 4A and 4F). When PSKR1-GFP was coexpressed with CNGC17-RFP, colocalization but no interaction was detected (Figures 4B, Table 1), as the fluorescence lifetime value (FLT) was unchanged in comparison to PSKR1-GFP alone (Table 1). By contrast, we observed colocalization and interaction of PSKR1-GFP with AHA1-RFP, AHA2-RFP, and BAK1-RFP (Figures 4C to 4E, Table 1). Finally, PSKR1-RFP colocalized with BRI1-GFP (Figure 4G), but the two receptors did not interact in the plasma membrane (Table 2).

Colocalization but no interaction of PSKR1 and CNGC17 was also observed when the reverse fusions CNGC17-GFP and PSKR1-RFP were coexpressed (Figure 5, Table 3). CNGC17-GFP colocalized and interacted with BAK1-RFP, AHA1-RFP, and AHA2-RFP (Figures 5C to 5G, Table 3). In particular, AHA1 showed a strong interaction with CNGC17 (Table 3). In summary, the data support specific interactions between PSKR1 and BAK1, AHA1, and AHA2 and also between CNGC17 and BAK1, AHA1, and AHA2 in yeast and in planta. The data further suggest that while PSKR1 and CNGC17 do not interact directly with each other, they may be constituents of a larger complex formed with BAK1, AHA1, and/or AHA2.
We further studied homomerization of CNGC17 and PSKR1 (Figure 6). FLIM analysis revealed a strong interaction between CNGC17-GFP and CNGC17-RFP, indicative of homodimerization or oligomerization (Figures 6A, 6B, and 6E). For PSKR1, no interaction was detected between PSKR1-Nub and PSKR1-Cub in the yeast assay (Figure 3A), whereas a weak interaction was observed with FLIM analysis (Figures 6C, 6D, and 6F).

Promotion of Seedling Growth and Protoplast Expansion by PSK Is Dependent on BAK1

PSKR1 shows a strong physical interaction with BAK1, suggesting that BAK1 might be required for PSK-mediated growth regulation. To test this hypothesis, 5-d-old seedlings of the T-DNA insertion lines bak1-3 and bak1-4 were grown on media supplemented with or without 1 nM PSK and root and hypocotyl lengths were compared with wild-type seedlings (Figure 7A). Roots of light-grown bak1-3 and bak1-4 seedlings were significantly shorter than wild-type roots and were unable to respond to PSK with enhanced growth unlike wild-type roots (Figure 7A). Roots and hypocotyls of etiolated bak1-3 and bak1-4 seedlings were marginally shorter than in the wild type (Figures 7B and 7D) and roots elongated less than wild-type roots in response to PSK (Figure 7B).

Protoplasts isolated from bak1-3 and bak1-4 seedlings expanded less in response to treatment with 1 nM PSK than wild-type protoplasts (Figure 7C), indicating that PSK-induced protoplast expansion was dependent on BAK1. To test if cGMP can act as a signal intermediate between PSKR1/BAK1 and CNGC17, we analyzed protoplasts from pskr1-3 and bak1-4 seedlings. pskr1-3 protoplasts do not expand when treated with PSK (Figure 7E; Stührwohldt et al., 2011) but do expand in response to cGMP (Figure 7E). Similarly, bak1-4 protoplasts, which do not expand in response to PSK (Figure 7C) showed a wild-type swelling response to 50 μM 8-Br-cGMP, supporting the view that cGMP-activated CNGC17 mediates protoplast expansion downstream of PSK and BAK1 (Figure 7E).

Taken together, we showed that PSK-induced protoplast expansion is dependent on PSKR1, BAK1, and CNGC17. Our results suggest that the PSKR1/BAK1 interaction contributes to growth promotion via the putative cation channel CNGC17 and H+-ATPase, and we provide evidence that cGMP acts as a signal intermediate between PSKR1 and CNGC17.
A Point Mutation in the GC Center of PSKR1 Impairs Receptor Function in Planta

PSKR1 has a GC center within subdomain IX of the kinase and was shown to produce cGMP in vitro and in vivo (Kwezi et al., 2011). Introducing the point mutation G923K in the soluble PSKR1 protein resulted in reduced cGMP synthesis. We generated a full-length PSKR1(G923K) receptor (Figure 8) and introduced it into the pskr1-3 pskr2-1 receptor-less background under the control of the 35S promoter (Supplemental Figure 3). The mutant receptor did not rescue the short-root phenotype of pskr1-3/pskr2-1 35S:PSKR1(G923K) lines. These results support the view that G923 in the GC center of PSKR1 is crucial for the growth-promoting activity of the PSK receptor.

DISCUSSION

PSK promotes cell growth in Arabidopsis seedlings and plants in a PSK receptor-dependent manner (Matsubayashi et al., 2006; Hartmann et al., 2013). Likewise, expansion of protoplasts is induced by PSK and is dependent on PSKR1 (Stührwohldt et al., 2011). Signaling of seedling and plant growth by PSK likely involves gene regulation, while protoplast expansion is a rapid response that occurs independently of de novo protein synthesis (Stührwohldt et al., 2011). The signal transduction pathway and downstream proteins that mediate long-term growth and protoplast expansion in response to PSK are not known. In general, plant cell growth or reversible cell expansion, for instance of guard cells, requires an increase in turgor, which can result from cation uptake and subsequent osmotically driven water influx. Irreversible cell growth further depends on altered cell wall properties that allow for controlled yielding of the cell wall. Acidification of the apoplastic space via activation of the PM localized H+-ATPase (termed AHA in Arabidopsis) by auxin (Duby and Boutry, 2009) or brassinosteroids (Caesar et al., 2011) activates cell wall loosening protein activities that allow for cell expansion commonly referred to as acid growth. AHA activation also changes the ion equilibrium across the PM, favoring cation influx. In this study, we provide evidence that PSK promotes cell expansion via cGMP as a second messenger that activates the cation channel CNGC17 as its target, cGMP was previously shown to promote swelling of tobacco protoplasts (Volotovski et al., 1998) and stoma opening in Tradescantia albilora, while inhibition of GC caused stoma closure, supporting a role for GC activity in guard cell expansion (Phamawati et al., 1998, 2001). We further show that the PSK receptor PSK1 interacts with the PM-localized ATPase isoforms AHA1 and AHA2, which in turn physically interact with CNGC17, thus providing indirect linkage between PSK1 and the cGMP target CNGC17. Strong downregulation of CNGC17 transcript levels in cngc17 was not compensated for by elevated expression of other subgroup III CNGCs, but CNGC15, CNGC16, and CNGC17, along with CNGC2 and CNGC20, were upregulated in response to cGMP in Arabidopsis plants, indicating positive control of genes that encode cGMP targets (Maathuis, 2006).

Interaction studies revealed binding of PSK1 to AHA1 and AHA2, the two most highly expressed isoforms of the Arabidopsis AHA gene family with 11 members in total. Recently, Caesar et al. (2011) reported activation of AHA1 and hyperpolarization of the plasma membrane by BR1 in a brassinolide (BL)-dependent manner. Interestingly, cell wall expansion required hyperpolarization of the plasma membrane and was not accomplished by cell wall acidification alone. Hyperpolarization and cell wall acidification synergistically induced cell wall expansion, while application of BL had a yet stronger effect, indicating that cell expansion requires several changes, including changes in electrochemical properties that are triggered by BR1. It is conceivable that the interaction of PSKR1 with AHA1 and AHA2 likewise induces AHA activity with plasma membrane hyperpolarization, apoplastic acidification, and cell wall extension as a result.

AHA1 and AHA2 were shown to not only interact with PSKR1 but also with the nCNG1-gated cation channel CNGC17. Furthermore, CNGC17 is coexpressed with PSK receptor genes, supporting a functional relationship between the encoded proteins. CNGCs are voltage-independent nonselective cation channels. Arabidopsis CNGC1, CNGC3, and CNGC4 transport Na+ and K+ (Leng et al., 1999; Balagué et al., 2003; Gobert et al., 2006). Calcium permeability was shown for CNGC2 (Hua et al., 2003), CNGC10 (Ali et al., 2007), and CNGC18 (Frietsch et al., 2007). However, for the majority of the 20 members of the Arabidopsis CNGC

Table 1. FLT (in ns) of PSKR1-GFP as a Control in Comparison to PSKR1-GFP Coexpressed with CNGC17-RFP, AHA1-RFP, AHA2-RFP, or BAK1-RFP in Tobacco Cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>FLT (in ns)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSKR1-GFP</td>
<td>2.43</td>
<td></td>
</tr>
<tr>
<td>CNGC17-RFP</td>
<td>2.43</td>
<td></td>
</tr>
<tr>
<td>PSKR1-GFP AHA1-RFP</td>
<td>2.33*</td>
<td>0.6344</td>
</tr>
<tr>
<td>PSKR1-GFP AHA2-RFP</td>
<td>2.33*</td>
<td>0.6344</td>
</tr>
<tr>
<td>PSKR1-GFP BAK1-RFP</td>
<td>2.12*</td>
<td>0.6344</td>
</tr>
<tr>
<td>Mean FLT</td>
<td>2.43</td>
<td></td>
</tr>
<tr>
<td>± SD</td>
<td>±0.02</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.6344</td>
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</tbody>
</table>

The lower the FLT, the stronger is the protein interaction. The asterisk indicates a significant difference to the control (two-sample Student’s t test). Values are means ± SD of five replicates. The results were confirmed in an independent biological experiment with another five replicates.

Table 2. FLT (in ns) of BRI1-GFP and BRI1-GFP Coexpressed with PSKR1-RFP

<table>
<thead>
<tr>
<th>Protein</th>
<th>FLT (in ns)</th>
<th>P value</th>
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<tbody>
<tr>
<td>BRI1-GFP</td>
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<td></td>
</tr>
<tr>
<td>PSKR1-RFP</td>
<td>2.41</td>
<td></td>
</tr>
<tr>
<td>Mean FLT</td>
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<td></td>
</tr>
<tr>
<td>± SD</td>
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<tr>
<td>P value</td>
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<td></td>
</tr>
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</table>

Values are means ± SD (two-sample t test) of five replicates. The results were confirmed in an independent biological experiment.

family, detailed electrophysiological characterization is lacking (Kaplan et al., 2007). In general, CNGCs are characterized by six transmembrane helices, a pore between helix five and six, and a C-terminal cytoplasmic extension that carries the cNMP binding site as well as a CaM binding domain that overlaps with the cNMP binding site (Köhler and Neuhaus, 2000; Talke et al., 2003). For CNGC2, binding of cNMP and CaM to their overlapping domains was shown to result in competition such that CaM binding inactivated channel activity (Hua et al., 2003). Recently, CaM binding to an IQ motif that is localized more proximal to the C-end was described for CNGC20, which would not interfere with cNMP binding (Fischer et al., 2013). Regardless of the site of CaM binding, the current data indicate that CNGCs are subject to feedback regulation by Ca2+/CaM at the protein level.

Protoplasts displayed rapid expansion in response to PSK and in response to cGMP. CNGC17 was required for protoplast expansion, indicating that cation influx through CNGC17 directly or indirectly drives PSK-induced and cGMP-induced water influx. Expansion of maize (Zea mays) protoplasts in response to PSK was dose-dependent on K+ in the medium (Stührwohldt et al., 2011). K+ uptake via CNGC17 would result in a direct increase in osmotic potential and subsequent water uptake. A change in osmotic potential could also be brought about by CNGC17-mediated Ca2+ influx and Ca2+-regulated cation channel opening. Protoplasts that lacked PSKR1 expanded in response to cGMP in a wild-type manner, indicating that cGMP acts downstream or independently of PSK/PSKR1. This evidence supports a function of cGMP as a second messenger in PSK signaling during protoplast expansion.

A putative GC catalytic domain was identified within subdomain IX of the PSKR1 kinase based on sequence comparison with known GCs (Kwezi et al., 2011). Ectopically expressed soluble PSKR1 kinase was shown to generate cGMP at a low level. Furthermore, Arabidopsis leaf protoplasts that were transformed with full-length PSKR1 produced more basal cGMP than untransformed protoplasts, and cGMP production was enhanced by PSK but not by unsulfated PSK pentapeptide. A GC core was also identified in the related LRR-RLKs Arabidopsis PepR1 (Qi et al., 2010) and BRI1 (Kwezi et al., 2007) and GC activity for both recombinantly expressed soluble proteins was demonstrated (Kwezi et al., 2007; Qi et al., 2010). On the other hand, a recently published analysis of the BRI1 crystal structure combined with activity assays of recombinantly

| Table 3. FLT (in ns) of CNGC17-GFP Coexpressed with PSKR1-RFP, BAK1-RFP, AHA1-RFP, or AHA2-RFP in Tobacco Cells |
|-----------------|-----------------|-----------------|-----------------|
|                 | CNGC17-GFP     | CNGC17-GFP     | PSKR1-RFP       |
| Mean FLT        | 2.42           | 2.36           |
| ± SD            | ±0.05          | ±0.07          |
| P value         | 0.1409         |                |
|                 | CNGC17-GFP     | CNGC17-GFP     | BAK1-RFP        |
| Mean FLT        | 2.45           | 2.29*          |
| ± SD            | ±0.03          | ±0.10          |
| P value         | 0.0            |                |
|                 | CNGC17-GFP     | CNGC17-GFP     | CNGC17-GFP      |
| Mean FLT        | 2.51           | 2.19*          | 2.36*           |
| ± SD            | ±0.09          | ±0.11          | ±0.08           |
| P value         | 0.0002         | 0.0124         |

Values are means ± SD of five replicates. The results were confirmed in independent biological experiments with five replicates each; asterisk indicates a significant difference to the control (two-sample Student’s t test).
expressed soluble BRI1 protein concluded that BRI1 does not possess GC activity (Bojar et al., 2014). While it seems likely that PSKR1 directly synthesizes cGMP that binds to and activates CNGC17, it is also conceivable that cGMP is generated by an as yet unidentified GC upon PSKR1 activation to drive CNGC17-dependent protoplast expansion.

Interaction studies further revealed binding of PSKR1 to BAK1. BAK1 acts as a coreceptor for a number of LRR receptor kinases, including BRI1 and FLS2 (Chinchilla et al., 2009; Postel et al., 2010; Kim et al., 2013), and binds to the bacterial effector proteins AvrPto and AvrPtoB. This latter interaction prevents binding of BAK1 to the immune receptor FLS2 and activation of immune responses (Shan et al., 2008). It was proposed that BAK1 acts as an adaptor protein that generally ensures activity of RLKs (Postel et al., 2010). The LRR-RLK superfamily consists of more than 600 members that are classified in 16 families. PSKR1 groups with BRI1 in family X (Morillo and Tax, 2006). For BRI1, the best-studied LRR-RLK binding of the ligand brassinosteroid was shown to release the inhibitory protein BRI1 kinase inhibitor 1 (BK1), which initiates a series of transphosphorylations between BRI1 and BAK1 that result in receptor activation (Jiang et al., 2013). Using live-cell imaging, BRI1 was shown to interact with BAK1 also in the absence of a ligand, and signal initiation was proposed to take place from preassembled heterodimeric receptor complexes (Bücherl et al., 2013). Our finding that BAK1 binds to PSKR1 with high affinity is therefore not surprising and

**Figure 7.** Root Growth and Protoplast Expansion in Response to PSK Are Reduced in bak1-3 and bak1-4 Compared with the Wild Type.

(A) Root lengths (±SE, n = 65, P < 0.001, ANOVA, Tukey’s test) of wild-type, bak1-3, and bak1-4 seedlings grown with or without 1 μM PSK for 5 d at long-day conditions. Numbers in gray bars indicate the percent increase in root length.

(B) Root lengths (±SE, n = 61, P < 0.001, ANOVA, Tukey’s test) of etiolated wild-type, bak1-3, and bak1-4 seedlings grown with or without 1 μM PSK for 5 d. Numbers in gray bars indicate the percent increase in root length.

(C) Protoplasts from bak1-3 and bak1-4 hypocotyls are impaired in PSK-dependent expansion compared with the wild type.

(D) Hypocotyl lengths of etiolated seedlings grown for 5 d.

(E) Protoplasts from bak1-4 and pskr1-3 seedlings show a wild-type swelling response to 50 μM 8-Br-cGMP. At time = 0 min, 1 nM PSK or 50 μM 8-Br-cGMP was added as indicated. Results are averages (±SE) of up to 11 protoplasts analyzed per treatment and genotype.

(A), (B), and (D) Results were obtained from two independent biological experiments. Different letters indicate significantly different values.

**Figure 8.** The G923K Mutation in the GC Center of PSKR1 Results in Receptor Inactivation in Planta.

(A) Schematic diagram of PSKR1 with its N-terminal signal peptide (red), extracellular LRRs, the PSK binding island domain, the single transmembrane helix (TM), the juxtamembrane domains (gray) adjacent to the TM, and the intracellular kinase (blue) starting at position 733 with the designated GC center shown in green. The receptor has a total length of 1008 amino acids. The exchange of a glycine by a lysine at position 923 (G923K) is indicated.

(B) Representative photographs of 5-d-old wild-type, pskr1-3 pskr2-1 (r1r2), and 35S:PSKR1(G923K)-3 seedlings.

(C) Primary root lengths were analyzed in 5-d-old seedlings of the wild type, pskr1-3 pskr2-1 (r1r2), the GFP-tagged wild-type receptor complementation line 35S:PSKR1-GFP-1, and three independent 35S:PSKR1(G923K) lines (averages ± SE, n = 24 for 35S:PSKR1-GFP-1, and n ≥ 64 for all other lines). Different letters indicate significantly different values (P < 0.001, Tukey’s test).
The semicircles indicate experimentally shown protein interactions. (A) The semicircles indicate experimentally shown protein interactions. (B) PSKR1 possesses kinase activity, binds to CaM (Hartmann et al., 2014), and was described as a GC (Kwezi et al., 2011). PSKR1 interacts with BAK1 and with the H+-ATPases AHA1 and AHA2. BAK1, AHA1, and AHA2 in turn bind to the cyclic nucleotide-gated cation channel CNGC17 that is subject to regulation by cGMP and CaM (Zelman et al., 2012; Fischer et al., 2013). AHAs, CNGC17, and the promiscuous coreceptor BAK1 are predicted to form a functional core module that links proton extrusion to cation uptake across the plasma membrane. We hypothesize that different receptor kinases, such as PSKR1 or BRI1, can bind to this core complex, which mediates cell expansion. The broken line indicates possible cation permeabilities for CNGC17.

This study shows that CNGC17 interacts with H+-ATPase, possibly resulting in a functional module that links proton extrusion to cation influx. This transport protein complex interacts with BAK1 via CNGC17. In addition, PSKR1 binds to the H+-ATPase and to BAK1 (Figure 9). Some if not all of these interactions are conserved between the PSK receptor PSKR1 and the brassinosteroid receptor BRI1: (1) BRI1 interacts with BAK1; (2) BRI1 interacts with AHA1 (Caesar et al., 2011); (3) since BAK1 interacts with CNGC17, and given that the interactions occur at the same time rather than independently of each other, it follows that not only PSKR1 but also BRI1 can physically connect with CNGC17 via BAK1 and AHA. This would suggest that a membrane-anchored protein nanocluster exists that brings together a proton pump, a cation channel, and the promiscuous coreceptor BAK1. This core complex can connect to different receptors such as BRI1 or PSKR1. Binding of BL to BRI1 results in proton pump activation, cell wall acidification, wall relaxation, and cell expansion (Caesar et al., 2011). Cell expansion was not previously reported with BRI1. BRI1 interacts with AHA1 (Caesar et al., 2011); (3) since BAK1 is known to bind to various proteins, interaction with a channel protein has not been described previously. Since both PSK and BR control cell elongation, they may both rely on CNGC17 to do so.

This study shows that CNGC17 interacts with H+-ATPase, possibly resulting in a functional module that links proton extrusion to cation influx. This transport protein complex interacts with BAK1 via CNGC17. In addition, PSKR1 binds to the H+-ATPase and to BAK1 (Figure 9). Some if not all of these interactions are conserved between the PSK receptor PSKR1 and the brassinosteroid receptor BRI1: (1) BRI1 interacts with BAK1; (2) BRI1 interacts with AHA1 (Caesar et al., 2011); (3) since BAK1 interacts with CNGC17, and given that the interactions occur at the same time rather than independently of each other, it follows that not only PSKR1 but also BRI1 can physically connect with CNGC17 via BAK1 and AHA. This would suggest that a membrane-anchored protein nanocluster exists that brings together a proton pump, a cation channel, and the promiscuous coreceptor BAK1. This core complex can connect to different receptors such as BRI1 or PSKR1. Binding of BL to BRI1 results in proton pump activation, cell wall acidification, wall relaxation, and cell expansion (Caesar et al., 2011). Cell expansion was not induced to the same degree by cell wall acidification and hyperpolarization as it was by BL, indicating that BL triggers additional responses. These may come from CNGC17-mediated influx of osmotically active K+, of the second messenger Ca2+, or both. Interestingly, in immune signaling, CNGC2 was suggested to mediate cytosolic Ca2+ elevation in response to the peptide signal Arabidopsis Pep1 in a PepR1-dependent manner (Ali et al., 2007; Qi et al., 2010). CNGCs may thus be general targets of LRR-RLKs that possess GC activity. Recent studies showed that PSK not only regulates growth but also modulates immune responses through PSKR1 (Igarashi et al., 2012; Mosher et al., 2013). It is tempting to speculate that CNGC17 may not only play a role in growth regulation but also in immunity.

In conclusion, we propose that plant cell growth is subject to regulation by a plasma membrane-bound protein nanocluster that is composed in a modular fashion of a conserved core element that contains transport proteins, a promiscuous coreceptor, and a variable receptor that provides ligand specificity.

**METHODS**

**Plant Material, Growth Conditions, and Protoplast Analysis**

Experiments with *Arabidopsis thaliana* were performed on ecotype Columbia-0. The *pskr1-3 T-DNA* insertion line, the double knockout line *pskr1-3 pskr2-1*, and the 35S:PSKR1-GFP-1 line were previously described (Kutschmar et al., 2009; Stührwohldt et al., 2011; Hartmann et al., 2014). The *cngc17 T-DNA* insertion line SALK_041923 and homozygous seeds for the BAK1 T-DNA insertion lines, bak1-3 (SALK_116202C), and bak1-4 (SALK_034523C) were obtained from NASC (Nottingham Arabidopsis Stock Centre, University of Nottingham, Nottingham, UK). The T-DNA in *cngc17* is inserted in the fourth exon just after the coding sequence for the sixth transmembrane region and preceding the large regulatory cytoplasmic C terminus. For growth experiments, Arabidopsis seeds were surface-sterilized for 20 min in 1 mL 2% (w/v) sodium hypochlorite, washed five times with autoclaved water, and laid out under sterile conditions on square plates. Seedlings were grown in the dark at 22°C for 5 d or under long-day conditions at 22°C for 5 d, at 80 µM on plates containing 0.5x strength Murashige and Skoog media (Murashige and Skoog, 1962) and 1.5% (w/v) sucrose solidified with 0.38% (w/v) Gelrite (Duchefa). The media were supplemented with PSK (NeoMPS) as indicated.

Protoplasts were isolated from hypocotyls of 5-d-old etiolated Arabidopsis seedlings by digesting the tissues with cellulase Onozuka RS (Duchefa) and pectolyase (Kikkoman) for 3 h as described (Stührwohldt et al., 2011). Only vital protoplasts with strong cytoplasmic streaming were used for the experiments. Protoplast volumes were calculated from the circumference that was determined at each time point indicated assuming a spherical shape.

**qPCR, RT-PCR, and Cloning Procedures**

Total RNA was isolated from whole seedlings or shoots and roots of 5-d-old wild-type Columbia-0 and *cngc17* seedlings with Tri-Reagent following manufacturer’s instructions (Sigma-Aldrich) and treated with DNase I (MBI Fermentas). For RT-PCR analysis, RNA was reverse transcribed with oligo (dt) primers (MBI Fermentas). *CNGC14, CNGC15, CNGC16, CNGC17, CBGC18, and ACTIN2* cDNAs as a loading control were amplified with the gene-specific primers listed in Supplemental Table 1.

qPCR analysis of *CNGC14, CNGC15, CNGC17*, and *CBGC18* was performed with 10 ng cDNA each using the Rotor-Gene SYBR Green PCR Kit (Qiagen) with *ACTIN2* and *GAPC1* as control genes using the primers listed in Supplemental Table 1. The relative transcript abundance was
calculated based on the ΔΔCP method (Pfaffl, 2001) in relation to each reference gene and averaged from three independent biological replicates.

35S:CNGC17 lines were generated in the cngc17 background by amplifying the CNGC17 full-length coding sequence. To generate the PSKR1(G923K) receptor variant, a site-directed mutation of the full-length PSKR1 receptor was performed via overlap extension PCR. The open reading frames of CNGC17 and PSKR1(G923K) were each ligated into pB7WG2 downstream of the cauliflower mosaic virus 35S promoter via an LR reaction (Gateway cloning; VIB) (Karimi et al., 2002). Independently transformed lines were identified and analyzed as described previously (Kutschmar et al., 2009).

**Protein Interaction Studies**

Mating-based split ubiquitin assays were performed as described (Grefen et al., 2009). For FRET analysis, the sequences of BR1, BAK1, PSKR1, AHA1, AHA2, and CNGC17 were expressed as C-terminal chromophore fusions in pH7FWG2 (GFP) or pB7RWG2 (RFP) (Karimi et al., 2002). These binary vectors and p19 as gene silencing suppressor were transformed into Agrobacterium tumefaciens binary vectors and p19 as gene silencing suppressor were transformed into lines were generated in the 35S:PSKR1(G923K) complementation lines. The lifetime $\tau$ [ns] of either the donor only expressing cells or the donor-acceptor pairs was measured with a pulsed laser as an excitation light source of 473 nm and a repetition rate of 40 MHz (PicoQuant Sepia). The lifetime $\tau$ of the donor dimer was determined using the SP8 laser scanning microscope (Leica Microsystems) with LAS AF software and monoexponential curve fitting, correction for the instrument response function, and implications for brassinosteroid signal initiation. Plant Physiol. 191: 1191–1195.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers PSKR1 (AT2G02220), CNGC14 (AT2G24610), CNGC15 (AT2G28260), CNGC16 (AT3G48010), CNGC17 (At4g30360), CNGC18 (At5g14870), cngc17 T-DNA insertion line SALK_041923, BAK1 (At4g33430), bak1-3 T-DNA insertion line SALK_116202C, bak1-4 T-DNA insertion line SALK_034523C, and pskr1-3 T-DNA insertion line SALK_008585.

**Statistical Analysis**

Data were statistically analyzed using Minitab. Comparison of means was analyzed for statistical significance with an ANOVA (Tukey’s test) or a two-sample Student’s t test. Constant variance and normal distribution of data were verified before statistical analysis and the P value was set to P < 0.001 if one or both conditions were not achieved. The P value for the Pearson product moment correlation is indicated in the figure legends.

**Supplemental Data**

Supplemental Figure 1. CNGC gene expression and 35S:CNGC17 complementation lines.

Supplemental Figure 2. PSKR1 and CNGC17 interactions detected with the yeast mating-based split-ubiquitin system.

Supplemental Figure 3. 35S:PSKR1(G923K) complementation lines in the receptor null pskr1-3 pskr2-1 (tr12) background express the mutant receptor gene.

Supplemental Table 1. Primers used for RT-PCR and qPCR analyses.

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Phytosulfokine Regulates Growth in Arabidopsis through a Response Module at the Plasma Membrane That Includes CYCLIC NUCLEOTIDE-GATED CHANNEL17, H⁺-ATPase, and BAK1

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