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Plant Phytosulfokine Peptide Initiates Auxin-Dependent Immunity through Cytosolic Ca²⁺ Signaling in Tomato

Running title: PSK initiates Ca²⁺- and auxin-dependent immunity

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ABSTRACT

The disulfated pentapeptide, phytosulfokine (PSK), which is an important signaling molecule, has recently been implicated in plant defenses to pathogen infections, but the mechanisms involved remain poorly understood. Here, using surface plasmon resonance and gene silencing approaches, we show that SIPSKR1 rather than SIPSKR2, functions as the major PSK receptor in immune responses of tomato (*Solanum lycopersicum*). Silencing of PSK signaling genes rendered tomato more susceptible to infection by the economically-important necrotrophic pathogen *Botrytis cinerea*. Analysis of tomato mutants defective in defense hormone biosynthesis or signaling demonstrated that PSK-induced immunity requires auxin biosynthesis and associated defense pathways. Using aequorin-expressing tomato plants, we provide evidence that PSK perception by SIPSKR1 elevates cytosolic $[Ca^{2+}]$, leading to auxin-dependent immunity responses via enhanced binding activity between SiCaMs and the auxin biosynthetic SiYUCs. Thus, the data presented here demonstrate that PSK perceived mainly by SIPSKR1 increases cytosolic $[Ca^{2+}]$, leading to auxin-mediated pathways that enhance immunity to *B. cinerea*. The discovery of this PSK signaling pathway provides new insights into PSK-mediated control of plant immunity.

INTRODUCTION

Within natural environments, plants are exposed to attack by a wide variety of herbivores and microbial pathogens. Biotic threats to crops pose a significant risk to agriculture and can result in tremendous economic losses to the farmer. During evolution, plants have acquired a sophisticated innate immune system that serves to mitigate the adverse effects of pathogen attack. However, accurate signal perception is a prerequisite for the elicitation of appropriate defense responses. This is achieved by the presence of an array of cell surface-localized pattern recognition receptors (PRRs), particularly receptor-like kinases (RLKs) and receptor-like proteins (RLPs) that detect apoplastic elicitors. PRRs such as FLAGELLIN-SENSING 2 (FLS2), FLAGELLIN-SENSING 3 (FLS3), EF-Tu receptor (EFR), and Chitin Elicitor receptor kinase 1 (CERK1) directly sense pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) (Chinchilla et al., 2006; Zipfel et al., 2006; Miya et al., 2007; Hind et al., 2016). Upon recognition, these receptors trigger both local and systemic pathogen-defense signaling cascades leading to basal immunity and non-host resistance (Böhm et al., 2014). In addition to PAMPs/MAMPs, plant cell surface receptors

are also involved in the perception of endogenous plant compounds, referred to as damage-associated molecular patterns (DAMPs), to trigger immunity (Boutrot and Zipfel, 2017). In particular, the plant secreted disulfated pentapeptide, phytosulfokine [PSK; (SO₃H)-Ile-Tyr (SO₃H)-Thr-Gln] is thought to be a DAMP acting in the immune response.

PSK is generated by the processing of precursors of ~80 amino acids that are encoded by PSK gene families, which are ubiquitous in higher plants. PSK precursors undergo tyrosine sulfation by a tyrosylprotein sulfotransferase (TPST) in the cis-golgi followed by proteolytic cleavage in the apoplast (Srivastava et al., 2008; Komori et al., 2009). Mature PSK peptides are recognized at the cell surface by the membrane-bound receptor, PSKR, which belongs to the leucine-rich repeat receptor-like kinase (LRR-RLK) class (Matsubayashi et al., 2002). However, the signaling mechanisms and pathways that PSK activates are unknown.

AtPSKR1 and AtPSKR2 were shown to be PSK receptors in Arabidopsis. These receptors contain a conserved extracellular LRR domain and a cytoplasmic kinase domain (Matsubayashi et al., 2006; Amano et al., 2007). The extracellular domains consist of 21 LRRs with an island domain that is required for PSK perception (Matsubayashi et al., 2002; Matsubayashi et al., 2006; Amano et al., 2007). Recognition between the PSK-PSKR pair functions as a master switch for a complex, but poorly defined, intracellular signaling pathway. Moreover, the plant PSKR family and their ligand-binding properties have not been extensively characterized other than in Arabidopsis and *Daucus carota* (carrot), and little is known about the molecular steps that link PSK to downstream signaling events.

The cytoplasmic kinase domain of PSKR1 overlaps a canonical guanylate cyclase (GC) core. Overexpression of AtPSKR1 in protoplasts resulted in an increase in the endogenous levels of guanosine 3',5'-cyclic phosphate (cGMP), suggesting that Arabidopsis PSKR1 has GC activity (Kwezi et al., 2011). Similar to AtPSKR1, the cytosolic domains of other LRR-RLKs such as the Arabidopsis Pep receptor (AtPepR1) and the brassinosteroid (BR) receptor (AtBRI1) have a GC core with GC activity. Moreover, the generation of cGMP from GTP has been demonstrated by analysis in vitro of the recombinant AtPepR1 and AtBRI1 proteins (Kwezi et al., 2007; Qi et al., 2010). cGMP is a potential activating ligand for cyclic nucleotide-gated channels (CNGCs) in plants. These cation channels are thought to facilitate Ca²⁺ and other cation fluxes (Ladwig et al., 2015). Intriguingly, Pep-AtPepR1 signaling leads to the expression of pathogen-defense genes, which was suggested to be mediated by the cGMP-activated Ca²⁺-conducting channel CNGC2 as well as the elevation of cytosolic Ca²⁺ levels (Qi et al., 2010; Ma et al., 2012). Similarly, BR perception by the receptor AtBRI1 leads to immediate cGMP-dependent cytosolic Ca²⁺ release in vivo, a

process that was abolished when the CNGC2 gene was mutated in Arabidopsis (Zhao et al., 2013a). Interestingly, study in Arabidopsis have demonstrated that PSK promotes cell expansion via a cGMP-dependent pathway, which also activates a cation channel protein encoded by CNGC17 (Ladwig et al., 2015). It is thus reasonable to hypothesize that the PSK receptor, PSKR1, induces an intracellular Ca^{2+} burst at the cell surface, which leads to an immune response in cells where this pathway is triggered.

PSK signaling pathways frequently act together with phytohormones to fine-tune plant responses to external and metabolic stimuli through interactions that can be either synergistic or antagonistic (Mosher et al., 2013; Rodiuc et al., 2016). For example, PSKR1 decreases the resistance of Arabidopsis plants to the biotrophic pathogen *Pseudomonas syringae* but enhances defenses against the necrotrophic pathogen *Alternaria brassicicola*. Such observations were suggested to be related to suppression of salicylic acid (SA)-mediated defense responses (Mosher et al., 2013). PSK has also been demonstrated to suppress ethylene (ET) production and thus being involved in regulating Arabidopsis copper homeostasis (Wu et al., 2015). PSK requires auxin to stimulate non-embryogenic proliferation in carrot cell cultures (Eun et al., 2003).

Necrotrophic fungal pathogens such as the grey mold fungus *Botrytis cinerea* are a major threat to food security worldwide. *B. cinerea* has a remarkable host range, encompassing over 200 plant species. This pathogen alone causes annual losses of several hundreds of millions of US dollars worldwide, mainly because of its adverse effects on tomato production (Dean et al., 2012). To date, accessions with complete resistance to this pathogen has not yet been identified in tomato. Understanding the molecular basis of PSK-triggered immunity to *B. cinerea* therefore not only has intrinsic scientific value with significant translational opportunities in tomato, but it also has the potential to provide new knowledge that can lead to enhanced disease resistance in a wide range of crops. Here, we present the first evidence for a PSK-SIPSKR1 signaling pathway, which involves intracellular Ca^{2+} release, leading to downstream auxin-dependent signaling cascades that trigger appropriate immune responses against *B. cinerea* in tomato.

RESULTS

PSK signaling confers immunity against *B. cinerea* in tomato

To investigate the function of PSK in the tomato immune system acting against *B. cinerea*,

exogenous PSK-induced defense responses were induced by applying 0.5-20 μM PSK. The application of PSK decreased fungal growth, as measured by *B. cinerea* actin mRNA accumulation in a concentration-dependent manner, the effect being maximal at the 10 and 20 μM concentrations (Supplemental Figure 1A). Based on preliminary dose-dependent trial experiments, the 10 μM concentration was selected as the optimal level required to induce the required response in the following experiments. Leaves were sprayed with either PSK, desulfated PSK peptide (dPSK) or water (control) 12 h before pathogen inoculation. Chlorophyll a fluorescence imaging was used to determine the damage response to photosynthesis, assessed by changes in the quantum yield of photosystem II (ΦPSII) to *B. cinerea* infection. While ΦPSII was significantly decreased 2 days post-inoculation (dpi) with *B. cinerea*, this parameter remained higher in PSK-treated plants (Figures 1A and 1C). These observations are consistent with a lower level of pathogen infection in PSK-treated plants compared with dPSK- or non-pretreated controls, as determined by *B. cinerea*-specific actin mRNA accumulation and the extent of cell death, assessed by trypan blue staining (Figures 1B and 1D).

Eight PSK precursor genes were identified in tomato based on homology to the *Arabidopsis* genes (Supplemental Figure 1B). At the transcriptional level, the expression of four of these precursor genes (SIPSK3, SIPSK3L, SIPSK4 and SIPSK7) as well as the single copy tyrosine sulfation processing gene SITPST, was significantly induced upon *B. cinerea* infection (Figure 1E). Transcript abundance of these genes was reduced using virus-induced gene silencing (VIGS) to examine their respective roles in innate immunity. This approach decreased the levels of target gene transcripts by up to 75% compared to empty vector controls (TRV:0), with little or no effect on the expression of non-target homologous genes (Supplemental Figure 1C; Supplemental Data Set 1). However, there was an exception in that the TRV:SIPSK3 plants exhibited significantly lower levels of SIPSK3L transcripts under conditions of *B. cinerea* infection (Supplemental Figure 1C). This may be due to the degree of similarity between the two PSK precursors, resulting in co-silencing of the SIPSK3 and SIPSK3L genes in the TRV:SIPSK3 plants. However, this was not the case in the TRV:SIPSK3L plants, where SIPSK3L gene silencing was specific (Supplemental Figure 1C). Upon *B. cinerea* inoculation, even though gene-silencing in the TRV:SIPSK4 and TRV:SIPSK7 plants did not influence responses to *B. cinerea* inoculation, the susceptibility of TRV:SIPSK3 and TRV:SIPSK3L, as well as the TRV:SITPST plants were significantly enhanced, as shown by the significant decrease in ΦPSII , more dead cells in the leaves and increases in leaf *B. cinerea* actin accumulation (Figures 1F to 1I). Thus, SIPSK3L and

SITPST contribute positively to defense. It is also possible that SIPSK3 might participate in PSK-mediated defense responses. Taken together, these findings suggest that PSK signaling is vital for the induction of tomato immunity against *B. cinerea*.

PSK receptor identification and its biological functions in tomato immunity

Based on overall amino acid similarity to *Arabidopsis* PSKR1/2 and *D. carota* PSKR, Solyc01g008140.3 (SIPSKR1) and Solyc07g063000.3 (SIPSKR2) were determined as putative tomato PSKR paralogs. The homologous PSKRs belong to the large LRR-RLKs family, each of which contains conserved extracellular tandem copies of LRR, an island domain, a transmembrane domain, and a cytoplasmic kinase domain (Supplemental Figures 2A and 2B; Supplemental Data Set 2). To determine the sub-cellular localization of the tomato PSKR paralogs, the binary vectors 35S::PSKRs fused to GFP and 35S::SIFLS2-mCherry as a plasma membrane localization marker, were transiently co-expressed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens*-mediated transformation (Robatzek et al., 2006). SIPSKR1-GFP and SIPSKR2-GFP co-localized with SIFLS2-mCherry at the plasma membrane (Figure 2A).

The binding affinities of PSK to SIPSKR1 and SIPSKR2 were determined using surface plasmon resonance (SPR) analysis. The recombinant-expressed extracellular portions of these two proteins were immobilized onto the surface of a CM5 sensor chip via amine coupling. Concentration-dependent binding was recorded following the application of PSK or dPSK. Both SIPSKR1 and SIPSKR2 interacted with PSK. The binding affinity constant (KD) values were in the similar micromolar range to that described for PSK and the recombinant DcPSKR protein (Wang et al., 2015). PSK exhibited a slightly stronger binding affinity to SIPSKR1 than to SIPSKR2. The association rate constant (k_a) for the SIPSKR1-PSK interaction was higher than that of the SIPSKR2-PSK interaction (Figure 2B). SPR assays confirmed the important role of the sulfate group in mediating PSK recognition. The naturally-occurring form of PSK displayed the stronger binding affinity and higher k_a values with regard to both SIPSKR1 and SIPSKR2 compared to dPSK. This result is in agreement with the observation that dPSK elicits defense responses against *B. cinerea* in tomato but to a lower extent than PSK (Figures 1A to 1D). Similarly, the application of relatively high concentrations of dPSK also triggered a weak PSK response in *Arabidopsis* (Kutschmar et al., 2009). A recent study demonstrated that PSK stabilizes the DcPSKR island domain, which in turn recruits a somatic embryogenesis receptor-like kinase (SERK)

to form a stable PSKR-SERK complex, leading to allosteric activation of PSKR (Wang et al., 2015). The silencing of SIPSKR1 impaired leaf immunity, whereas silencing SIPSKR2 had no significant effects. The co-silencing of the SIPSKR1/R2 genes did not lead to any further changes compared to the silencing of SIPSKR1 alone, confirming the limited role for SIPSKR2 in defenses against *B. cinerea* (Figures 2C to 2F; Supplemental Figure 2C; Supplemental Data Set 3). Furthermore, *B. cinerea* infection induced a slight but significant increase in SIPSKR1 transcript abundance, but again such changes were not observed in the expression of the SIPSKR2 gene (Supplemental Figure 2C). Taken together, these observations suggest that SIPSKR1, rather than SIPSKR2, plays a crucial role in PSK perception and immunity to *B. cinerea*.

PSK-induced immunity requires downstream auxin biosynthesis and associated defense pathways

Considerable cross talk between PSK signaling pathways and hormone pathways has been suggested to occur in the regulation of plant growth and stress responses (Eun et al., 2003; Mosher et al., 2013; Wu et al., 2015; Rodiuc et al., 2016). The relative levels of pathogen defense-related hormones (Glazebrook, 2005; Kazan and Manners, 2009) were therefore analyzed under mock- and *B. cinerea*-inoculated conditions. Significant increases in the levels of phytohormones, including SA, jasmonic acid (JA), ET, and indole-3-acetic acid (IAA), were observed in response to *B. cinerea* (Figure 3A). However, only IAA contents were increased in response to the PSK treatment in both mock- and *B. cinerea*-inoculated plants. In contrast, the levels of other hormones were either constant or decreased in response to PSK application (Figure 3A). The changes in the transcript abundance of marker genes for the signaling-related pathways of these hormones are consistent with the observed changes in hormone contents (Figure 3B).

The responses of mutants, which are defective in hormone accumulation or signaling, also provide evidence for the roles of phytohormones in PSK-induced immunity. For example, tomato NahG plants do not accumulate SA because of salicylate hydroxylase overexpression (Brading et al., 2000). The *jai1-1* mutants of tomato are defective in CORONATINE-INSENSITIVE1 (COI1) and show impaired JA-signaling (Li et al., 2004). Never ripe (Nr), which is mutated at the dominant tomato ethylene receptor NEVER RIPE, shows greatly reduced sensitivity to ethylene (Tieman et al., 2000). The *diageotropica* (*dgt*) mutants are defective in a type A cyclophilin protein, leading to suppression of the

TIR1/AFB auxin receptor-induced signaling cascade (Lavy et al., 2012). The *jai1-1*, *Nr* and *dgt* mutants exhibited increased susceptibility to *B. cinerea* (Figures 3C and 3D), whereas no changes in susceptibility were observed in the *NahG* plants. Strikingly, the application of PSK promoted plant defenses to the same extent in the WT and the *NahG* plants, as well as in the *jai1-1* and *Nr* mutant lines. However, the PSK treatment had no effect on the susceptibility of the *dgt* plants to *B. cinerea* (Figures 3C and 3D).

We next verified the involvement of auxin pathways in PSK-induced immunity using the gene-silenced tomato plants described above. Blocking the expression of the PSK synthesis and signaling component genes, *SIPSK3*, *SIPSK3L*, *SITPST*, and *SIPSKR1* led to lower IAA contents and to a higher level of disease susceptibility (Figures 4A and 4B; Supplemental Figure 3A). This increased susceptibility could be largely reversed by the exogenous application of nanomolar amounts of NAA (Figures 4A and 4B). *SIPSKR1* silencing compromised tomato immunity, an effect that could be complemented by the application of NAA but not PSK (Figures 4C and 4D; Supplemental Figure 3B). Interestingly, minor changes in the transcript abundance of genes encoding PSK signaling components were observed following NAA application under both mock- and *B. cinerea*-inoculated conditions (Figure 4E). These results strongly suggest that auxin functions downstream of PSK-*SIPSKR1* signaling in tomato immunity against *B. cinerea*.

PSK triggered cytosolic Ca²⁺ signaling in the immune response

PSK-induced Ca²⁺ signaling was determined by monitoring the effect of PSK on cytosolic Ca²⁺ levels in tomato leaf discs that express the Ca²⁺ reporter protein, aequorin. Cytosolic Ca²⁺ concentrations [Ca²⁺] were rapidly increased upon PSK application compared with the dPSK or control treatments (Figure 5A; Supplemental Figure 4A), suggesting that Ca²⁺ channels are activated by PSK in planta. In confirmation of this hypothesis, PSK did not cause cytosolic Ca²⁺ release in the presence of the Ca²⁺ channel inhibitors, ruthenium red (RR) or verapamil (Ver) (Figure 5A). PSK-induced immunity against *B. cinerea* was also compromised by these inhibitors (Figures 5C to 5F). Furthermore, the observed PSK-mediated increase in cytosolic [Ca²⁺] was impaired in TRV:*SIPSKR1* but not TRV:*SIPSKR2* plants (Figure 5B; Supplemental Figure 4B). Taken together, these observations suggest that cytosolic Ca²⁺ signaling functions in PSK-*PSKR1* binding-induced events. These findings provide evidence of a novel signaling cascade elicited in tomato defense responses against *B. cinerea*.

SlCaMs binding to the auxin biosynthetic protein SlYUCs mediates PSK-induced immunity against *B. cinerea*

Having established that both auxin and cytosolic Ca^{2+} signaling are required for PSK-induced immunity against *B. cinerea*, we explored the connection between these two signaling pathways further. Calmodulins (CaMs) are Ca^{2+} -binding proteins, whose affinities to target proteins and hence activities are modified upon Ca^{2+} binding. This process translates changes in local $[\text{Ca}^{2+}]$ into a specific physiological responses (McCormack et al., 2005; Hartmann et al., 2014). There are six homologous CaM genes encoding a total of four CaM protein isoforms in tomato. SlCaM3, SlCaM4 and SlCaM5 are identical in amino acid sequence. SlCaM1, SlCaM2 and SlCaM6 are also highly conserved and share 98%, 99%, and 91% amino acid identity with SlCaM3/4/5, respectively (Supplemental Figure 5A). Of these, SlCaM2 exhibits the highest transcript levels in tomato leaves and other organs (Zhao et al., 2013b). Plant YUCCA (YUC) genes encode flavin-containing monooxygenases that are rate-limiting enzymes in the typical two-step pathway of auxin biosynthesis, which might be required for auxin-modulated pathogen defense responses (Dai et al., 2013; Hentrich et al., 2013). Based on the Arabidopsis YUCs amino acid sequences, nine SlYUC homologs were identified in tomato (Supplemental Figure 5B). Three of these, i.e. SlYUC2, SlYUC8, and SlYUC9 are barely detectable at the transcriptional level in leaves (Supplemental Figure 5C). All the other six SlYUC isoforms are predicted to contain putative CaM binding motifs. The four SlCaM genes (SlCaM1, SlCaM2, SlCaM3, and SlCaM6) and the six SlYUC isoforms (SlYUC1, SlYUC3, SlYUC4, SlYUC5, SlYUC6, and SlYUC7) were cloned and expressed in *N. benthamiana* and used in bimolecular fluorescence complementation (BiFC) assays (Figure 6A; Supplemental Figure 6A). Taking the most abundant SlCaM2 isoform (Zhao et al., 2013b) as the target protein, BiFC analysis demonstrated that the SlYUC1, SlYUC3, SlYUC5, and SlYUC6 proteins, but not the SlYUC4 or SlYUC7 proteins, show fluorescence signals when co-expressed with SlCaM2 (Figure 6A). It should be noted that the expression of SlYUC7 is relatively low at both transcriptional and protein levels (Supplemental Figure 5C; Supplemental Figure 6A). Thus, it is still not known whether SlYUC7 could interact with SlCaM2, and further studies are required to elucidate this issue. Furthermore, among these SlYUC genes, SlYUC6 transcript abundance was significantly induced upon *B. cinerea* infection (Supplemental Figure 5C). Hence, this gene product was used as a target protein to further examine interactions with

the other three SlCaM protein isoforms (SlCaM1, SlCaM3, and SlCaM6). As shown in Figure 6A, SlCaM1, SlCaM3 and SlCaM6 were also able to bind to SIYUC6, even though the signal was a little weaker with regard to SlCaM6. The SlCaMs- and SIYUCs-dependent fluorescence signals largely co-localized with those of SIFLS2-mCherry in the plasma-membrane, whereas SIYUC6 specifically localized (Figure 6A and Supplemental Figure 6B). Strikingly, the fluorescence signals were strengthened under PSK-treated conditions in the BiFC assays (Figure 6B). The interactions between the SlCaM2 and SIYUC6 proteins were further verified using co-immunoprecipitation (Co-IP) following expression of these two proteins with different tags in *N. benthamiana* (Figure 6C). Intriguingly, SlCaM2-SIYUC6 binding was increased by the PSK and CaCl₂ treatments. In contrast, binding was weakened in the presence of the Ca²⁺ channel inhibitor RR. These findings suggest that PSK modulates SlCaM2 in such a way as to enhance the binding coefficient towards SIYUC6.

We further investigated the contribution of the SlCaMs-SIYUCs interaction to PSK-induced immunity. We constructed a TRV:SlCaM2 vector based on the targets of the SlCaM homologs (Supplemental Data Set 4). This vector efficiently silenced the expression of most of the SlCaMs genes (Supplemental Figure 7). The TRV:SlCaM2 plants showed weaker defenses towards *B. cinerea* infection than the TRV:0 controls. The lower level of immunity observed in these plants was accompanied by lower IAA contents (Figures 7A to 7D). Furthermore, SlCaM2 silencing compromised PSK-induced immunity and IAA accumulation, an effect that could be complemented with NAA but not PSK (Figures 7A to 7D). Thus, PSK-induced IAA accumulation and immunity were largely dependent on the functions of the Ca²⁺ sensor SlCaMs.

DISCUSSION

Peptide signaling pathways have been well studied in animals and shown to fulfill many important functions. In contrast, peptide signaling underpinning plant responses to environmental stimuli or developmental triggers has received much less attention and remains poorly characterized. Here we present several lines of evidence demonstrating that the tomato PSK peptide acts as an immunity-regulating signal against the necrotrophic pathogen *B. cinerea*. The data not only extend our understanding of the PSK receptor family in tomato, but they also show that perception of PSK by SIPSKR1 initiates a cytosolic Ca²⁺ signaling cascade, leading to the binding of SlCaMs to SIYUCs that is associated with auxin-dependent immunity against *B. cinerea*. We present a new model in which the Ca²⁺

signal triggered by the PSK signaling peptide modulates auxin biosynthesis, leading to innate immune responses to this major plant pathogen (Figure 8). We present several lines of evidence in support of this conclusion which we discuss below.

Firstly, the expression of several tomato PSK precursor genes was induced in response to *B. cinerea* inoculation (Figure 1E). The application of the synthetic PSK peptide resulted in enhanced defenses against *B. cinerea* relative to controls and dPSK treatments (Figures 1A to 1D). Moreover, silencing of the peptide precursor genes (SIPSK3 and SIPSK3L), the tyrosine sulfation gene (SITPST), and the receptor gene (SIPSKR1) significantly impaired leaf defense responses, leading to enhanced disease symptoms (Figures 1F to 1I; 2C to 2F). These results support the conclusion that the PSK signal peptide is essential for immunity to the necrotrophic pathogen *B. cinerea* in tomato. The positive contribution of PSK signaling to resistance against *B. cinerea* has not been reported previously, although PSK has been implicated in defenses against another necrotrophic fungal pathogen *A. brassicicola* in *Arabidopsis* (Mosher et al., 2013).

Secondly, the characteristics of the PSK receptor in tomato were described for the first time. LRR-RLK SIPSKR1 and SIPSKR2 share a high sequence homologies and structural identities to AtPSKR1/2 (Matsubayashi et al., 2006; Amano et al., 2007) and to DcPSKR (Matsubayashi et al., 2002) (Supplemental Figures 2A and 2B; Supplemental Data Set 2). SPR analysis demonstrates that PSK has a higher KD and k_a with respect to the recombinant extra cellular region of the SIPSKR1 than to SIPSKR2 (Figure 2B). The affinity values obtained in this study are in the similar micromolar range to those reported for the PSK-DcPSKR interaction that was measured by microscale thermophoresis (MST) method (Wang et al., 2015). They are however, weaker than the [³H]PSK-PSKR interactions measured using ligand-based affinity chromatography of microsomal fractions from either carrot (Matsubayashi et al., 2002) or *Arabidopsis* (Matsubayashi et al., 2006). The discrepancies in reported affinities probably indicate that the cellular environment provides a more favorable medium for interactions between PSK and its receptor proteins compared to that used in in vitro assays for studies on recombinant proteins. Even though SIPSKR2 interacted with PSK and was shown to be involved in PSK perception (Figure 2B), the gene silencing approaches used in this study confirm that only SIPSKR1 is the major transducer of the PSK signal in the regulation of *B. cinerea* defenses. SIPSKR2 silencing did not lead to any significant effects on plant responses to *B. cinerea* (Figures 2C to 2F). Similarly, *Arabidopsis* loss-of-function and gain-of-function studies demonstrated that PSK perception requires AtPSKR1 for the regulation of root growth, while AtPSKR2 had a more

marginal role (Amano et al., 2007). In accordance with the current study, mutants lacking *AtPSKR2* showed similar responses to wild type *Arabidopsis* with regard to pathogens such as *A. brassicicola* and *P. syringae* (Igarashi et al., 2012; Mosher et al., 2013). Therefore, *SIPSKR2* functions may be largely inactive or below the level of detection in relation to plant immunity.

Thirdly, the data presented here demonstrate that auxin biosynthesis and associated signaling are required for PSK-induced immunity against *B. cinerea*. Within the context of phytohormone-mediated immunity, SA-dependent resistance is effective largely against biotrophs and hemi-biotrophs, whereas JA- and ET-mediated responses are predominantly effective against necrotrophs (Glazebrook, 2005). While auxin is a classical growth-regulating hormone in plants, it has been shown to positively modulate plant immunity in response to necrotrophic pathogens (Llorente et al., 2008; Kazan and Manners, 2009; Qi et al., 2012). SA has been suggested to be linked to the PSK signaling, attenuating responses to the hemi-biotrophic *P. syringae* and PAMPs (Igarashi et al., 2012; Mosher et al., 2013; Rodiuc et al., 2016). However, few studies have investigated PSK-induced phytohormone modulation in plant-necrotrophic pathogen interactions. The observed susceptibility phenotype of the *Arabidopsis pskr1* mutant to the necrotroph *A. brassicicola* led us to speculate that SA, JA or ET pathways may be involved in defense signaling (Mosher et al., 2013). The data presented here demonstrate the absence of significant changes in SA or JA levels or in the transcript accumulation of marker genes related to the signaling pathways of these phytohormones following PSK application (Figures 3A and 3B). A previous transcriptome study using *Arabidopsis* gain-of-function overexpressing lines and loss-of-function mutants also reported that signaling through *PSKR1* does not significantly affect SA- or JA-related gene expression (Rodiuc et al., 2016). In addition, ET signaling is important in plant immunity against *B. cinerea*. Pretreatments with irreversible ethylene perception inhibitors resulted in significant increases in pathogen susceptibility in tomato (Díaz et al., 2002). However, ET generation was suppressed by PSK application in the present study (Figure 3A), a finding that agrees well with a previous study in *Arabidopsis* (Wu et al., 2015). These findings suggest that ET signaling has only a minor role in PSK-induced immunity against *B. cinerea*. Moreover, PSK-induced immunity was not changed in the *NahG*, *jai1-1* or *Nr* mutants (Figures 3C and 3D). Taken together, these findings suggest that SA, JA, and ET signaling are not required for PSK-induced immunity against *B. cinerea* in tomato.

Based on the observations reported here, we propose that auxin signaling functions

downstream of PSK signaling leading to immunity against *B. cinerea* in tomato. This conclusion is based on several lines of evidence: i) the application of PSK increased auxin levels and the abundance of transcripts associated with auxin signaling under both mock- and *B. cinerea*-inoculated conditions (Figures 3A and 3B). ii) PSK-induced immunity against *B. cinerea* is compromised in the auxin signaling mutant *dgt* compared to the WT (Figures 3C and 3D); iii) Silencing of the *SIPSK3*, *SIPSK3L*, *SITPST* and *SIPSKR1* genes substantially decreased *B. cinerea*-induced IAA accumulation (Figure 4A). Silencing of the PSK-related genes (*SIPSK3*, *SIPSK3L*, *SITPST*, and *SIPSKR1*) induced susceptibility to *B. cinerea* in a manner that was effectively complemented by the application of physiological levels of NAA without any effect on the PSK transcript abundance (Figures 4B and 4E); iv) *SIPSKR1* silencing compromised-immunity was complemented by the application of NAA but not by PSK (Figures 4C and 4D). Auxin is involved in most aspects of plant growth and development (Benjamins and Scheres, 2008), which is also required for PSK-induced cell proliferation in asparagus (Matsubayashi et al., 1999) and in carrot (Eun et al., 2003). In agreement with the current study, *Arabidopsis* auxin signaling mutants *axr1*, *axr2* and *axr6* that are impaired in the auxin-stimulated SCF (Skp1–Cullin–F-box) ubiquitination pathway, exhibited increased susceptibility to *B. cinerea* and some other necrotrophic fungi (Llorente et al., 2008). The *asa1-1* and *cyp79b2/b3* *Arabidopsis* mutants, which are defective in auxin biosynthesis, were more susceptible to infection by *A. brassicicola*, another type of necrotrophic pathogen, than wild type plants (Qi et al., 2012). Therefore, the activation of downstream auxin defense pathways may underpin the effects of PSK on growth, enhancing growth while promoting immunity against necrotrophic pathogens, including *B. cinerea*.

The final proof in support of the novel molecular signaling pathway triggered by PSK is the demonstration that cytosolic Ca^{2+} and the binding of sensor CaMs to auxin biosynthetic proteins are crucial components of the Ca^{2+} -activated auxin signaling pathway that combats *B. cinerea* infection. The generation of PSK- and *SIPSKR1*-dependent Ca^{2+} accumulation contributes to the innate response to *B. cinerea* (Figure 5). CaMs are prototypical calcium sensors that translate local changes in $[\text{Ca}^{2+}]$ into physiological responses (McCormack et al., 2005). Whether cytosolic Ca^{2+} signaling regulates auxin biosynthesis is unknown. However, this study has shown that SiCaMs bind to the rate-limiting SIYUCs of the auxin biosynthesis pathway (Figure 6), providing a potential mechanism by which cytosolic Ca^{2+} signaling may modulate auxin synthesis. Most importantly, the interaction between SiCaM2 and SIYUCs was shown to be responsive to PSK (Figures 6B and 6C), a finding that is also essential to PSK-induced IAA generation and immunity against *B. cinerea* (Figure 7). In

accordance with this observation, previous studies have revealed that SiCaM2 overexpression enhanced *B. cinerea* defenses in tomato fruit (Peng et al., 2014). In addition, the PSK-induced cytosolic Ca^{2+} wave and the elicitation of immunity were completely inhibited by the addition of calcium channels blockers (Figure 5). This suggests that the observed increases in cytosolic $[\text{Ca}^{2+}]$ may be generated through the PSKR1 GC activity-induced opening of the CNGC channel (Ladwig et al., 2015). It should be noted that CaMs not only act as Ca^{2+} sensors that bind SIYUCs, but they can also bind directly to the PSKR1 receptor at the kinase subdomain (Hartmann et al., 2014; Fischer et al., 2017). Binding of AtPSKR1 to CaMs within the kinase subdomain is required for AtPSKR1 functioning in the regulation of growth responses of Arabidopsis roots (Hartmann et al., 2014). The data presented here do not allow any conclusions to be drawn with regard to whether SIPSKR1 is directly involved in Ca^{2+} signal generation, or whether this receptor acts as an indirect sensor. Interestingly, prior work with Arabidopsis demonstrated that supplying physiologically-relevant concentrations of calcium inhibited PSKR1 kinase activity, while enhancing its GC activity in vitro. These findings suggest that calcium acts as a PSKR1 bimodal switch between the overlapping kinase and GC activities (Muleya et al., 2014). A similar protein structure with dual kinase/GC activities has previously been reported for AtBRI1 (Kwezi et al., 2007) and for wall-associated kinase-like 10 (Meier et al., 2010), with similar predictions for other kinases. Based on these observations, we conclude that cytosolic Ca^{2+} levels and CaMs, triggered by PSK signaling, initiate the finely-tuned regulation of PSKR activities that are able to respond to different stimuli.

In conclusion, the data presented here show that the signal transduction cascade by which PSK triggers immunity to the necrotrophic pathogen *B. cinerea*, requires SIPSKR1. This in turn triggers cytosolic Ca^{2+} signaling and leads to increases in auxin synthesis and associated auxin-dependent immunity in tomato (Figure 8). Since orthologs of PSK precursors have been identified across the plant kingdom, it will be interesting to investigate whether the perception of PSK ligands by their receptors in other species uses a similar mechanism for regulating immunity to necrotrophic pathogens, as well as for the control of plant growth. Manipulation of peptide-induced defenses is an attractive disease management strategy that could potentially be used to enhance disease resistance in many diverse plant species.

METHODS

Plant materials and VIGS

The tomato (*Solanum lycopersicum* L.) lines used in most of the studies were mainly in the MoneyMaker (MM) background. However, some studies were also conducted using several wild-type (WT) tomato lines that were used as controls, as appropriate, depending on the mutant background. Specifically, tomato seeds of the JA-signaling mutant *jai1-1* and its WT progenitor Castlemart (CM) were kindly provided by Dr. C. Li (Chinese Academy of Sciences, Beijing, China). Homozygous *jai1-1* seedlings were selected from F2 populations as described previously (Li et al., 2004). The ET-signaling mutant Nr and its WT line cv. Pearson, as well as the auxin signaling insensitive mutant *dgt* and its isogenic WT line cv. VFN8 were obtained from the Tomato Genetics Resource Center (University of California, Davis, CA, USA). Seeds of the NahG transgenic line (in which overexpression of salicylate hydroxylase abolishes SA accumulation) and its WT control line MM were from the laboratory of Dr. J.D.G. Jones (Sainsbury Laboratory, Norwich, UK). The MM line expressing aequorin to monitor the effects of PSK treatment on cytosolic Ca²⁺ concentrations was obtained from Dr. Gerald A. Berkowitz (University of Connecticut, Connecticut, USA). Tomato seeds were sown in sterilized soil in 72-well trays and germinated at 25 °C. After a 2-week germination period, the seedlings were transplanted into plastic pots (diameter, 10.5 cm; depth, 17.5 cm; one plant per pot) containing soil and perlite in controlled-environment growth chambers (Conviron, Winnipeg, Canada). The photosynthetic photon flux density (PPFD) was 500 μmol m⁻² s⁻¹, the photoperiod was 14/10 h (day/night), the day/night air temperature was 25/20 °C, and the relative humidity was 88%.

VIGS was performed by infiltration of fully expanded cotyledons of 10 day-old tomato (MM) seedlings with bipartite tobacco rattle virus (TRV) vectors using a mix of pTRV1 and pTRV2. PCR-amplified cDNA fragments of the target genes were cloned into pTRV2. In the case of co-silencing of *SIPSKR1* and *SIPSKR2*, both fragments were cloned into pTRV2 by different multiple cloning site. The resulting plasmid was transformed into *A. tumefaciens* GV3101. The empty pTRV2 vector was used as control. The success of the VIGS protocol was evaluated according to the method of Liu et al. (2002) using the expression of the phytoene desaturase (PDS) gene, which causes photo-bleaching, as a marker for silencing in tomato. The infiltrated plants were grown under a 14 h photoperiod at 22 °C. Three to 4 weeks later, transcript abundance of targeted genes were analyzed by qRT-PCR in each plant. Samples from the upper first 1~2 fully expanded leaves were collected by punching discs. Only plants showing significant silencing were used for

experiments. The primers used for VIGS cloning and the qRT-PCR assay are listed in Supplemental Data Set 5. The nucleotide sequence alignment of homologous genes based on tomato database (available at: <http://solgenomics.net/>) and online VIGS tool (<http://vigs.solgenomics.net/>) revealed no significant identical sequence between VIGS targets and non-target homologous genes (see Supplemental Data Sets 1, 3, and 4).

Between 5 and 10 four-week-old tomato plants at about five-leaf stage were used for each treatment in all the VIGS or non-VIGS experiments. Except where mentioned, samples were randomly collected from lateral leaflets from the uppermost 1~2 fully expanded leaves. For the VIGS experiments, the transcript levels of target and non-target homologous genes were determined using the same samples as those used for the *B. cinerea* actin transcript assays (See Supplemental Figures 1C, 2C, 3A, 3B, and 7). Single intact leaflets were used for the disease symptom assays, with samples taken from the corresponding opposite leaflets for the assays of silencing efficiency and *B. cinerea* actin transcript.

Pathogens, elicitor treatment and disease symptom assays

Tomato leaves were inoculated with *B. cinerea* (BO5-10 strain) suspensions at a density of 2×10^5 spores mL⁻¹, and mock inoculations were performed using media buffer (Zhang et al., 2015). The inoculation was carried out by spraying the inoculum suspension on the whole leaf portion. For the elicitor treatment, unless otherwise noted, plant leaves were sprayed with water or fresh solutions of 10 μM PSK (NeoMPS, Strasbourg, France), 10 μM desulfated PSK (dPSK) (ChinaPeptides, Shanghai, China), 10 nM NAA (Sigma), 20 μM RR (Sigma) or 20 μM Ver (Sigma), individually or in combination. For experiments combining elicitor treatment and *B. cinerea* inoculation, the leaves were inoculated with *B. cinerea* 12 h after elicitor pretreatment.

After pathogen inoculation, disease symptoms were assessed by quantifying *B. cinerea* actin mRNA accumulation by qRT-PCR (Zhang et al., 2015), trypan blue staining (Bai et al., 2012), or by analysis of chlorophyll fluorescence with an Imaging-PAM Chlorophyll Fluorometer (IMAG-MAXI, Heinz Walz, Effeltrich, Germany). The quantum efficiency of light-adapted leaves (Φ PSII) was calculated as $F_m' - F / F_m'$ (Genty et al., 1989).

RNA isolation, transcript analysis and qRT-PCR

RNA was extracted using an RNA extraction kit (Axygen) followed by DNase digestion

(Promega), and reverse transcribed using a ReverTra Ace quantitative (qPCR) RT Kit (Toyobo), according to the manufacturer's instructions. qRT-PCR was performed using the LightCycler 480 Real-Time PCR System (Roche Diagnostics). Each reaction (20 μ L) consisted of 10 μ L of SYBR Green PCR Master Mix, 8.2 μ L of water, 1 μ L of cDNA, and 0.4 μ L of forward and reverse primers. PCR was performed using 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C. The specific primers employed for target genes and internal control actin gene are described in Supplemental Data Set 5.

PSK receptor recombinant protein expression, purification and PSK binding activity analysis

Protein expression and purification were performed according to previous work (Manohar et al., 2015). Briefly, the extracellular regions of the potential PSKR protein-encoding genes, SIPSKR1 and SIPSKR2, were cloned and inserted into the pET28a vector to enable the expression of recombinant proteins with an N-terminal His₆ tag. The error-free clones were confirmed by sequencing and then transformed into *Escherichia coli* strain BL-21 (DE3). Expression of the proteins was induced by isopropyl- β -D-thiogalactopyranoside, and the proteins were purified by affinity chromatography on a Ni-NTA His-binding resin (Novagen).

SPR analysis of the PSK binding activity was performed with a Biacore T200 instrument (GE Healthcare) with a CM5 sensor chip (GE Healthcare). Activation, deactivation, and preparation of the coupled flow cell as well as the ligand-binding assay were performed essentially as described previously (Song et al., 2014). Briefly, the recombinant SIPSKR1- and SIPSKR2-encoding proteins were immobilized in parallel-flow channels of the CM5 sensor chip using an amine coupling kit (GE Healthcare). To test PSK binding to potential receptors, serial concentrations of PSK or dPSK (diluted in 0.01 M PBS, pH 7.4) were injected into the flow system. Experiments were conducted with PBS (pH 7.4) as the running buffer, and the analyte was injected at a flow rate of 30 μ L/min. The association time was 90 s, the dissociation time was 180 s, and the chip was regenerated for 30 s with 50 mM NaOH. Equilibration of the chip with the running buffer for another 60 s was performed before the next injection. The kinetic constants of binding were obtained using a 1:1 Langmuir binding model in BIA evaluation software.

Phytohormone content measurement

The phytohormones SA, JA and IAA were extracted from tomato leaves as described previously with minor modifications (Durgbanshi et al., 2005; Wu et al., 2007). Briefly, frozen tomato leaves (100 mg) were homogenized with 1 mL of ethyl acetate spiked with D5-JA, D5-IAA and D4-SA (OIChemIm) as internal standards to a final concentration of 100 ng mL⁻¹. After shaking for 12 h in the dark at 4 °C, the homogenate was centrifuged at 18,000 g for 10 min at 4 °C. The supernatant was collected, and the pellet was re-extracted with another 1 mL of ethyl acetate, shaken for 2 h and centrifuged. The supernatants from the two centrifugation steps were combined and evaporated to dryness under N₂ gas. The residue was re-suspended in 0.5 mL of 70% (v:v) methanol and centrifuged at 18,000 g for 2 min at 4 °C. The final supernatants were pipetted into glass vials and then analyzed by HPLC-MS/MS using the same method described previously (Wang et al., 2016).

Ethylene production was measured as described previously (Yin et al., 2012). Briefly, tomato seedlings were sealed in 500-mL rubber-topped flasks for 1 h at 20 °C, and then 1 mL of head-space gas was removed and injected into a gas chromatograph (Lunan Chemical Engineering Instrument Co. Ltd., model SP 6800, Shandong, China) fitted with a GDX-502 column. The temperatures of the injector, detector and oven were 110, 140 and 90 °C, respectively.

Cytosolic Ca²⁺ measurements

Cytosolic Ca²⁺ levels were evaluated as described previously (Tanaka et al., 2010; Zhao et al., 2013a) with some modifications using aequorin-expressing MM tomato lines. Briefly, leaf discs (0.3 cm diameter) collected from different plants were transferred individually to a 96-well microplate and incubated overnight in 50 µL of reconstitution buffer in the dark to allow binding between CTZ and aequorin. The buffer contained 12.5 µM coelenterazine-cp (CTZ-H) (LUX Innovate), 1 mM KCl, 1 mM CaCl₂ and 10 mM MgCl₂, adjusted to pH 5.7 with Tris base. After overnight incubation, 50 µL of PSK solution or water control was added to the wells. Ruthenium red and verapamil were added to the leaves 30 min before adding PSK. Luminescence was measured using a Microplate Luminometer (Titertek Berthold, Germany) with a 0.5 min interval reading time over a period of 35 min. At the end of each experiment, the remaining aequorin was discharged by the addition of an equal volume of solution containing 2 M CaCl₂ in 30% (v:v) ethanol. Luminescence values were calibrated as calcium concentrations according to previous study (Knight et al., 1996).

Transient protein expression and protein-protein interaction assays

Subcellular localization-associated genes were cloned under control of the 35S CaMV promoter using pCAMBIA2300 vectors with GFP tag at the C-terminus. BiFC assay was performed as previously described (Yang et al., 2007). BiFC vectors p2YC and p2YN were generously provided by Dr. C. Mao (Zhejiang University, China), p2YC-SiCAMs and p2YN-SiYUCs were constructed to be fused with C-terminal hemagglutinin (HA) tag upstream of the YFP sequences. Meanwhile, pCAMBIA2300-35S::SIFLS2-mCherry was co-expressed as membrane location marker. *A. tumefaciens*-mediated transient expression in *N. benthamiana* leaves was performed as described (Liao et al., 2015). An *A. tumefaciens* suspension carrying a given construct was infiltrated into young, fully expanded *N. benthamiana* leaves using a needleless syringe. At 48 h after infiltration, subcellular localization of GFP, YFP, or mCherry-tagged proteins in leaves was determined with a Zeiss LSM710NLO confocal microscope, excitation/emission wavelengths were for 488 nm/500-530 nm for GFP, 514 nm/520-560 nm for YFP, and 561 nm/580-620 nm for mCherry.

Co-IP was performed as in previous studies with minor modifications (Li et al., 2014). Binary vector pCAMBIA2300-35S::SiCaM2-HA and pCAMBIA2300-35S::SiYUC6-FLAG were expressed in *N. benthamiana* leaves for about 48 h by *A. tumefaciens*-mediated transformation with empty vector as control, and then infiltrated with 10 μ M PSK or other chemicals for 2 h. Each set of FLAG-tagged soluble protein immunoprecipitation was operated in 1 mL Co-IP buffer [50 mM Tris-HCl (pH7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Triton, 1 \times protease inhibitor (Sigma), 2.5 μ L 0.4 M DTT, 2 μ L 1 M NaF and 2 μ L 1 M Na₃VO₃ added before using] with 10 μ L of α -FLAG agarose beads (Sigma). After 3 h gently shaking at 4 °C, the agarose beads were washed four times with Co-IP washing buffer [50 mM Tris-HCl (pH7.5), 150 mM NaCl, 5 mM EDTA, 0.1% Triton] and once only with 50 mM Tris-HCl (pH7.5). The collected agarose beads were used for analyzing the immunoprecipitated proteins by Western blot with α -FLAG or α -HA antibodies. Besides, 50 μ L samples in Co-IP buffer were assemble for protein loading control before adding agarose beads. Primers used for cloning into binary vectors are listed in Supplemental Data Set 5.

Statistical analysis

At least three independent biological replicates were sampled for each determination. Unless otherwise stated, each biological replicate consisted of an independent sample that was pooled of two leaves, each taken from a different plant. The experiments were independently performed two or three times. The data obtained were subjected to analysis of variance using SAS software, version 8 (SAS Institute Inc., Cary, NC, USA), and the means were compared using Tukey's test at the 5% level.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The effect of PSK concentration on immunity, and the transcript abundance of PSK biosynthesis-related genes in VIGS tomato plants (supports Figure 1).

Supplemental Figure 2. Analysis of PSK receptor sequences, and the transcript abundance of SIPSKR_s in VIGS tomato plants (supports Figure 2).

Supplemental Figure 3. The transcript abundance of PSK signaling-related genes in VIGS tomato plants (supports Figure 4).

Supplemental Figure 4. The effect of PSK concentration on cytosolic Ca²⁺ levels, and the transcript abundance of SIPSKR_s in VIGS tomato plants (supports Figure 5).

Supplemental Figure 5. Analysis of tomato CaM and YUC homologs (supports Figure 6).

Supplemental Figure 6. Co-expression of SiCaMs and SiYUCs in *N. benthamiana* leaves (supports Figure 6).

Supplemental Figure 7. Effects of SiCaM2 silencing on the transcript abundance of SiCaM homologs in tomato plants (supports Figure 7).

Supplemental Data Set 1. Nucleotide sequence alignments based on mRNA region of tomato PSK precursor homologs (supports Figures 1 and 4; Supplemental Figures 1 and 3).

Supplemental Data Set 2. Amino acid sequence alignments of PSKR homologs from tomato, *Arabidopsis* and *Daucus carota* (supports Figure 2 and Supplemental Figure 2).

Supplemental Data Set 3. Nucleotide sequence alignments based on mRNA region of tomato PSKR homologs (supports Figure 2 and Supplemental Figure 2).

Supplemental Data Set 4. Nucleotide sequence alignments based on mRNA region of tomato CaM homologs (supports Figure 7 and Supplemental Figure 7).

Supplemental Data Set 5. Primers used in this study.

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AUTHOR CONTRIBUTIONS

K.S. conceived and designed the experiments; H.Z., Z.H., C.L., C.Z., W.J., S.S. X.L., and K.S. performed the experiments; H.Z., Z.H. and K.S. analyzed the data; X.X., X.C., J.Z., Y.Z. and J.Y. provided technical and intellectual support; J.Y. provided suggestions for the manuscript preparation; C.F. and K.S. wrote the paper with contributions from other authors.

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FIGURE LEGENDS

Figure 1. PSK signaling confer tomato plants immunity against *B. cinerea*.

(A-D) Tomato defense to *B. cinerea* is promoted by exogenous PSK. Four-week-old tomato plants were treated with 10 μ M PSK, 10 μ M dPSK, or water as control 12 h before *B. cinerea* inoculation. (A) Representative chlorophyll fluorescence imaging of the photochemical quantum yield of photosystem II (Φ PSII) at 2 days post *B. cinerea* inoculation (dpi). (B) Representative images of trypan blue staining for cell death in leaves at 2 dpi. Scale bar = 250 μ m. (C) The quantification data of Φ PSII at 2 dpi. (D) Relative *B. cinerea* actin transcript abundance in infected leaves at 1 dpi.

(E) Effects of *B. cinerea* inoculation on the transcript abundance of PSK precursor genes and tyrosine sulfation processing gene SITPST in leaves at 0.5 dpi. The transcript abundance of each gene under mock-inoculated condition were defined as 1. nd, not detected. The asterisk indicates a significant effect of *B. cinerea* inoculation with tomato plants.

(F-I) Effects of PSK-biosynthesis related genes silencing on tomato innate immunity against *B. cinerea*. (F) Representative chlorophyll fluorescence imaging of Φ PSII at 2 dpi. (G) Trypan blue staining for cell death in leaves at 2 dpi. Scale bar = 250 μ m. (H) The quantification data of Φ PSII at 2 dpi. (I) Relative *B. cinerea* actin transcript abundance in infected leaves at 1 dpi.

The results in (C, D, E, H, and I) are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test). The above experiments were repeated three times with similar results.

Figure 2. Characterization of tomato PSK receptors.

(A) Subcellular localization of SIPSKR1 and SIPSKR2. SIPSKRs-GFP and SIFLS2-mCherry (marker for plasma membrane localization) plasmids were transiently co-expressed into *N. benthamiana* leaves. The GFP and mCherry signals were visualized under confocal microscopy after 48 h infiltration. Scale bar = 50 μ m.

(B) Surface plasmon resonance analysis of the binding of PSK to potential tomato PSK receptors, SIPSKR1 and SIPSKR2. The curves represent the concentrations of the injected PSK and dPSK. From bottom to top, 0.39, 0.78, 3.125, 6.25, 12.5, and 25 μ M were used for PSK. An additional 50 μ M concentration was also used for dPSK. The recombinant extracellular portion of the SIPSKR1 and SIPSKR2 was immobilized onto the sensor chip.

The obtained kinetic constants for specific binding are shown in each panel. RU, resonance units.

(C-F) Effects of SIPSKR1- and/or SIPSKR2-silencing on tomato innate immunity against *B. cinerea*. **(C)** Representative chlorophyll fluorescence imaging of the photochemical quantum yield of photosystem II (Φ PSII) at 2 days post *B. cinerea* inoculation (dpi). **(D)** Representative images of trypan blue staining for cell death in leaves at 2 dpi. Scale bar = 250 μ m. **(E)** The quantification data of Φ PSII at 2 dpi. **(F)** Relative *B. cinerea* actin transcript abundance in infected leaves at 1 dpi.

The results in **(E, F)** are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test). The above experiments were repeated two times with similar results.

Figure 3. PSK promotes endogenous IAA accumulation and the PSK-induced tomato immunity against *B. cinerea* is blocked in auxin signaling mutant.

(A) Effects of PSK application on endogenous leaf hormone contents (SA, JA, ET, and IAA). Four-week-old tomato plants were treated with 10 μ M PSK, dPSK, or water as control 12 h before *B. cinerea* inoculation, and leaf samples were collected at 0.5 days post *B. cinerea* inoculation (dpi).

(B) Effects of PSK application on the transcript abundance of hormone signaling-related marker genes in leaves at 0.5 dpi. The elicitor application is as in **(A)**. SA-related genes SIPR1b, JA-related genes SICO11, ET-related genes SIERF1, Auxin-related genes SIARF5.

(C) Effects of PSK application on trypan blue staining for cell death in hormone signaling-defective and control plants at 2 dpi. Tomato wild type (WT), mutants, or transgenic lines were treated with 10 μ M PSK or water control 12 h before *B. cinerea* inoculation. The following tomato lines were used: SA accumulation-defective transgenic NahG and its WT line cv. Moneymaker (MM), JA-signaling mutant *jai1-1* and its WT line cv. Castlemart (CM), ET-signaling mutant Nr and its WT line cv. Pearson, auxin signaling-insensitive mutant *dgt* and its WT line VFN8. Scale bar = 250 μ m.

(D) Relative *B. cinerea* actin transcript abundance in infected hormone-related mutants in the presence or absence of PSK at 1 dpi, the elicitor application is as in **(C)**.

The results in **(A, B, and D)** are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test). The above experiments were repeated three times with similar results.

Figure 4. Auxin functions downstream of PSK-SIPSKR1 signaling in tomato immunity against *B. cinerea*.

(A) The changes of IAA content in PSK signaling component gene-silenced tomato plants. Leaf samples were collected at 0.5 days post *B. cinerea* inoculation (dpi).

(B) Effects of NAA application on leaf *B. cinerea* actin transcript abundance in target gene-silenced tomato plants at 1 dpi. PSK signaling component gene-silenced and TRV:0 control plants were treated with 10 nM NAA or water control 12 h before *B. cinerea* inoculation.

(C-D) SIPSKR1 silencing-compromised immunity was complemented by NAA but not by PSK. (C) Trypan blue staining for cell death as affected by SIPSKR1 silencing and application of PSK and NAA. Tomato SIPSKR1-silenced plants were treated with 10 μ M PSK or 10 nM NAA 12 h before *B. cinerea* inoculation, and leaf samples were collected at 2 dpi. Scale bar = 250 μ m. (D) Relative *B. cinerea* actin transcript abundance in infected leaves at 1 dpi.

(E) The effects of 10 nM NAA application on the transcript abundance of PSK signaling component genes in leaves under both mock- and *B. cinerea*-inoculated condition, samples were taken at 0.5 dpi.

The results in (A, B, D, and E) are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments (P < 0.05, Tukey's test). The experiments in (A, B, and E) were repeated three times, and others were repeated two times with similar results.

Figure 5. Cytosolic Ca²⁺ elevation is induced and required for PSK-induced tomato immunity against *B. cinerea*.

(A-B) PSK-induced cytosolic Ca²⁺ elevation in leaves of aequorin-expressing tomato plants as affected by Ca²⁺ channel inhibitors (A) or SIPSKRs genes silencing (B). Tomato leaf discs were preincubated for 30 min with ruthenium red (RR) or verapamil (Ver) at 20 μ M, and 10 μ M PSK ligand was then added at time 0. The signals shown at 0.5-min intervals are the mean values \pm SD, (n = 10~12). In (A), totally 50 leaf discs obtained from at least 5 plants were used for experiment and each treatment had 10 leaf discs; In (B), each 12 leaf discs obtained from independent 5 plants served as one treatment.

(C-F) Effects of Ca²⁺ channel inhibitor on tomato innate immunity against *B. cinerea*. The four-week-old tomato plants were treated with 10 μ M PSK, 20 μ M each Ca²⁺ channel

inhibitor, or water as control 12 h before *B. cinerea* inoculation. **(C)** Representative chlorophyll fluorescence imaging of the photochemical quantum yield of photosystem II (Φ PSII) at 2 days post *B. cinerea* inoculation (dpi). **(D)** Representative images of trypan blue staining for cell death in leaves at 2 dpi. Scale bar = 250 μ m. **(E)** The quantification data of Φ PSII at 2 dpi. **(F)** Relative *B. cinerea* actin transcript abundance in infected leaves at 1 dpi. The results in **(E and F)** are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test).

The above experiments were repeated three times with similar results.

Figure 6. SiCaMs binds to auxin biosynthetic protein SIYUCs.

(A) BiFC analyses of the binding between SiCaM2 and SIYUCs (left panel), and between SiCaMs and SIYUC6 (right panel). Both spliced YFP constructs and SIFLS2-mCherry (marker for plasma membrane localization) plasmids were transiently co-expressed into *N. benthamiana* leaves. The YFP and mCherry signals were visualized under confocal microscopy after 48 h infiltration. Scale bar = 50 μ m.

(B) The changes of BiFC fluorescence signal between p2YC-SiCaM2 and p2YN-SIYUC6 with or without 2 h of PSK (10 μ M) application. Scale bar = 50 μ m. The fluorescence signal intensity from three independent repeats was quantified and the data are shown as mean \pm SD (n = 3). Asterisks indicates a significant effect of PSK application ($P < 0.05$, Tukey's test).

(C) Co-IP analysis of association between HA-tagged SiCaM2 and FLAG-tagged SIYUC6 with or without application of 10 μ M PSK, 20 μ M CaCl₂, and 20 μ M Ca²⁺ channel inhibitor ruthenium red (RR) for 2 h. Total proteins were extracted from leaves transiently expressed with the SiCaM2-HA, SIYUC6-FLAG construct alone or their combinations after 48 h infiltration. The extracted proteins were immunoprecipitated with an anti-FLAG antibody and the presence of SiCaM2-HA and SIYUC6-FLAG in the immune complex was determined by immunoblot (IB) with the indicated antibody. The Co-IP band intensity (top) from three independent repeats was quantified by Image J software. The data are shown as mean \pm SD (n = 3). Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test).

The experiments in **(A, B)** were repeated three times, and experiments in **(C)** were repeated two times with similar results.

Figure 7. SiCaM2 silencing compromises the PSK-induced immunity and IAA accumulation.

(A) Representative chlorophyll fluorescence imaging of the photochemical quantum yield of photosystem II (Φ PSII) as affected by SiCaM2 silencing, and application of PSK and NAA. Tomato SiCaM2-silenced plants were treated with 10 μ M PSK or 10 nM NAA 12 h before *B. cinerea* inoculation, and leaf samples were collected at 2 days post *B. cinerea* inoculation (dpi).

(B) The quantification data of Φ PSII at 2 dpi.

(C) Relative *B. cinerea* actin transcript abundance in infected leaves at 1 dpi.

(D) The changes of IAA content in SiCaM2-silenced tomato plants at 0.5 dpi, as affected by exogenous PSK and NAA application.

The results in (B to D) are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test). The above experiments were repeated three times with similar results.

Figure 8. A working model of PSK-induced immunity against *B. cinerea* in tomato plants. PSK plays as a damage-associated molecular pattern (DAMP), its precursors and protein processing are activated upon *B. cinerea* inoculation. At the apoplast, PSK signaling peptide is mainly perceived by its receptor PSKR1, which transduce the signal into cytoplasm by initiating cytosolic Ca^{2+} influx. The transient cytosolic Ca^{2+} are further transduced to CaMs which binds to YUCs, promoting auxin biosynthesis and associated signaling to combat *B. cinerea* infection.

Supplemental Data

Supplemental Figure 1. The effect of PSK concentration on immunity, and the transcript abundance of PSK biosynthesis-related genes in VIGS tomato plants (supports Figure 1).

(A) The dose-dependent alleviatory effect of PSK on leaf *B. cinerea* transcript abundance. Four-week-old tomato plants were treated with indicated concentrations of PSK 12 h before *B. cinerea* inoculation, and leaf samples were taken at 1 day post *B. cinerea* inoculation (dpi) for relative leaf *B. cinerea* actin transcript abundance assay.

(B) Phylogenetic analysis of PSK precursor genes from tomato and *Arabidopsis*. Amino acid sequence alignment and tree construction were performed with MEGA program. A consensus neighbor-joining tree was obtained from 1000 bootstrap replicates of aligned sequences. The percentage at branch represents the posterior probabilities of amino acid sequences.

(C) Relative transcript abundance of homologous PSK precursor genes and SITPST in VIGS tomato plants. After initial silencing efficiency test 3~4 weeks after *A. tumefaciens* infiltration, tomato plants were subject to *B. cinerea* inoculation, and leaf samples were collected at 1 dpi. The samples are same with those used for *B. cinerea* actin assay in Figure **II**. The transcript abundance were expressed as a ratio of the mean levels in TRV:0 control plants, which were defined as 1.

The results in (A and C) are presented as the mean values \pm SD; $n = 3$. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test). The experiments in (A) were repeated two times, and experiments in (C) were repeated three times with similar results.

Supplemental Figure 2. Analysis of PSK receptor sequences, and the transcript abundance of SIPSKRs in VIGS tomato plants (supports Figure 2).

(A) Phylogenetic analysis of PSK receptors from tomato, *Arabidopsis* and *Daucus carota*. Amino acid sequence alignment and tree construction were performed with MEGA program. A consensus neighbor-joining tree was obtained from 1000 bootstrap replicates of aligned sequences. The percentage at branch represents the posterior probabilities of amino acid sequences.

(B) Schematic of SIPSKR1/R2 protein. The diagram shows the signal peptide (SP),

extracellular leucine-rich repeats (LRRs), an island domain, a transmembrane domain (TM), and a cytoplasmic Ser/Thr kinase domain.

(C) Relative transcript abundance of SIPSKRs in VIGS tomato plants. After initial silencing efficiency test 3~4 weeks after *A. tumefaciens* infiltration, tomato plants were subject to *B. cinerea* inoculation, and leaf samples were collected at 1 dpi. The samples are same with those used for *B. cinerea* actin assay in Figure 2F. The transcript abundance were expressed as a ratio of the mean levels in TRV:0 control plants, which were defined as 1. The results are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test). The experiments were repeated two times with similar results.

Supplemental Figure 3. The transcript abundance of PSK signaling-related genes in VIGS tomato plants (supports Figure 4).

(A) Relative transcript abundance of SIPSK3, SIPSK3L, SITPST, and SIPSKR1 in VIGS tomato plants.

(B) Relative transcript abundance of SIPSKR1 in VIGS tomato plants.

After initial silencing efficiency test 3~4 weeks after *A. tumefaciens* infiltration, tomato plants were subject to pharmacological treatment and *B. cinerea* inoculation, and leaf samples were collected at 1 dpi. The samples in (A) are same with those used for *B. cinerea* actin assay in Figure 4B. The samples in (B) are same with those used for Figure 4D. The transcript abundance were expressed as a ratio of the mean levels in TRV:0 control plants, which were defined as 1. The results are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test). The experiments in (A) were repeated three times, and experiments in (B) were repeated two times with similar results.

Supplemental Figure 4. The effect of PSK concentration on cytosolic Ca²⁺ levels, and the transcript abundance of SIPSKRs in VIGS tomato plants (supports Figure 5).

(A) The dose-dependent effect of PSK on cytosolic Ca²⁺ elevation in leaves of aequorin-expressing tomato plants. The signals shown at 0.5-min intervals are the mean values \pm SD generated from 8 leaf discs per treatment.

(B) Relative transcript abundance of SIPSKR1 and SIPSKR2 in VIGS tomato plants. Approximately three weeks after *A. tumefaciens* infiltration, each plant was taken with an

uppermost expanded leaflet for target gene silencing efficiency analysis by qRT-PCR. Five plants with efficient silencing from each TRV: SIPSKR1 and TRV: SIPSKR2 group were selected and transcript abundance of target genes are shown as the mean values \pm SD (n = 5). These selected plants were further used for cytosolic Ca²⁺ assay in Figure 5B, and leaf discs samples for cytosolic Ca²⁺ assay were taken from the corresponding opposite leaflet for the silencing efficiency assay. The transcript abundance were expressed as a ratio of the mean levels in TRV:0 control plants, which were defined as 1. Different letters indicate significant differences between treatments (P < 0.05, Tukey's test).

The experiments in (A) were repeated two times, and experiments in (B) were repeated three times with similar results.

Supplemental Figure 5. Analysis of tomato CaM and YUC homologs (supports Figure 6).

(A) Amino acid sequence alignments of tomato CaM homologs with Clustal X program.

(B) Phylogenetic analysis of YUCs from tomato and Arabidopsis. Amino acid sequence alignment and tree construction were performed with MEGA program. A consensus neighbor-joining tree was obtained from 1000 bootstrap replicates of aligned sequences. The percentage at branch represents the posterior probabilities of amino acid sequences.

(C) Effects of *B. cinerea* inoculation on the relative transcript abundance of SIYUCs in leaves at 0.5 dpi. The transcript abundance of SIYUC1 in mock-inoculated condition were defined as 1. The results are presented as the mean values \pm SD; n = 3. nd, not detected. The asterisk indicates a significant effect of *B. cinerea* inoculation with tomato plants. The experiments were repeated two times with similar results.

Supplemental Figure 6. Co-expression of SICaMs and SIYUCs in *N. benthamiana* leaves (supports Figure 6).

(A) Co-expression of p2YC-SICaMs and p2YN-SIYUCs proteins were detected by immunoblot analysis with an anti-HA antibody. Samples of infiltrated areas were collected from *N. benthamiana* leaves after 48 h *A. tumefaciens* infiltration. Rubisco (RBC) was stained with Ponceau for protein loading control (bottom). The black boxes indicate specific SIYUCs and SICaMs bands.

(B) SIYUC6 localizes to plasma membrane. SIYUC6-GFP and SIFLS2-mCherry (marker for plasma membrane localization) plasmids were transiently co-expressed into *N.*

benthamiana leaves. The GFP and mCherry signals were visualized under confocal microscopy after 48 h infiltration. Scale bar = 50 μ m.

The experiments were repeated two times with similar results.

Supplemental Figure 7. Effects of SlCaM2 silencing on the transcript abundance of SlCaM homologs in tomato plants (supports Figure 7).

After initial silencing efficiency test 3~4 weeks after *A. tumefaciens* infiltration, tomato plants were subject to *B. cinerea* inoculation, and leaf samples were collected at 1 dpi. The samples are same with those used for *B. cinerea* actin assay in Figure 7C. The transcript abundance were expressed as a ratio of the mean levels in TRV:0 control plants, which were defined as 1. The results are presented as the mean values \pm SD; n = 3. Different letters indicate significant differences between treatments ($P < 0.05$, Tukey's test). The experiments were repeated three times with similar results.

Supplemental Data Set

Supplemental Data Set 1. Nucleotide sequence alignments based on mRNA region of tomato PSK homologs (supports Figures 1 and 4; Supplemental Figures 1 and 3).

Supplemental Data Set 2. Amino acid sequence alignments of PSKR homologs from tomato, Arabidopsis and Daucus carota (supports Figure 2 and Supplemental Figure 2). The conserved domains were predicted by SMART (<http://smart.embl-heidelberg.de>).

Supplemental Data Set 3. Nucleotide sequence alignments based on mRNA region of tomato PSKR homologs (supports Figure 2 and Supplemental Figure 2).

Supplemental Data Set 4. Nucleotide sequence alignments based on mRNA region of tomato CaM homologs (supports Figure 7 and Supplemental Figure 7).

Supplemental Data Set 5. Primers used in this study.