EWR1 as a SCOOP peptide activates MIK2-dependent immunity in Arabidopsis

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ABSTRACT

Phytocytokines are plant peptide signals perceived by plasma membrane-localized receptors in regulating plant immunity. It was recently reported that the phytocytokine SERINE-RICH ENDOGENOUS PEPTIDE12 (SCOOP12) is recognized by the receptor kinase MALE DISCOVERER 1-INTERACTING RECEPTOR-LIKE KINASE 2 (MIK2) and activates plant immune responses and resistance to pathogens in Arabidopsis. Here, we show that Arabidopsis ENHANCER OF VASCULAR WILT RESISTANCE 1 (EWR1) and four EWR1 close propeptide homologs encode functional SCOOP peptides, which are able to activate immune responses via MIK2 and BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and SOMATIC EMBRYOGENESIS RECEPTOR KINASE 4 (SERK4).

Introduction

The perception of immunogenic patterns by pattern recognition receptors (PRRs) initiates pattern-triggered immunity (PTI), which plays a critical role in plant resistance to potential pathogens. Plant plasma membrane-resident PRRs activate plant immunity by recognizing microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), and phytocytokines (Gust et al. 2017; Hou et al. 2019; Zhou and Zhang 2020; Tanaka and Heil 2021; Hou et al. 2021a).

Phytocytokines are plant endogenous peptides, which are usually produced in the cytosol and released into the apoplast when plants encounter pathogen infections (Luo 2012; Hou et al. 2021a). Once released into the apoplast, phytocytokines are usually perceived by cell surface-resident receptor-like kinases (RLKs), which contain an extracellular domain, a transmembrane region, and a cytoplasmic kinase domain (Shiu and Bleecker 2001; Couto and Zipfel 2016; Escocar de Azvedo Manhaes et al. 2021). In the model plant Arabidopsis, PEPI RECEPTOR 1 (PEPR1) and PEPR2 recognize PLANT PEPTIDE ELICITORS (Peps) (Yamaguchi et al. 2006; Yamaguchi et al. 2010), RECEPTOR-LIKE 7 (RLK7) recognizes PAMPs-INDUCED PEPTIDE 1 (PIP1) and PIP2 (Hou et al. 2014), and GFG1 INSENSITIVE 3 (RGI3) individually or together with RGI4 recognizes RGF7 and RGF9/GLV2 (Steigmann et al. 2021; Wang et al. 2021). Upon phytocytokine perception, the leucine-rich repeats RLK (LRR-RLK) receptors usually heterodimerize with SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) LRR-RLKs, e.g. BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1)/SERK3 and SERK4 and trigger downstream PTI responses, including phosphorylation of the receptor-like cytoplasmic kinases (RLCKs), the elevation of cytosolic Ca2+ concentration, transient apoplastic reactive oxygen species (ROS) burst, the activation of mitogen-activated protein kinases (MAPks) and calcium-dependent protein kinases (CDPKs), reprogramming of defense gene expression, callose deposition, production of immune-related hormones and antimicrobial components, and plant growth inhibition (Yu et al. 2017; Zhou and Zhang 2020; DeFalco and Zipfel 2021).

SCOOPs are a family of Brassicaceae-specific peptides, which are derived from the C-terminus of precursors or/and resistance to pathogens (Gully et al. 2019; Rhodes et al. 2021; Hou et al. 2021b). SCOOP peptides have been confirmed to trigger hallmark PTI responses or/and resistance to pathogens (Gully et al. 2019; Rhodes et al. 2021; Hou et al. 2021b). SCOOPs are recognized by the LRR-RLK MALE DISCOVERER 1-INTERACTING RECEPTOR-LIKE KINASE 2 (MIK2), induce the dimerization between MIK2 and BAK1/SERK4, and activate immune responses, such as MAPK activation, ROS production, and cytosolic Ca2+ concentration increase (Rhodes et al. 2021; Hou et al. 2021b). Disruption of SCOOP-MIK2 signaling in Arabidopsis leads to a significant increase in susceptibility to the vascular wilt fungus Fusarium oxysporum (Van der Does et al. 2017; Coleman et al. 2021; Hou et al. 2021b).

In this study, we identified another five SCOOP peptides (SCOOP24-28), all of which are active for activating plant immune responses in Arabidopsis but not in tobacco and tomato. The SCOOP activation of plant immune responses and/or resistance to F. oxysporum is disrupted by the loss-of-function mutation of MIK2. Tobacco leaves expressing MIK2 obtain an ability to activate ROS production upon SCOOP treatments.
Table 1. Peptide sequences used in this study.

| Peptide Name | Sequences (N→C) |
|--------------|-----------------|
| flg22        | QRLSTGRINSAKEDDAAGLOIA |
| SCOOP12      | MGSGASGGPVRSOSSOAGGR   |
| SCOOP24      | KIRVPKSKPDDROW        |
| SCOOP25      | IIESRPSKRPAPQC        |
| SCOOP26      | KGFTGSGFSPAPHPG       |
| SCOOP27/EWR1 | KTVKVSRSPPAKGW        |
| SCOOP28      | EIRTPSGRIPAPFPQ       |

Materials and methods

Plant materials and growth conditions

The Arabidopsis thaliana accession Columbia-0 (Col-0) was used as wild-type (WT). T-DNA insertion mutants of mik2-1, mik2-2, and bak1-5/serk4-1 were described previously (Hou et al. 2021b). A transgenic line expressing p35S::Aequorin was provided by Dr. Marc Knight (Durham University, UK). Arabidopsis plants used for ROS detection were grown in soil at 20–23°C, and 75–100 µM m−2 s−1 light with a 12-h light/12-h dark photoperiod. Arabidopsis seedlings used for analyses of root growth inhibition and cytosolic Ca2+ concentration increase were grown on half-strength Murashige and Skoog (1/2MS) plates containing 0.5% (w/v) sucrose, 0.75% (w/v) agar, and 2.5 mM MES, pH 5.7, under the same conditions as plants grown in soil. Cherry tomatoes (Lycopersicon esculentum) were grown in glass bottles with 1/2MS under a 14-h light/10-h dark photoperiod at 23°C. Tobacco (Nicotiana benthamiana) were grown on soil under a 14-h light/10-h dark photoperiod at 23°C.

Peptide synthesis

Peptides were synthesized at Scilight-Peptide (Beijing, China). The sequences of synthesized peptides were listed in Table 1.

Root growth assay

Cold stratified seeds were surface-sterilized with 70% (v/v) ethanol for 5 min and were sown on 1/2MS plates with or without 1 µM peptides. Ten-day-old seedlings grown on plates vertically in a growth chamber were photographed, and the root length of seedlings was measured using Image J (http://rsb.info.nih.gov/ij/).

Transient gene expression in N. benthamiana leaves

Agrobacterium tumefaciens GV3101 carrying pCAMBIA1300-Ubi::Mik2-FLAG or a blank vector cultivated overnight was suspended into a concentration of OD600 = 0.3 with incubation buffer (0.1% sucrose, 2.5 mM MES, 100 µM acetosyringone, pH 5.7) and infiltrated into N. benthamiana leaves using a 1-µL needleless syringe.

ROS assay

ROS burst was determined by a luminol-based assay. Leaf discs from four-week-old Arabidopsis or N. benthamiana plants or cotyledon fragments from one-week-old tomato seedlings were incubated in 200 µL ddH2O overnight in a 96-well plate. Then, ddH2O was replaced by 200 µL of reaction solution containing 50 µM of luminol, and 10 µg/mL of horseradish peroxidase (Sigma-Aldrich) supplemented with or without 100 nM peptide. Luminescence was measured immediately after adding the solution with a luminometer (Navigator, Promega) with a 30-second interval for 30 min. The total values of ROS production were indicated as means of the relative light units (RLU).

Measurement of cytosolic Ca2+ concentration

One-week-old seedlings expressing p35S::Aequorin grown vertically on ½MS plates were incubated in a 96-well plate containing 200 µL solution with 1 mM KCl and 1 mM CaCl2. Aequorin was reconstituted by treating the seedlings with coelenterazine-h (Promega, Beijing, China) in the dark overnight at a final concentration of 10 µM. Luminescence was measured with a luminometer (Navigator, Promega) with a 30-second interval for 20 min. The values for cytosolic Ca2+ concentrations were indicated as means of RLU.

Fusarium oxysporum disease assay

Fusarium oxysporum disease assay was followed as described previously with modifications (Wang et al. 2020). F. oxysporum strain Fo5176 was cultivated in liquid potato dextrose medium for 3 days at 28°C on a rotary shaker at 120 rpm. The cultivated fungi were filtered with eight layers of sterilized gauze and spores were collected by centrifugation at 1000 g. Spores were washed twice and resuspended with sterile water to adjust to a final concentration of 10⁶ spores/mL. Ten-day-old Arabidopsis seedlings grown on 1/2MS plate were planted into a sterilized gas-permeable vessel chamber containing 160 g clay, 120 mL 1/2MS liquid medium, and 0.3 g sucrose (pH 5.7). After ten-day growth, each seedling was inoculated with 1 mL of spore suspension with or without 1 µM peptides by dripping around the root. The seedlings were imaged and the seedling survival rates were counted 10 days after inoculation.

Results

Identification of new SCOOPs

In previous studies, 23 SCOOP family members have been identified in the Arabidopsis genome (Gully et al. 2019; Hou et al. 2021b). However, some SCOOP homologs may not have been identified through a comprehensive homology search using BLAST, due to the low identity of the amino acid sequence between SCOOPs. As reported previously, we treated ten-day-old Arabidopsis seedlings with 1 µM SCOOP12 for one and six hours and determined the gene expression change compared to non-treated seedlings through RNA sequencing analysis (Hou et al. 2021b). We identified 63 small genes encoding secreted peptide precursors which are upregulated by SCOOP12 peptide in Arabidopsis seedlings (Figure 1(a)). Eight of these genes encode PROSCOOP12 and PROSCOOP12 homologous proteins. Among the PROSCOOPs upregulated by SCOOP12, PROSCOOP12 is not most induced by SCOOP12, although PROSCOOP12 was reported to be highly induced by aggression by different pathogens (Gully et al. 2019). It suggests that SCOOP12 might regulate plant resistance to pathogens.
by initiating multiple SCOOP-mediated immune signaling. In addition, we found four genes whose locus is closely linked (AT3G13432, AT3G13433, AT3G13435, and AT3g13437) in the list of the SCOOP12-upregulated genes. AT3g13437 was named EWR1 and has been reported to be involved in plant resistance to vascular wilt pathogens (Yadeta et al. 2014). Like SCOOPs, EWR1 is a Brassicaceae-specific gene. It promotes us to see if EWR1, as well as the three EWR1-linked proteins, belong to the SCOOP family. Sequence alignment does not show significant sequence similarity between these peptide precursors and PROS-COOPs. However, the four small proteins, like PROS-COOPs, harbor a typical SxS motif (where S is serine and x is any amino acid) in their C-termini (Figure 1(b)). These SxS motifs share high sequence similarity with SCOOPs (Figure 1(c)), implying that they might belong to SCOOP family. Through blast searching using amino acid sequences of EWR1 and EWR1-linked genes in the TAIR database (https://www.arabidopsis.org/), we identified a homolog of AT3G13433 encoded by AT2g25297.2 transcript. AT2g25297.2 also contains a SCOOP peptide (Figure 1(c)). Together, we identified another five SCOOP peptide candidates in Arabidopsis, and named them as SCOOP24 (AT3g13432), SCOOP25 (AT3g13433), SCOOP26 (AT3g13435), SCOOP27/EWR1 (AT3g13437), and SCOOP28 (AT2g25297) (Figure 1(c)).

**SCOOP24-28 activate plant immune responses**

To test if these peptides display activities similar to SCOOP12, we synthesized peptides corresponding to SCOOP24, SCOOP25, SCOOP26, SCOOP27, and SCOOP28, and analyzed their activities for immune activation. We found that all these peptides are able to differentially induce hallmarks of PTI responses, including ROS production (Figure 2(a,b)) and cytosolic calcium increase (Figure 2(c,d)), and root growth inhibition, at a concentration of 100 or 1000 nM (Figure 2(e,f)). In contrast to SCOOP12, the activities for ROS production and cytosolic calcium increase of all the peptides, especially SCOOP26, are lower (Figure 2(b,d)). The root growth inhibition by SCOOP27 is dose-dependent (Figure 2(g,h)). SCOOP27 activities for root inhibition are also weaker than Pep1 and SCOOP12 even at a higher concentration (Figure 2(i,j)). The SCOOP27 peptide is well dissolved. The relatively weak activity of the peptide may be determined by its sequence characteristics and stability. However, SCOOP25 and SCOOP26 show comparable activities for root growth inhibition to SCOOP12 and more potent activities than other peptides (Figure 2(e,f)). It is consistent with our previous report that the activities for immune activation by SCOOPs do not always match that of root growth inhibition (Hou et al. 2021b).
To determine if the SCOOP-triggered immune activation relies on the SCOOP receptor MIK2 and coreceptor BAK1/SERK4, we tested SCOOP27-induced ROS production in mik2 and bak1/serk4 mutants. The results indicated that the SCOOP27-induced ROS production is fully abolished in mik2-1, mik2-2, and bak1-5/serk4 mutants (Figure 3(a)). SCOOP27 as well as SCOOP12 cannot induce ROS production in tobacco and tomato (Figure 3(b)). In contrast, flg22 strongly induced ROS in the Solanaceous plants as reported previously (Figure 3(b)) (Robatzek et al. 2007). Then, we constructed a binary vector harboring carboxyl-terminally FLAG-tagged MIK2 under CaMV 35S promote and transiently expressed MIK2 in tobacco leaves using Agrobacterium-mediated transient transformation. We found that SCOOP12 and SCOOP27 are able to dramatically induce ROS production in tobacco leaves expressing MIK2 but not in control plants (Figure 3(c)). Moreover, we found that the SCOOP27 suppression on root growth inhibition is also abolished in mik2 and bak1-5/serk4 mutants (Figure 3(d,e)). Together, our results suggest that SCOOP27 functions in a MIK2- and BAK1/SERK4-dependent manner.

A previous report showed that SCOOP27/EWR1 overexpression confers Arabidopsis resistance to vascular wilt pathogens, including Verticillium dahlia and F. oxysporum (Yadeta et al. 2014). The mik2 mutants are also markedly...
more susceptible to *F. oxysporum* infections compared to WT plants (Van der Does et al. 2017; Coleman et al. 2021; Hou et al. 2021b). To determine if SCOOP27/EWR1 triggers a MIK2-dependent pathogen resistance, we pretreated roots of *Arabidopsis* WT and *mik2* seeds with or without SCOOP27 peptides and tested the seedling resistance to *F. oxysporum* f. sp. *conglutinans* strain Fo5176. *mik2* seedlings show no different growth phenotype and survival rate compared to WT plants in a sterile growth state (Figure 3 (f,g)). As reported previously, *mik2* mutants are more susceptible to Fo5176 than WT plants (Figure 3(f,g)). SCOOP27 co-inoculation significantly enhanced the plant resistance to Fo5176 in WT plants but not *mik2* mutants (Figure 3(f,g)). Therefore, SCOOP27-triggered a MIK2-dependent resistance to Fo5176 in *Arabidopsis*.

Discussion

Twenty-three SCOOP peptides have previously been identified as phytohormones, which activate MIK2-mediated immunity in *Arabidopsis*. In this study, we identified another five SCOOP peptides through analysis of the SCOOP12-upregulated genes and homologous blast using new identified SCOOPs. All these newly identified SCOOPs are active in the activation of plant immune responses. Therefore, a total of 28 SCOOPs have been identified in *Arabidopsis* so far. All these SCOOP peptides are about 15–20 amino acids in length with a SxS motif and are derived from the carboxyl ends of corresponding secreted peptide precursors. However, these SCOOP precursor genes seem to belong to different gene families although they all share a relatively conserved SxS motif. Even for the SxS motifs, sequence similarity among different SCOOP members is also low except for two completely conserved serines. That is why the five SCOOP members have not been identified previously. Likewise, it is possible that other unidentified SCOOPs may exist in other peptide precursors or other protein families in *Arabidopsis*.

Peptide signals are usually perceived by LRR-RLKs or LRR-receptor-like proteins (LRR-RLPs) (Olsson et al. 2019). In *Arabidopsis*, more than 10 peptide-receptor pairs have been identified to be involved in the regulation of various plant developmental processes and plant stress responses. Most of these peptide families have fewer than 10 members, and different peptide members in the same family are often recognized by more than one receptor. For example, eight Pep1 homologous peptides are perceived by two receptors, PEPR1 and PEPR2, and nine ROOT MERISTEM GROWTH FACTORS (RGFs) are perceived by five receptors, RG11–RG15 (Huffaker et al. 2006; Yamaguchi et al. 2006; Matsuoka et al. 2010; Yamaguchi et al. 2010; Ou et al. 2016). However, *Arabidopsis* encodes a big...
SCOOP family which contains 28 members, and all SCOOP peptides tested previously and in this study trigger responses through one LRR-RLK, MIK2, though *Arabidopsis* also encodes a close MIK2 homolog MIK2L (Hou et al. 2021b). In addition, SCOOPs are orphan genes and evolved through rapid duplication (Arendsee et al. 2014; Gully et al. 2019). These clues indicate that SCOOPs play very important roles in plants and thus are under strong selection pressures. However, the SCOOP-MIK2 function in the regulation of plant immunity and other physiological processes remains largely unknown. Therefore, much effort is needed to invest toward understanding the SCOOP-MIK2 function in further studies.

SCOOP27 is also known as EWR1, which was identified as an enhancer of plant resistance to vascular wilt pathogens. In line with this, our study indicated SCOOP27 triggers *Arabidopsis* resistance to the vascular wilt pathogen *F. oxysporum* through MIK2 (Figure 3(f)). It was reported that SCOOP27/EWR1 overexpression enhances tobacco resistance to vascular wilt pathogens (Yadeta et al. 2014). However, no ROS production is induced by the SCOOP27 peptide in tobacco and tomato (Figure 3(b)), suggesting that SCOOP27 may not trigger pathogen resistance in *Solanaceae* plants. It also suggests that SCOOP27 might not be equivalent to the full length of EWR1 in plant resistance. It is possible that another peptide motif in EWR1 is perceived by tobacco to activate immunity or EWR1 owns anti-microbe activities as suggested previously (Yadeta et al. 2014).

**Disclosure statement**

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**Shuguo Hou** has broad expertise in plant molecular biology and biochemistry. He is particularly interested in the molecular endogenous peptide signals involved in plant immune regulation. Some recent work in his group concerning the SCOOP and SCREW peptide functions in plant immune modulations has been published in *Nature* and *Nature Communications*.

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