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The SCOOP12 peptide regulates defense response and root elongation in Arabidopsis thaliana

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Abstract

Small secreted peptides are important players in plant development and stress response. Using a targeted in silico approach, we identified a family of 14 Arabidopsis genes encoding precursors of serine-rich endogenous peptides (PROSCOOP). Transcriptomic analyses revealed that one member of this family, PROSCOOP12, is involved in processes linked to biotic and oxidative stress as well as root growth. Plants defective in this gene were less susceptible to Erwinia amylovora infection and showed an enhanced root growth phenotype. In PROSCOOP12 we identified a conserved motif potentially coding for a small secreted peptide. Exogenous application of synthetic SCOOP12 peptide induces various defense responses in Arabidopsis. Our findings show that SCOOP12 has numerous properties of phytocytokines, activates the phospholipid signaling pathway, regulates reactive oxygen species response, and is perceived in a BAK1 co-receptor-dependent manner.

Keywords: Arabidopsis, DAMP, defense signaling, oxidative stress, phytocytokines, root development, secreted peptide.

Introduction

In order to counter constant pathogen aggression, plants have developed sophisticated perception and defense systems. These plant responses are regulated by complex networks involving regulatory proteins and hormones, and are associated with massive changes in gene expression (Buscaill and Rivas, 2014). Among the involved players, it has been shown that small secreted peptides play an important role through their direct interaction with pathogens or through their function in development and cell–cell communication involving ligand–receptor interactions (Murphy et al., 2012; Marniroli and Maestri, 2014; Gust et al., 2017). The secreted peptides derive from protein precursors having a shared N-terminal signal peptide which targets the protein to the secretory pathway. They can be categorized into two major classes: (i) the small post-translationally modified peptides (PTMPs) which are the targets of post-translational maturation and are produced...
through proteolytic processing; and (ii) the cysteine-rich peptides (CRPs) characterized by an even number of cysteine residues involved in intramolecular disulfide bonds (Tavormina et al., 2015). Although they are mainly involved in plant growth and developmental processes, it has been shown that numerous genes encoding secreted peptides are also involved in plant-pathogen interactions (Albert, 2013). For instance, the CRP class includes the antimicrobial peptides such as knottins and defensins, which interact with and disrupt the pathogen cell membrane (Goyal and Mattoo, 2014). Regarding PTMPs, families such as the phytosulfokines (PSKs), CLE/CLV3, IDA/IDL, or PSY are players in processes regulating a large families such as the phytosulfokines (PSKs), CLE/CLV3, IDA/IDL, or PSY are players in processes regulating a large diversity of plant–pathogen interactions (Lee et al., 2015). Although they are mainly involved in plant growth and biochemistry (CRPs) characterized by an even number of cysteine residues, they can also participate in defense reactions. Oligalacturonides and cutin monomers are examples of non-peptidic DAMPs which are released upon fungal infection (Fauth et al., 1998). Their perception by neighboring cells also elicits the immunity response (De Lorenzo et al., 2011). The small peptide AtPep1 is a well-documented DAMP (Bartels and Boller, 2015). A first induction of AtPep1 and other peptides of this family by wounding or pathogen attack has a positive feedback on the expression of its own precursors as well as defense marker genes, that is thought to amplify defense signaling pathways (Huffaker and Ryan, 2007).

It is considered that only a small fraction of the gene space likely to encode signaling peptides has been described, and their diversity appears to be largely underestimated (Matsubayashi, 2014). Indeed, the Arabidopsis genome contains >1000 genes harboring secreted peptide features whose biological function is currently unknown (Lease and Walker, 2006, 2010). This lack of data can be explained by the fact that this type of gene has only recently been detected due to their small size and their low sequence conservation (Silverstein et al., 2007). Furthermore, the frequent functional redundancy inside these gene families (Matsubayashi, 2014) renders mutant knock-out approaches less successful. The mining of previously published transcriptomes is an efficient way to explore this unknown gene space and decipher functions of new genes for which, without reference, the inference of function by similarity cannot be applied. Based on transcriptome meta-analysis and bioinformatics predictions in a ‘guilt by association’ approach, we identified a peptide family, of which at least one member is involved in plant immunity and root development. This work describes the identification of a gene family specific to the Brassicaceae genus encoding putative secreted peptides. The functional characterization of PROSOOP12, one of its members in Arabidopsis, shows that this small gene could act as modulator in the response to different pathogen aggressions and in root development, presumably via controlling reactive oxygen species (ROS) detoxification. We illustrate that the small endogenous SCOOP12 peptide displays most properties of phytocytokines, processed and actively transported players in endogenous danger signals without cellular damage (Gust et al., 2017).

Materials and methods

Plant material

Plant material was wild-type Arabidopsis thaliana L. Heynh cultivar 6 Columbia (Col-0) as well as the cultivar Wassilewskija (Ws) and the mutants prosoop12 (T-DNA line FLAG_394H10 in the Ws background; primers used for genotyping are detailed in Supplementary Table S1 at JXB online), bak1-4 (T-DNA line SALK_116202), fls2 (Gómez-Gómez and Boller, 2000), and pep1/pep2 described by Flury et al. (2013). The prosoop12 mutant in the Col-0 background was created using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) approach. We searched prosoop12 gene-specific single guide RNA (sgRNA) and potential off-target sites in the Arabidopsis Col-0 genome using the Crispor Tefor program (http://crispor.tefor.net). The 20 base sgRNA with the sequence AAGAATTTGACCCATTTTTG was used. Soil-grown plants used for Enervia amylovora and Alternaria brassicicola inoculations as well as all in vitro plants (on Murashige and Skoog (MS) medium) were grown under short-day conditions (photoperiod of 8 h light at 22 °C/16 h dark at 21 °C, with 70% relative humidity). Plants used for all other assays were grown under long-day conditions (photoperiod of 16 h light at 22 °C/8 °C dark at 21 °C, with 60% relative humidity). Bas sia napus (Darmor-bzh) and Solanum lycopersicum (Sweet Baby) were grown under short-day conditions.

Plant inoculation with E. amylovora

Ws, Col-0, and the prosoop12 mutant in both genotypes were grown for 5 weeks on soil. Four leaves of 20 plants were infiltrated with bacterial suspensions of the wild-type strain of E. amylovora CFBP1430 at a concentration of 10^7 colony-forming units (cfu ml^-1) in sterile water or were mock treated using a needleless syringe. Symptom severity was scaled as described in Degraeve et al. (2008). For symptom rating (for Ws and prosoop12-Ws), at least 12 rosette leaves were used per condition in two biological replicates. Maximal symptoms appeared at 24 h or 48 h post-inoculation (hpi) depending on biological replicates. Therefore, representative experiments are presented at either 24 hpi or 48 hpi. For bacterial counting (for Col-0 and prosoop12-Col-0), samples were taken 3 d post-infection using a cork borer (d=5 mm) to cut one leaf disc per infected leaf. Leaf discs were ground in sterile water, diluted, and plated as droplets of 10 µl on LB plates. Plates were incubated, and colonies were counted the next day. Bacteria of 32 leaves of the wild type and prosoop12 were extracted and quantified.

Seed contamination and leaf infection by A. brassicicola

Fifty surface-sterilized seeds per Petri dish of Ws and prosoop12 were immersed in a solution containing A. brassicicola (strain abra43) with 10^5 conidia ml^-1 for 1 h and dried under sterile conditions. Leaves of Ws wild type and prosoop12 mutant were inoculated with 5 µl of an A. brassicicola solution, with a concentration of 10^5 conidia ml^-1. Symptoms were observed 6 d after infection. Necrotic areas were quantified using Imagej. The experiments were repeated three times.

Protection assay

Mature leaves of A. thaliana plants were infiltrated by needleless syringe infiltration with the indicated elicitor peptide or control solution and were kept under long-day growth conditions for 24 h. The Pseudomonas syringae pv tomato DC3000 strain was grown in overnight culture on YEB medium plates supplemented with appropriate antibiotics. Cells were harvested from the plate, re-suspended in sterile 10 mM MgCl2, and diluted to an OD600 of 0.02. The bacterial solution was infiltrated into
the pre-treated leaves with a needleless syringe. Plants were maintained at high humidity. Samples were taken using a cork borer (d=8 mm) to cut one leaf disc per infected leaf. Leaf discs were ground in 10 mM MgCl₂, diluted to the indicated concentration, and plated as droplets of 10 µl on YEB plates with the appropriate selection. Plates were incubated at 28 °C, and colonies were counted 2 h after infection (0 dpi) as well as 1 d and 2 d post-infection. Eight plants were infected for each pre-treatment and sampling time point. The experiment was performed twice with similar results.

Transcriptomic analysis
Microarray analysis was performed with the CATMA array v5 (Hilton et al., 2004). Leaves were collected 24 h after inoculation from two independent biological replicates. Total RNA was extracted using the Qiagen RNeasy kit according to the supplier’s instructions. RNA integrity, cDNA synthesis, hybridization, and array scanning were performed as described in Lurin et al. (2004). cDNA from leaves inoculated with E. amylovora were hybridized against cDNA of leaves inoculated with water collected at the same time point. Statistical analysis was based on two dye swaps as described in Gagnot et al. (2008). To determine differentially expressed genes, a paired t-test on the log ratios was performed. Spots displaying extreme variance were excluded. The raw P-values were adjusted by the Bonferroni method, which controls the family wise-error rate. We considered as differentially expressed those genes with a Bonferroni P-value ≤0.05 Gagnot et al. (2008).

Determination of gene expression by qPCR
Detached leaves of 3-week-old plants were collected and floated for 2 h in elicitor or control solution. After the treatment, material was frozen and ground in liquid nitrogen. RNA from 100 mg of tissue was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel Hoerdt, France). The DNase treatment was performed according to the manufacturer’s recommendations. For PCR, cDNA was synthesized from 10 ng of total RNA extract with oligo(dT) primers using Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s recommendations (Promega). For quantitative real-time reverse transcription–PCR (qPCR) in a 96-well format, the Chromo4™ manufacturer’s instructions (Promega) were followed. For quantitative real-time reverse transcription–PCR (qPCR) in a 96-well format, the Chromo4™ System (Bio Rad) was used. Expression was normalized to that of the gene ACR12 (AT5G04740, because of its constant transcription profile upon elicitor treatment) using the qGene protocol (Muller et al., 2002). All the gene-specific primers used are detailed in Table S1.

Seedling growth inhibition assay
Seedlings were germinated on MS agar and grown for 5 d before transferring one seedling per well to 24-well plates containing 500 µl of MS medium or MS medium supplied with the indicated elicitor peptide to a final concentration of 1 µM (six replicates per elicitor peptide treatment). Photos were taken, and fresh weight and root length were measured after a further 8 d. The root length of proscoop12 and wild-type plants was determined on vertical MS plates.

Elicitor peptides
Peptides of flg22 (QLRLSTGSRINSKDAADGQIA), A. thaliana Plant Elicitor Peptide 1 (AT5Pep1) (ATKVKAQQRKGEVSSGRPGQHIN), SCOOP12 (PVRSSQASQAGGR), SCOOP12 (GRPRSSAGSSQVQ), SCOOP12 S5/7A (PVRSAQASQAGGR), SCOOP12 S5A (PVRSAQASQAGGR), and SCOOP12 S7A (PVRSSQASQAGGR) were obtained from Eurogentec SA (Angers, France) and diluted in water to the final concentration used for the assays.

Measurement of reactive oxygen species
For ROS assays, leaf discs of 3-week-old soil-grown plants were placed into each well of a white 96-well plate (Thermo Scientific, Waltham, MA, USA) in 0.1 ml of water and kept in the dark overnight. For elicitation and ROS detection, horseradish peroxidase and luminol were added to a final concentration of 10 µg ml⁻¹ and 100 µM, respectively. Luminescence was measured directly after addition of elicitor peptides in a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany).

Cell culture conditions
Arabidopsis thaliana cells were grown in a liquid MS-based (Duchefa-Kalys, France) growth medium (pH 5.6) with the addition of 2,4-dichlorophenylacetic acid (0.2 mg l⁻¹), sucrose (30 g l⁻¹), and KH₂PO₄ (0.2 g l⁻¹). Cells were grown under continuous light (200 µE m⁻² s⁻¹) on a rotary shaker and subcultured weekly to fresh medium. For radiolabeling experiments, 7-day-old cell suspensions were used.

Radioisotope labeling of phospholipids
Arabidopsis cells were aliquoted (7 ml) in individual flasks and kept for 3 h under mild rotation for equilibration. Radioisotope labeling was done by the addition of 53 MBq l⁻¹ [³²P]orthophosphate. Lipids were extracted according to Lepage et al. (2009). Lipids were separated by TLC using an acidic solvent system composed of chloroform:acetone:acetic acid:methanol:water (10:4:2:2:1, v/v/v/v/v) (Lepage, 1967) or in a solvent system composed of chloroform:methanol:ammonia:water (90:70:1:16, v/v/v) (Munnuk et al., 1994). Radiolabeled spots were quantified by autoradiography using a Storm phosphorimager (Amersham Biosciences, UK). Individual phospholipids were identified by co-migration with non-labeled standards visualized by primuline staining or by phosphate staining.

Accession numbers
Transcriptome data are available at the Gene Expression Omnibus with the accession number GSE22683. The samples used (including biological repetitions) are: GSM562282, GSM562283, GSM562284, GSM562285, GSM562286, GSM562287, GSM562288, GSM562289, GSM562294, GSM562295, GSM562296, and GSM562297.

Results
Identification of the PROSCOOP gene family
Meta-analysis of CATMA microarray data (Gagnot et al., 2008) has previously highlighted several hundred non-annotated small protein–coding genes of unknown function in Arabidopsis (Aubourg et al., 2007). Further, we investigated the whole CATMA resource available at this time in order to identify new genes induced by various stresses for further functional analyses. Among them, AT5G44585 caught our attention because of its highly informative profile: this gene was differentially expressed in 136 experiments (21% of the whole set), being strongly induced in response to a large panel of biotic or oxidative stresses, E. amylovora infection being one of the top stresses. Biological contexts were extracted from each CATdb experiment (http://tools.ips2.u-psud.fr/CATdb) and
classified into eight classes (Fig. 1; Supplementary Table S2). It is noteworthy that no less than 70% of the complete transcriptomic response of AT5G44585 could be summarized with three keywords: pathogen response, oxidative stress, and root growth. Generally, we found this gene to be strongly up-regulated in most biotic and oxidative stress conditions, while it was down-regulated in conditions aiming at diminishing oxidative stress. Furthermore, AT5G44585 exhibited a constitutive expression in roots in growth conditions but is down-regulated in numerous conditions affecting root elongation such as nitrogen starvation (Krapp et al., 2011). This advocated for further exploration of this gene in oxidative stresses, root development, and in response to pathogen infections.

The screening of the Arabidopsis genome revealed that AT5G44585 belongs to a small family of 14 unknown homologous genes with similar intron–exon structure (two or three exons), encoding proteins ranging from 72 to 117 amino acids. Analysis of the N-terminal regions using the SIGNALP v4.1 (Nielsen, 2017) and the PREDOTAR v1.04 (Small et al., 2004) software revealed a signal peptide targeting proteins to the endoplasmic reticulum to be present in all members of the family. DeepLoc v1.0 (Almagro et al., 2017) predicts an extracellular localization for the 14 proteins, with scores ranging from 0.88 to 1. The 14 genes are organized in two tandemly arrayed clusters on chromosomes 1 and 5 (Fig. 2A). The largest 37 kb long gene cluster on chromosome 5 contains numerous vestiges of transposable elements (Helitron type) from 0.88 to 1. The 14 genes are organized in two tandemly arrayed clusters on chromosomes 1 and 5 (Fig. 2A). The largest 37 kb long gene cluster on chromosome 5 contains numerous vestiges of transposable elements (Helitron type) which could have impacted evolution of this family through proteolytic processing. For these reasons, this newly identified family has been named PROSCOOP, for putative precursors of SCOOP peptides (Serine riCh endOgenOus Peptide). The genes are termed PROSCOOP1–PROSCOOP14 (AT5G44585 being PROSCOOP12) and the corresponding mature peptides are named SCOOP1–SCOOP14 (Fig. 2A).

Previously reported RNA sequencing (RNA-seq) approaches (Hruz et al., 2008) allowed us to broaden our transcriptome analysis to the PROSPOC family members that were missing on the microarrays (only four of them are present in the Affymetrix Ath1 chip). We could confirm the regulation of their transcription in several stress conditions and organs (Fig. 2B). These data show a large diversity of transcriptional profiles in this family, suggesting its involvement in different biological functions. Notably, PROSCOOP12 shows a distinct transcription profile as it is among the minority of paralogs to be highly induced by aggression by different pathogens and expressed in the whole root system.

In order to assess the evolutionary conservation of the PROSCOOP family, an extensive BLASTP search for homologs in GenBank was carried out. We identified this family in several Brassicaceae genomes reaching from Eutrema salsugineum to Camelina sativa, and the number of identified homologs in these genomes ranged from 1 to 13. Outside the Brassicaceae genus, no similar proteins could be detected despite low stringency searches. The phylogenetic tree built from the multiple alignment of the 74 identified PROSCOOP homologs shows that gene duplications occurred before speciation of the eight different Brassicaceae species (Supplementary Fig. S1).

In order to identify divergent yet still conserved smaller regions, the MEME algorithm (Bailey et al., 2015) was used, excluding full-length alignments, on the 74 identified homologs. This sensitive approach allowed the identification of two significantly conserved 11 amino acid long motifs (Fig. 3). These motifs are good candidates for functional mature peptides (or a part of them) following the putative proteolytic processing of the corresponding precursor. Indeed, both motifs are proline, serine, arginine, and glycine rich, as in previously described PTMP families such as CLV3/CLE (Betsuyaku et al., 2011), IDA (Vie et al., 2015), PIP (Hou et al., 2014), and CEP (Roberts et al., 2013). Motif 1 is more ubiquitous than motif 2 since it was detected in 72 sites (e-value of 9.8e–213) compared with 39 sites (e-value of 3.4e–179) out of the 74 PROSCOOP homologs. Therefore, we have focused our downstream functional analysis on motif 1 (Fig. 3), named SCOOP hereafter.
Characterization of a new Arabidopsis phytocytokine

Fig. 2. The PROSCOOP family. (A) Gene organization: coding exons and introns are represented by blue boxes and blue broken lines, respectively. Remains of transposable elements (Helitron type) are represented by orange boxes, and the green box indicates a putative non-coding RNA of unknown function. The TAIR gene names and corresponding PROSCOOP nomenclature are indicated. PROSCOOP2 and PROSCOOP3 are not annotated in the last TAIR version but are confirmed by the ESTs EG446167, EG448031, EG446890, and CB253842. (B) Transcription of the PROSCOOP family: significant (P-value <0.05) differential expression induced by specific perturbations (upper panel) and transcription level in different Arabidopsis organs (lower panel) are based on RNA-seq data obtained from the Genevestigator platform (Hruz et al., 2008). The PROSCOOP12 gene is indicated by a red frame.
PROSCOOP12 is co-expressed with genes involved in hormone signaling and defense

In order to make a first assessment of the potential biological relevance of PROSCOOP12 and to predict its putative functional partners, we further mined previously published Arabidopsis transcriptome data (Gagnot et al., 2008). Based on the assumption that genes with related biological functions are likely to be co-expressed (Schöner et al., 2007), we used the results of the Gaussian mixture model-based clustering method from the GEM2Net resource (Maugis et al., 2009; Zaag et al., 2015). The PROSCOOP12 gene was found to be co-expressed with 83 genes in a set of experimental samples comprising biotic stress triggered by necrotrophic bacteria and fungi. This cluster of 83 genes has been enriched by the integration of functional partners based on co-citations, protein–protein interactions, and common biological pathways using TAIR, the Arabidopsis interactome (Arabidopsis Interactome Mapping Consortium, 2011), and the STRING database (Szklarczyk et al., 2017). This step resulted in a network of 117 genes (Supplementary Tables S3A, B) mainly focused on hormone crosstalk [in particular salicylic acid (SA)/jasmonic acid (JA) signaling], pattern-triggered immunity (PTI), brassinosteroid and phenylpropanoid pathways, and nitrogen metabolism (Supplementary Fig. S2). Out of 117 genes, 53 are involved in response to stimulus (GO:0050896, fdr 1.31e-11); among them, 26 genes are classified in defense response (GO:0006952, fdr 5.72e-10) and 14 in transmembrane signaling receptor activity (GO:0004888, fdr 1.41e-09). Numerous key players in defense were found to be clustered with PROSCOOP12, such as the NIMIN1, IOS1, NHL6, MLO12, FRK1, LECRKA4.1, CRK13, and HA2 genes and the WRKY11, -14, -18, -22, -60, and -70 transcription factor genes. This relational network contains two other genes encoding PTMPs, namely PROVIR10 and PSK4, and two PTMP receptor kinases, PSKR1 and PSY1R, that are involved in root development and modulation of SA/JA defense responses (Mosher et al., 2013). PROVIR10 has been found to correlate positively with disease triggered by necrotrophic pathogens (Dobón et al., 2015) and PSK4 encodes a phytosulfokine, one of the peptide growth factors involved in disease establishment (Rodiu et al., 2016). This approach led us to explore the role of PROSCOOP12 and its SCOOP12 peptide regarding fungal and bacterial infections.

PROSCOOP12 is involved in pathogen defense and root development

PROSCOOP12 transcription was induced in the presence of different pathogens, Eniwia amylovora being one of the highest inducers (Fig. 1; Supplementary Table S2). Necrogenic pathogens are known to induce a response rather different from biotrophic pathogens in regards to ROS production (Venisse et al., 2001). Therefore, in comparison with the responses of this gene to other oxidative stresses, we expected a high correlation.
We hypothesized that infection with the necrogenic bacterium \textit{E. amylovora} and the necrotrophic fungus \textit{A. brassicicola} were suitable conditions to test a putative effect of the lack of function. This hypothesis was reinforced with the analysis of co-expressed putative partners, and its putative role as a secreted DAMP. Screening Arabidopsis mutant collections (Dérozier et al., 2011), we identified a T-DNA mutant \textit{proscoop12} in theWs background. Homozygous mutant plants did not transcribe \textit{PROSCOOP12} (Supplementary Fig. S3). Compared with wild-type plants, \textit{proscoop12} displayed a higher tolerance to \textit{E. amylovora}-induced cell death as observed by a reduction of necrotic symptoms in leaves (Fig. 4A). This phenotype has only been observed in \textit{wrky70} (Moreau et al., 2012). Like \textit{WRKY70}, \textit{PROSCOOP12} acts as a negative regulator of defense against this bacterium. The transcription factor \textit{WRKY70} is known to positively regulate \textit{WRKY60} and it is involved in the JA/SA crosstalk (Li et al., 2004). Notably, these two genes have been found clustered with \textit{PROSCOOP12} in our gene network analysis (Supplementary Fig. S2). We then performed a microarray transcriptomic comparison of \textit{proscoop12} versus the wild type following bacterial inoculation. The results show that 3731 genes were differentially expressed in the wild type in response to \textit{E. amylovora}, and 4125 in \textit{proscoop12}. Despite the difference in symptom intensity, the vast majority of the

![Fig. 4. Mutant phenotype in response to \textit{E. amylovora} and \textit{A. brassicicola} infections. (A) Effect of \textit{E. amylovora} infection on the \textit{proscoop12} mutant. The symptom scale used (0–3) is illustrated on the right. The asterisk indicates a significant difference from symptom severity in wild-type leaves inoculated with \textit{E. amylovora} (Mann–Whitney test, $\alpha=0.05$). (B) Distribution of the 126 genes up-regulated in \textit{proscoop12} versus the wild type inoculated with \textit{E. amylovora} according to their functional annotation. The complete results of this transcriptome approach are given in Supplementary Table S4. (C) Effect of \textit{A. brassicicola} seed infection on \textit{proscoop12} during germination 2, 3, and 8 d post-imbibition. Significant differences according to Student’s $t$-test results: $^*P<0.05$.](https://academic.oup.com/jxb/article-abstract/70/4/1349/5306346)
bacteria-responsive genes did not display significant differences in both lines. Indeed, only 131 genes displayed a significantly different expression (Bonferroni P-value 5%) between wild-type- and proscoop12-infected plants (Supplementary Table S4): 126 up-regulated and 5 down-regulated genes, these latter corresponding only to hypothetical proteins or pseudogenes.

The 126 up-regulated genes that may contribute to the difference in symptoms between proscoop12 and the wild type were challenged by functional annotation adding literature references to Gene Ontology (GO) terms to provide additional information (Supplementary Table S4; summarized in Fig. 4B). Indeed, 45% of them are connected to defense response (such as HR4, SQP1, AED1, MKK2, HD2B, and NPR3) and/or protection against oxidative stress (such as ALDH24B, BiP2, APX1, ATOM1, APR1, and PER50). Moreover, 18% were related to response to other stresses, mainly oxidative stress, and 10% could have indirect links with stress since they are involved in processes such as cell wall modifications or proteolysis. Only 13% could not be related to the phenotype, often because their function is currently unclear. Finally, the remaining 14% are unknown genes. The high percentage of genes directly related to protection against oxidative stress supports the hypothesis of a relationship between PROSCOOP12 and the control of ROS production.

The response of proscoop12 to a necrotrophic fungus infection was assessed using the Arabidopsis–A. brassicicola pathosystem (Pochon et al., 2012). Alternaria brassicicola inoculation of rosette leaves produced similar symptoms in wild-type and proscoop12 genotypes (Supplementary Fig. S4). Because seedling infection by A. brassicicola is mainly caused by seed transmission, we have also observed the fungal colonization during germination of infected seed lots under controlled conditions.

Two days after sowing, proscoop12 showed a significantly lower rate of germinating seeds prone to A. brassicicola infection compared with the wild type (Fig. 4C).

Because our transcriptome analysis suggested that PROSCOOP12 may play a role in root development (Figs 1, 2B), we compared the root lengths of wild-type and proscoop12 plants. Indeed, proscoop12 plants developed significantly longer roots than control plants (Fig. 5A, B). No significant difference was observed between the wild type and proscoop12 regarding the seedling fresh weight (Fig. 5C).

A second proscoop12 line was obtained in the Col-0 background using a CRISPR/Cas9 approach. The frameshift obtained in the first exon disrupts the coding frame 10 amino acids after the editing event, upstream of the conserved motif. The phenotypes previously observed with the Ws proscoop12 mutant were confirmed in this Col-0 mutant line (Supplementary Fig. S5).

The SCOOP12 peptide has the main features of DAMPs

The structural features of the PROSCOOP12 protein suggested that it should be classified as a secreted PTMP. At the functional level, its transcriptional behavior suggested that it may play a role as a DAMP. Indeed, the induction of PROSCOOP12 expression by a large panel of biotic stresses and the root phenotypes identified in the proscoop12 mutant revealed some analogies with the AtPROPEP1 and AtPROPEP2 genes which are the precursors of the AtPep1 and AtPep2 peptides, respectively, well-characterized DAMPs (Bartels and Boller, 2015). Likewise, both genes are also induced by biotic stress (Huffaker et al., 2006), and the AtPep1 DAMP is involved in root development since

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**Fig. 5.** Phenotypic comparison between proscoop12 and wild-type plants. (A, B) Root growth phenotypes determined after 10 d. Student’s t-test revealed that the different root length between the wild type and mutant is highly significant (*P<0.05). (C) Seedling fresh weight determined after 10 d. Bars show the combination of two biological repetitions (25 seedling each) and error bars show ±SE of the mean.
the overexpression of AtPROPEP1 and AtPROPEP2 causes significantly longer roots (Huffaker et al., 2006). Therefore, we wanted to test if PROSCOOP12 encodes a peptide that may act as a DAMP by comparing it with AtPep1.

The SCOOP12 peptide induces immune responses in Arabidopsis

Based on the identification of the conserved motif 1 (Fig. 3), a putative mature peptide SCOOP12 was defined (PVRSSQSSQAGGR) from PROSCOOP12 and synthesized in order to explore its biological function. Despite the non-predictable post-translational modifications, we tested the exogenous application of the synthetic SCOOP12 peptide as previously described for CLE and RGF PTMP families (Matsuzaki et al., 2010; Murphy et al., 2012; Whitford et al., 2012). Treatment of plants with SCOOP12 induced a wide range of long- and short-term immune responses (Fig. 6). One of the fastest defense responses is the production of ROS (Torres et al., 2006). We show here that SCOOP12 induced a more intense ROS burst compared with AtPep1 but weaker than flg22 (Fig. 6A). Next, we wanted to study the effect of SCOOP12 on genes closely linked to early defense mechanisms. FRK1 has previously been shown to be induced by pathogens, elicitors, SA (Asai et al., 2002; Boudsocq et al., 2010), and AtPep1 (Flury et al., 2013). Furthermore, our co-expression network approach identified co-expression of PROSCOOP12 with FRK1 (Supplementary Fig. S2). Therefore, we measured the FRK1 expression level in detached leaves floating for 2 h in solutions supplemented by SCOOP12 or AtPep1. Compared with controls, AtPep1 and SCOOP12 treatments resulted in a 15-fold and 8.5-fold increase in FRK1 expression, respectively (Fig. 6B). The deposition of callose is also known to be triggered by DAMPs (Luna et al., 2011). Callose staining after 24 h of treatment with the elicitor peptides showed that SCOOP12 induces callose deposition, yet at a weaker level compared with flg22 or AtPep1 (Fig. 6C, D). One of the long-lasting defense responses is an inhibition of growth caused by the elicitor (Krol et al., 2010). Our results indicate that perception of SCOOP12 also leads to an arrest of growth. The effect is comparable with the flg22 and the AtPep1 DAMP (Fig. 6E–G).

In order to demonstrate the specificity of the SCOOP12 sequence, we synthesized a peptide based on a randomized version of the same amino acids and tested plant responses to this scrambled SCOOP12 (scSCOOP12). Furthermore, we synthesized peptides with double alanine replacements (SCOOP12 S5/A7) and single replacements (SCOOP12 SSA and SCOOP12 S7A) to test the importance of the two highly conserved serine residues on positions 5 and 7 of SCOOP12 (Fig. 3) for its activity. Plants treated with scSCOOP12 as well as with the modified peptides did not show seedling growth inhibition. Total seedling fresh weight as well as root length were not different from those of control plants (Fig. 7A). Finally, treatments with scSCOOP12, SCOOP12 S5/A7, and SCOOP12 SSA did not induce a ROS burst, and only SCOOP12 S7A resulted in a low, but still significant ROS burst (Fig. 7B). These results highlight the importance of the amino acid order and the highly conserved serine residues for the perception of SCOOP12 by the plant.

Next, we wanted to test the conservation of plant responses to SCOOP12. For that purpose, plants were selected in which we identified PROSCOOP homologs (B. napus, Supplementary Fig. S1) and plants that do not contain this gene family (Nicotiana benthamiana and S. lycopersicum). We measured ROS production following application of SCOOP12 in these plants and included flg22 as a positive control. We detected a ROS burst caused by flg22 in all four plant species. On the other hand, SCOOP12 only resulted in a ROS burst in A. thaliana and, at a lower, yet still significant, level in B. napus (Supplementary Fig. S6). SCOOP12 seems to be similar enough to its closest B. napus homolog (BNCDY22858 with the motif FAPGSSSGHGGGR) to trigger a ROS burst. Therefore, only the two plant species containing homologs of the PROSCOOP gene family members showed a response to SCOOP12 treatments.

Pre-treatment with the SCOOP12 peptide protects Arabidopsis against Pseudomonas infection

It has previously been shown that priming of plants with the flg22 elicitor as well as with oligogalacturonides could result in enhanced tolerance against subsequent bacterial infections. For instance, plants pre-treated with these elicitors showed significantly reduced lesion size following an infection with Botrytis cinerea (Raacke et al., 2006; Ferrari et al., 2007). Using a similar assay, we found that plants pre-treated with flg22 as well as with SCOOP12 and AtPep1 were less susceptible to P. syringae pv. tomato DC3000 infection (Fig. 8). The effect of the two endogenous peptides SCOOP12 and AtPep1 was weaker than that of flg22, which is consistent with the fact that flg22 induced a stronger defense response compared with SCOOP12 (Fig. 6A, C).

SCOOP12 and AtPep1 induce the expression of several PROSCOOP genes

It has previously been shown that small endogenous peptides can induce the expression of their own precursors, resulting in a positive feedback loop. For instance, expression of several PROPEP genes can be induced by different AtPep peptides (Huffaker and Ryan, 2007). This led us to investigate the change in the steady-state transcript level of all 14 PROSCOOP family members after SCOOP12 exposure. Moreover, we decided to add AtPep1 in our assay for comparison since it is also known to induce the transcription of another peptide precursor, pnpPPIP1 (Hou et al., 2014). The results show that PROSCOOP 2, 7, 8, 12, and 13 are up-regulated by the AtPep1 treatment (Supplementary Fig. S7). Most importantly, the direct precursor PROSCOOP12 is up-regulated by SCOOP12 in comparison with the control treatment (Supplementary Fig. S7L). Therefore, there is a positive feedback loop linking SCOOP12 to its precursor PROSCOOP12 but also of other members of the PROSCOOP family such as PROSCOOP7. However, SCOOP12 did not induce the expression of PROPEP1 (Supplementary Fig. S7O). These results suggest that there is a feedback loop of SCOOP12 to its precursor and to PROSCOOP7, and that AtPep1 is capable of inducing five members of the PROSCOOP family.
The BAK1 co-receptor is involved in SCOOP12 perception
A well-characterized co-receptor of several receptors of small peptides is BR11-associated kinase1 (BAK1). Interaction of BAK1 with receptor-like kinases that act as elicitor receptors was proposed to be due to conformational changes occurring after ligand binding which results in the formation of...
To test if BAK1 is involved in the perception of SCOOP12, a seedling growth inhibition assay was performed on bak1-4 plants. Compared with wild-type controls, bak1-4 plants did not display any significant growth inhibition upon SCOOP12 treatment (Fig. 9). The same approach was carried out on fls2 (the flg22 receptor) and pepr1/pepr2 plants. In contrast to BAK1, our results suggest that these receptors are not involved in the perception of SCOOP12 (Fig. 9).

**SCOOP12 rapidly activates phospholipid signaling pathways in Arabidopsis cell suspensions**

Lipid signaling pathways act as multifunctional regulatory mechanisms in plants. They incorporate several groups of inducible enzymes that convert membrane phospholipids into signaling molecules. Phosphatidic acid (PA) is a well-known biologically active lipid that is produced in response to numerous hormonal and stress signals including, notably, flg22 (van der Luit et al., 2000). We demonstrate that application of SCOOP12 induces an accumulation of PA in Arabidopsis cell suspensions (Fig. 10A). This effect is observed as early as 5 min following SCOOP12 application at a low concentration of 100 nM (Fig. 10B, C). The scSCOOP12 had no effect on PA accumulation. Two modes of PA accumulation are known: phospholipase D (PLD)-dependent via direct hydrolysis of membrane phospholipids and diacylglycerol kinase (DGK)-dependent via phosphorylation of diacylglycerol (DAG). In our experiment, a labeling protocol that favors visualization of DGK-derived PA was used (Arisz and Munnik, 2013).
Phosphatidylinositol 4,5-bisphosphate (PIP₂) is a substrate to phosphatidylinositol-specific phospholipase C (PI-PLC) that produces DAG. We have also observed that the level of PIP₂ is transiently reduced following SCOOP12 treatment (Fig. 10B). These results suggest that SCOOP12 initiates a signaling cascade implicating PI-PLC (causing the depletion of PIP₂) and subsequent production of PA via phosphorylation of DAG by DGK.

Discussion

Considered jointly, our transcriptome, mutant phenotyping, and peptide assay results allow us to propose a model explaining the roles of the SCOOP12 peptide in Arabidopsis (Fig. 11). The induction of numerous genes involved in the protection against oxidative stress such as peroxidases, glutathione transferase, and phenylpropanoid synthases in proscoop12 in response to E. amylovora infection (Supplementary Table S4) might indicate that its lack of expression could result in a decrease in H₂O₂ levels. This could impair the activity of BAK1 in receptor complexes and indicate that they may act as an intrinsic part of the SCOOP12 regulatory cascade in plants.

The negative action of SCOOP12 on the antioxidant response is consistent with the reduction of symptoms observed in the proscoop12 defective mutant in the presence of the necrogenic bacterium E. amylovora (Degrave et al., 2008). In this case, the suppression of PROSCOOP12 seems to enhance the protection against oxidative stress, thus hampering bacterial development in infected Arabidopsis leaves.

The comparison of the PROSCOOP family with other previously published genes encoding such secreted peptides highlights numerous shared features but also interesting specificities. At the structural level, the PROSCOOP proteins distinguish themselves by the absence of a highly conserved C-terminal region. Indeed, the motifs detected with the MEME tool are quite divergent compared with the other PTMP precursors (Matsubayashi, 2011). This divergence may explain the fact that no PROSCOOP homologs could be detected outside the Brassicaceae genomes. This restricted phylogenetic profile is opposite to the other described secreted peptides which are conserved in both monocots and eudicots. Furthermore, in...
contrast to the majority of the known PTMPs, the conserved motifs are not localized at the C-terminal extremity of their precursors, and their maturation could involve two steps of proteolytic processing or a trimming step (Matsubayashi, 2011). Out of the 14 Arabidopsis PROSCOOP proteins, three include two duplicated SCOOP motifs (Fig. 3), reminiscent of the previously described cases of the CEP and PIP families (Roberts et al., 2013; Vie et al., 2015) and also of the CLE18 protein in which each copy of the conserved CLE motifs has a specific function (Murphy et al., 2012). The motif composition classifies SCOOP in the superfamily of ‘SGP-rich peptide’ among PIP, CLE, IDA, PEP, and CEP families (Hou et al., 2014). At the functional level, the triggering of ROS burst, FRK1 transcription, and callose deposition moves SCOOP12 close to the cytosolic AtPEP and apoplastic PIP families (Huffaker et al., 2006). Our results suggest a functional link between AtPEP1 and SCOOP12 since both peptides induce the transcription of PROSCOOP12 (Supplementary Fig. S7L). This collaboration between different peptide families has also been described with AtPEP1 and PIP1 which act co-operatively to amplify triggered immunity. Furthermore, the signaling induced by AtPEP1 (Schulze et al., 2010), PIP1 (Hou et al., 2014), and SCOOP12 (Fig. 9) is dependent on the BAK1 co-receptor. In addition to their role as amplifiers of the immune response, these peptides are involved in root development but via different mechanisms. The overexpression of the PIP1 precursor or its exogenous application inhibits Arabidopsis root growth as described for CEP (Roberts et al., 2013) and SCOOP12 peptide (Fig. 6F). On the other hand, the constitutive overexpression of PROPEP1 increases root development (Huffaker et al., 2006) whereas AtPEP1 treatment inhibits root growth (Poncini et al., 2017). Acting as growth factors and in contrast to SCOOP12, the PTMPs PSK and PSY1 are involved in root elongation (Amano et al., 2007; Matsuzaki et al., 2010). These
comparisons show that despite common structural and functional characteristics, the SCOOP family is different from previously described secreted peptides. The divergence observed in the C-terminal sequence of PROSCOOP proteins suggests a broad range of biological functions through a diversity of receptors which will be the targets of future studies.

In conclusion, SCOOP12 belongs to a new family of putatively secreted peptides specific to the Brassicaceae species. At the functional level, such secreted peptides are classified as phytocytokines (such as RALFs, systemin, and PIPs) which are secondary endogenous danger signals. Indeed, this classification (Gust et al., 2017) distinguishes them from classical DAMPs (primary endogenous danger signals) which are passively released from injured tissue without biosynthesis and secretion processes. Nevertheless, the final processing of SCOOP12 is based on structural comparisons with analogous peptides and remains to be experimentally confirmed. Through its negative action on antioxidant responses and its positive effect on PA/ROS production (PLC pathway), SCOOP12 could play a role in the moderation of defense responses, as well as root elongation, to prevent unnecessary energy loss in a ‘trade-off’ fashion (Walters and Heil, 2007). The functions of such plant secreted peptides at the boundaries of development and stress signaling pathways open the way to future strategies that jointly consider product quality/quantity and new resistance traits.
Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Phylogenetic tree of PROSCOOP homologs.

Fig. S2. Relational annotation of genes co-expressed with PROSCOOP12 and their functional partners.

Fig. S3. Confirmation of absence of transcription in the pro-scoop12 T-DNA knock-out line by RT–PCR.

Fig. S4. Effect of A. brassicicola infection on pro-scoop12 leaves.

Fig. S5. Confirmation of pro-scoop12 mutant phenotype in a second genotype.

Fig. S6. ROS burst measurements on selected plant species treated with SCOOP12.

Fig. S7. Transcriptional response of the PROSCOOP gene family to SCOOP12 and AtPep1.

Table S1. Gene-specific primer sequences used for mutant genotyping and qPCR analysis of all the PROSCOOP genes.

Table S2. List of the 136 comparisons in which transcription of AT5G44585 was deregulated in CATdb (http://tools.ips2.u-psud.fr/CATdb)

Table S3. List of 117 genes involved in the relational annotation of PROSCOOP12 (in addition to Supplementary Fig. S2).

Table S4. Transcriptomic comparison of pro-scoop12 and wild-type plants during E. amylovora infection.

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