Database mining of plant peptide homologues

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Abstract  In plant-pathogen interactions, pathogens employ secreted molecules, known as effectors to overcome physical barriers, modulate plant immunity, and facilitate colonization. Among these diverse effectors, some are found to mimic the plant peptides, to target host's peptide receptors, and intervene in the peptide-regulated defense pathways and/or plant development. To better understand how pathogens have co-evolved with their plant hosts in order to improve disease management, we explored the presence of plant peptide mimics in microbes by bioinformatic analysis. In total, 36 novel peptide mimics belong to five plant peptide families were detected in bacterial and fungal kingdoms. Among them, phytosulfokine homologues were widely distributed in 22 phytopathogens and one bacterium, thereby constituted the largest proportion of the identified mimics. The putative functional peptide region is well conserved between plant and microbes, while the existence of a putative signal peptide varies between species. Our findings will increase understanding of plant-pathogen interactions, and provide new ideas for future studies of pathogenic mechanisms and disease management.

Key words: homologues, mimics, pathogen effectors, plant peptides.

In the co-evolutionary arms race between plants and pathogens, the plant possesses a large arsenal of immune receptors to sense and defend against microbial challenges. Pathogens, in turn, have evolved diverse secreted effectors to suppress the plant's immune responses, facilitate pathogen entry, and alter host physiology for pathogen colonization (Toruño et al. 2016). For example, the bacterial effector Hrp outer protein X1, secreted by the pathogen Pseudomonas syringae, can induce stomatal re-opening and enhance pathogen entry by degrading multiple JASMONATE ZIM DOMAIN transcriptional repressors, thereby, modulating jasmonic acid signaling (Gimenez-Ibanez et al. 2014). The fungal apoplastic effector Avirulence protein 2, a small cysteine-rich protein from the tomato pathogen Cladosporium fulvum, contributes to pathogenic virulence by inhibiting several Cys proteases that are required for basal host defense (van Esse et al. 2008). The identification of pathogen effectors and their host targets is crucial, not only to enhance our understanding of pathogenicity strategies but also to provide significant benefit to crop protection and breeding.

Growing evidence has been found for the existence of pathogen effectors that mimic plant hormones, especially peptides. Plant peptide hormones are made of several to hundreds of amino acids and play crucial roles in cell-to-cell communication for regulating many aspects of plant physiology (Hirakawa and Sawa 2019; Tavormina et al. 2015). By mimicking plant peptides, pathogens can escape immune recognition and manipulate the host's gene expression to facilitate colonization. For example, effectors that mimic CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE), C-TERMINALLY ENCODED PEPTIDE (CEP), and INFLOWER DEFICIENT IN ABSCISSION (IDA) peptides have been reported in sedentary plant-parasitic nematodes (Bobay et al. 2013; Eves-Van Den Akker et al. 2016; Guo et al. 2015; Kim et al. 2018; Lu et al. 2009; Wang et al. 2010, 2011). Elsewhere, the effector XA21-mediated immunity X has been identified to mimic PLANT PEPTIDE CONTAINING SULFATED TYROSINE (PSY) peptide to facilitate Xanthomonas oryzae pv. oryzae infection (Pruitt et al. 2017), and RAPID ALKALINIZATION FACTOR (RALF) homologues have been found across a number of phytopathogenic fungi (Thynne et al. 2017). Many pathogen effectors still remain undiscovered, and these peptide mimics might be acting as important virulence effectors in pathogenic strategies.

Abbreviations: CLE, clavata3/embryo surrounding region-related; CEP, c-terminally encoded peptide; IDA, inflorescence deficient in abscission; IDL, IDA-like; PSK, phytosulfokine; PEP, plant elicitor peptide; PSY, plant peptide containing sulfated tyrosine.

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With the growing availability of microbial whole-genome sequences and tools for bioinformatic analysis, we are increasingly able to compare genes from disparate organisms to identify potential peptide mimics that might be involved in phytopathogen emergence (Thynne et al. 2015). In this study, we examined the distribution of plant peptide homologues in microbes using Protein Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) analysis. The protein sequences of reported Arabidopsis peptide families, including CLE, CEP, IDA/IDL (IDA-like), PSY, RALF, PHYTOSULFOKINE (PSK), PLANT ELICITOR PEPTIDE (PEP), ROOT GROWTH FACTOR (RGF), HYDROXYPROLINE-RICH GLYCOPEPTIDE SYSTEMINS (HYPSYS), SYSTEMINS (SYS), PLANT DEFENSINs (PDFs), S-LOCUS CYSTEINE-RICH PROTEIN (SCR), were downloaded from The Arabidopsis Information Resource (http://arabidopsis.org) and used as queries to search for microbial peptide homologues in NCBI non-redundant protein database. By excluding the organism of Viridiplantae (taxid: 33090), we conducted the BLASTP search for the whole sequences and peptide motif sequences of each peptide family. All searches used the default BLOSUM62 scoring matrix, and an expect threshold of 10. After excluding the peptide homologues that have been annotated or reported, we used the filtered candidates as queries to search for additional genes to ensure the identification of as many homologues as possible. The signal peptide and cleavage site were examined by the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0/; Petersen et al. 2011). Peptide homologues were aligned with Multiple Sequence Alignment Program v.7 (https://mafft.cbrc.jp/alignment/software/; Katoh and Standley 2013). Phylogenetic trees were constructed using the neighbor-joining algorithm with 1,000 bootstrap replications in Molecular Evolutionary Genetics Analysis software 7 (Kumar et al. 2016). Two CLE sequences from Lotus japonicus (LjCLE-RS1 and -RS2) and five from the Globodera rostochiensis (GrCLEs) and Heterodera glycines (HgCLEs) were download from Universal Protein Resource (http://www.uniprot.org) and added in the phylogenetic analysis of CLE family.

The CLE family is one of the most extensively studied plant peptides. It is critical for cell-to-cell communication and is involved in multiple physiological processes (Hirakawa and Sawa 2019; Leasure and He 2012; Miyawaki et al. 2013; Yamada and Sawa 2013). In this study, we identified six novel CLE-like genes in the bacteria kingdom from the Actinobacteria, Proteobacteria, and Gemmatimonadetes phyla (Table 1) with protein size that ranges from 63 to 348 amino acids. While each gene contains a highly variable middle region and a conserved CLE domain, there are some variations in the existence of a signal peptide domain and in the location of the CLE domain, displaying an untypical CLE structure. The CLE homologues of Actinobacteria, Acidimicrobiaeae, and Thiotrichales bacteria, which have been reported as endophytes, lack an apparent signal peptide domain (Supplementary Figure S1). For Gemmatimonadetes bacterium, the CLE homologues

| Species name       | Protein ID       | Length (aa) | Presence of signal peptide |
|--------------------|------------------|-------------|-----------------------------|
| Bacteira Actinobacteria | HBW17759.1 | 348         | No                          |
|                    | OLB81324.1 | 248         | No                          |
| Acidimicrobia Acidimicrobiaeae | MBD52750.1 | 63          | No                          |
| Proteobacteria Gammaproteobacteria | MAX27851.1 | 75          | No                          |
| Thiotrichales | Gemmatimonadetes | OLB19964.1 | 301 | Yes                        |
|                    | PYO41156.1 | 310         | Yes                        |
possess a predicted hydrophobic signal peptide domain at the N-terminal, and a conserved CLE domain in the middle of the sequence (Supplementary Figure S2). The arginine located after the putative CLE domain in these homologues (Supplementary Figure S2) may support their function as a CLE peptide because the arginine is a typical amino acid to be cleaved by carboxyl peptidase (Tamaki et al. 2013). All the identified CLE homologues belong to the Arabidopsis A-type CLE peptide group (Figure 1; Wang and Fiers 2010). The arginine at the first position, proline at the fourth and ninth position, and glycine at the sixth positions are highly conserved in both the plant and bacterial CLE domains.

Phylogenetic analysis indicated that the putative CLE-like peptides of Actinobacteria (HBW17759.1), Acidimicrobiaceae and Thiotrichales bacteria were grouped with AtCLE19, AtCLE20 and AtCLE21 while those of Gemmatimonadetes and Actinobacteria (HBW17759.1) bacteria were closer to AtCLE1, AtCLE3, AtCLE4, LjCLE-RS1 and LjCLE-RS2, GrCLE4, and HgCLEs (Figure 1). CLE3 orthologues have been detected from soybean xylem, suggesting a role in long-distance signaling (Okamoto et al. 2015), and the Lotus japonicus CLE gene (LjCLE-RS1) has been reported to be involved in nodule formation as a systemic signal (Okamoto et al. 2013). Accordingly, the CLE homologues of Gemmatimonadetes and one of Actinobacteria (HBW17759.1) bacteria might act as systemic signals. Previous studies of soil bacterium Agrobacterium tumefaciens and arbuscular mycorrhizal fungus have revealed that their colonization is triggered and regulated by the plant’s CLE signaling pathways (Müller et al. 2019; Samorodova et al. 2018). In addition, CLE-like genes have been identified in many soil nematode

| Fungi and Bacteria species identified as containing PSK peptide homologues. |
|--------------------------------------------------|
| **Species name** | **Protein ID** | **Length (aa)** | **Presence of signal peptide** |
|--------------------------------------------------|
| **Fungi** | | | |
| **Basidiomycota** | | | |
| **Ustilaginomycotina** | OAJ06134.1 | 177 | Yes |
| **Tilletia indica** | OAJ16943.1 | 129 | Yes |
| **Tilletia controversa** | OAJ32324.1 | 71 | Yes |
| **Tilletia laevis** | KAE820126.1 | 71 | Yes |
| **Tilletia caries** | KAE8265338.1 | 71 | Yes |
| **Ascomycota** | | | |
| **Sordariomycetes** | | | |
| **Glomerellaceae** | CCF36476.1 | 74 | Yes |
| **Colletotrichum higginsianum** | XP_018154155.1 | 74 | Yes |
| **Colletotrichum higginsianum** | TIC090791.1 | 74 | Yes |
| **Colletotrichum orbiculare** | TDZ16180.1 | 136 | Yes |
| **Colletotrichum spinosum** | TDZ33132.1 | 113 | Yes |
| **Colletotrichum sidae** | TEA20895.1 | 113 | Yes |
| **Colletotrichum trifoli** | TDZ53646.1 | 113 | Yes |
| **Dothideomycetes** | | | |
| **Botryosphaeriales** | | | |
| **Lasiodiplodia theobromae** | KAB2573496.1 | 119 | Yes |
| **Diplodia corticola** | XP_020135590.1 | 133 | Yes |
| **Diplodia seriata** | KKY13961.1 | 103 | Yes |
| **Macrophomina phaseolina** | EKG09820.1 | 98 | Yes |
| **Capnodiales** | | | |
| **Mycosphaerellaceae** | | | |
| **Pseudocercospora fijiensis** | XP_007930253.1 | 146 | Yes |
| **Pseudocercospora musae** | KXT13808.1 | 131 | No |
| **Pseudocercospora euxmusae** | KXT01173.1 | 100 | Yes |
| **Ramularia collo-cygni** | XP_023628191.1 | 165 | Yes |
| **Cercospora berterae** | PPS0114.1 | 84 | Yes |
| **Cercospora beticola** | XP_023452928.1 | 102 | Yes |
| **Zymoseptoria brevis** | KJX92737.1 | 252 | Yes |
| **Zymoseptoria tritici** | SMR54594.1 | 235 | Yes |
| **Bacteria** | | | |
| **Proteobacteria** | | | |
| **Proteobacteria sp.** | RYZ80985.1 | 75 | No |
species (Lu et al. 2009; Wang et al. 2010, 2011), and studies have shown that GrCLEs and HgCLEs can interact with host cell-surface receptors, activate the plant’s CLE signaling pathways, and increase successful nematode infection (Lu et al. 2009; Wang et al. 2010). Silencing nematode CLE-like genes resulted in decreased parasitism which demonstrates the importance of CLE-like effectors for virulence (Bakhetia et al. 2007; Patel et al. 2008; Wang et al. 2011). The putative CLE peptides of Gemmatimonadetes and Actinobacteria (HBW17759.1) bacteria might function in the rhizosphere to control host plant physiology.

PSK homologues were found in 22 fungal species and one bacterial species (Table 2), consisting of 71 to 252 amino acids and containing at least one conserved YIYTQ motif in the sequence. A predicted signal peptide domain at the N-terminal was detected in most of the homologues. The 22 fungal species belong to the classes of Ustilaginomycotina (Basidiomycota), Sordariomycetes and Dothideomycetes (both Ascomycota), which are all phytopathogens. Our phylogenetic analysis indicated that these PSK homologues are separated from Arabidopsis PSKs, constituting evidence of two divergent groups (Figure 2). Group I contains sequences from five species of the genus Tilletia (Supplementary Figure S3), plant pathogens that affect grasses and especially wheat. The Tilletia spp. homologues had typical a PSK structure that includes a predicted signal peptide domain at the N-terminal and a conserved YIYTQ motif in the C-terminal (Supplementary Figure S3). Group II consists of sequences from multiple Ascomycota phytopathogens (Supplementary Figure S4). Members of this group not only contain a signal peptide domain at the N-terminal, but also feature at least two repeated PSK predicted peptide domains (Supplementary Figure S4) with the Zymoseptoria brevis homologue exhibiting the highest number at eight. In bacteria, one PSK homologue, from the Proteobacteria bacterium that isolated from phyllosphere metagenome, was detected (Table 2). This homologue comprises 75 amino acids with a conserved YIYTQ motif at the C-terminal, but it lacks a signal peptide at the N-terminal. PSK was originally isolated from conditioned medium of plant cell cultures (Matsubayashi and Sakagami 1996), and aside from its growth regulation function, its involvement in abiotic and biotic stress responses in plants has also been verified (Loivamäki et al. 2010; Matsubayashi et al. 2006). PSK signaling is, for example, vital for the induction of tomato immunity against the necrotrophic pathogen Botrytis cinerea (Zhang et al. 2018). The widespread of PSK homologues in economically significant phytopathogens presented in our study, might imply that fungi have co-opted host PSK signaling system to enhance their survival on plants.

IDA/IDL is a signaling peptide involved in the developmental control of floral organ abscission and root cap sloughing (Butenko et al. 2003; Shi et al. 2018). In this study, two IDA/IDL homologues were identified.
Phylogenetic analysis indicated that the relationships between the six Arabidopsis and two fungal IDA/IDL proteins are not close (Figure 3A). In Melampsora larici-populina, the main rust pathogen of many Populus species, the IDA/IDL homologue contains a conserved PIP motif at the C-terminal but lacks of a signal peptide domain at the N-terminal (Supplementary Figure S5). For Colletotrichum fructicola, a pathogen that affects many hosts worldwide, the IDA/IDL homologue contains a predicted signal peptide domain at the N-terminal and a conserved PIP motif at the C-terminal. A study of closely related Colletotrichum species has shown that Colletotrichum tofieldiae, a root endophyte on Arabidopsis thaliana, translocates inorganic orthophosphate (Pi) to the host and enhances plant growth and fitness under Pi deficient conditions, while the pathogenic Colletotrichum incanum significantly inhibits plant growth under low Pi conditions by inoculating the Arabidopsis root (Hiruma et al. 2016). Elsewhere, an IDA/IDL mimic has been reported in Meloidogyne incognita (Kim et al. 2018). Functional analysis has shown that the MiIDL1 gene produces an IDA mimic that could regulate cell separation and improve successful gall development on Arabidopsis roots. Whether the fungal IDA/IDL genes identified in our study have a role in their interactions with plants requires further verification.

Two PEP homologues, one bacterial and one fungal, were identified in our study (Table 3) with both containing a typical PEP motif at the C-terminal.

### Table 3. Fungi and Bacteria species identified as containing IDA/IDL, PEP or CEP peptide homologues.

| Plant peptide | Species name | Protein ID | Length (aa) | Presence of signal peptide |
|---------------|--------------|------------|-------------|----------------------------|
| **IDA/IDL**   | **Fungi**    |            |             |                            |
| Basidiomycota | Pucciniomycetes |          |             |                            |
| Melamporaceae | *Melampsora larici-populina* | XP_007419306.1 | 447          | No                         |
| Ascomycetes   | Sordariomycetes |          |             |                            |
| Glomerellaceae| *Colletotrichum fructicola* | XP_03184095.1 | 70           | Yes                        |
| **PEP**       | **Fungi**    |            |             |                            |
| Ascomycetes   | Saccharomycetes |          |             |                            |
| Metschnikowiaceae | *Metschnikowia sp. JCM 33374* | GEQ72778.1 | 196          | No                         |
| **Bacteria**  | Actinobacteria |          |             |                            |
| Corynebacteriales | *Mycellobacterium conceptionense* | WP_131827736.1 | 172         | No                         |
| **CEP**       | **Bacteria** |            |             |                            |
| Burkholderiaceae | *Ralstonia syzygii* | CCA88060.1 | 61           | No                         |

Figure 3. Phylogenetic trees of the IDA/IDL homologues (A), PEP homologues (B) and CEP homologues (C) in identified microbe species and Arabidopsis thaliana.
and lack of signal peptide domain at the N-terminal (Supplementary Figure S6). Phylogenetic analysis showed that Metschnikowia sp. JCM 33374 and Mycolicibacterium conceptionense PEP homologues were clustered with the Arabidopsis PEP5 protein and PEP7 protein, respectively (Figure 3B).

One bacterial CEP homologue was found in Ralstonia syzygii (Table 3, Supplementary Figure S7), and its phylogenetic tree indicated that it was grouped with Arabidopsis CEP5 protein (Figure 3C). CEP peptides play key roles in orchestrating nitrogen-demand signaling, root nodulation, and lateral root development (Taleski et al. 2018), and a CEP peptide mimic has been reported in the reniform nematode Rotylenchulus reniformis (Eves-Van Den Akker et al. 2016). Corresponding to the Arabidopsis CEP5 domain, the RrCEP1.1 domain of RrCEP1 could significantly up-regulate the host’s nitrate uptake, inhibit root elongation, and limit feeding site expansion at the same time, in order to control the overall drain on the host during infection. The identification of this CEP homologue and its further functional analysis may facilitate the disease management of Ralstonia syzygii in the future.

In conclusion, a number of plant peptide mimics were identified in a diverse range of microbes by bioinformatic analysis. One of the key findings here is that these microbes are economically significant plant pathogens, endophytic bacteria, or rhizosphere microbes, and all of them need to interact with their hosts. Potential peptide mimics found in our study may be involved in such host-microbe interactions, for example, acting as virulence effectors to facilitate host infection and/or disease. We anticipate that this study will increase our understanding of the extent of molecular mimicry in plant pathogens and provide new ideas for the study of pathogenic mechanisms and disease management in the future.

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