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Cell-to-cell signaling mediated by secreted signals and membrane-localized receptors is one of the critical mechanisms by which growth and development of multicellular organisms are cooperatively regulated. Signal molecules that specifically bind receptors are generally referred to as ligands. Because membrane-localized receptors act as master switches of complex intracellular signaling, identification of the ligand-receptor pair is one of the central issues of post-genome research. We are working to clarify the mechanisms by which plant development is regulated through identification of novel ligands such as small peptides and their specific receptors using *Arabidopsis* genome information, biochemical analysis and phenotypic observation.

I. Secreted peptide signals

Following complete sequencing of the *Arabidopsis* genome, a number of genes encoding small secreted peptides have been identified by *in silico* database analysis. Based on our own analysis, we identified 979 putative secreted peptide genes with an open reading frame (ORF) size between 50 and 150 amino acids in the *Arabidopsis* genome. These 979 ORFs include many functionally uncharacterized peptides. Although estimation of the total number of biologically relevant secreted peptide signals is difficult at present, the presence of many “orphan receptors” among receptor-like kinases in *Arabidopsis* suggest that a substantial number of intercellular signals remain to be identified.

One structurally characteristic group of peptide signals is “post-translationally modified small peptides”. These peptides are characterized by the small size of mature peptides (less than 20 amino acids) and the presence of post-translational modifications. In these peptide signals, peptide chain length and post-translational modifications are generally very important for their receptor binding activity and physiological functions.

1-1 Root meristem growth factor (RGF)

Root meristem growth factor (RGF) is a 13-amino-acid tyrosine-sulfated peptide involved in maintenance of the root stem cell niche in *Arabidopsis* identified by our group in 2010. RGF was identified in a search for sulfated peptides that recover root meristem defects of the loss-of-function mutant of tyrosylprotein sulfotransferase (*tpst-1*). TPST is a post-translational modification enzyme that catalyzes

tyrosine sulfation of secreted peptides and proteins. This approach is based on the assumption that the severe short root phenotype of the *tpst-1* mutant reflects deficiencies in the biosynthesis of all the functional tyrosine-sulfated peptides, including undiscovered peptide signals. RGFs are produced from ≈100-amino-acid precursor peptides via post-translational sulfation and proteolytic processing. RGF family peptides are expressed mainly in the stem cell area and the innermost layer of central columella cells, and diffuse into the meristematic region. RGF peptides regulate root development by stabilizing PLETHORA transcription factor proteins which are specifically expressed in root meristem and mediate patterning of the root stem cell niche (Figure 1).

To gain more insight into RGF signaling, we have developed a positive screening system to identify *Arabidopsis* mutants with altered response to RGF peptides. Several mutants that are less sensitive to RGF have been identified and are currently being further analyzed.

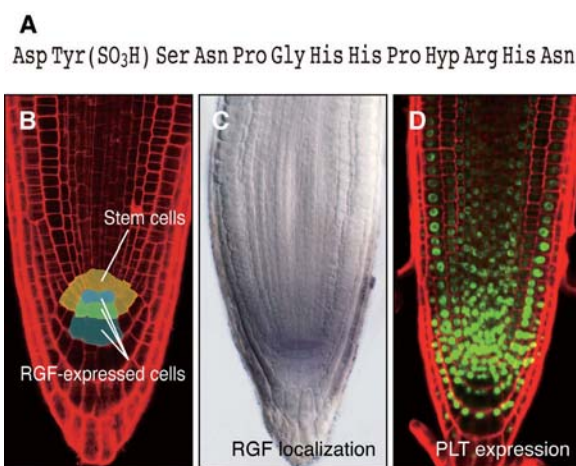


Figure 1. Mode of action of RGF peptide. (A) Structure of RGF1. (B) Expression domain of *RGF* genes. (C) Localization of RGF peptides visualized by anti-RGF1 antibody. (D) Expression pattern of PLETHORA (PLT2-GFP) transcription factor, a downstream target of RGF signaling.

1-2 Other novel peptide signal candidates

The common feature of known small post-translationally modified peptide signals is that they are encoded by multiple paralogous genes whose primary products are approximately 70- to 110-amino-acid cysteine-poor secreted polypeptides that share short conserved domains near the C-terminus. We have identified several novel polypeptide families that fulfill the above criteria by *in silico* screening and determined their mature structures by analyzing apoplastic peptide fractions by nano LC-MS/MS. Functional analysis of these peptides is now going on.

II. Post-translational modification mechanisms

Post-translational modifications are known to affect peptide conformation through steric interactions with the peptide backbone, thereby modulating the binding ability and specificity of peptides for target receptor proteins. To date, the following types of post-translational modification have

been identified in secreted peptide signals in plants: tyrosine sulfation and hydroxyproline arabinosylation (Figure 2).

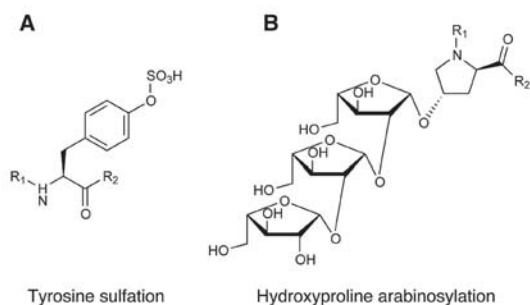


Figure 2. Post-translational modifications in secreted peptide signals in plants.

2-1 Tyrosine sulfation

Arabidopsis tyrosylprotein sulfotransferase (AtTPST) is a Golgi-localized 62-kDa transmembrane protein identified by our group in 2009. AtTPST is expressed throughout the plant body, and the highest levels of expression are observed in the root apical meristem. A loss-of-function mutant of *AtTPST* (*tpst-1*) displayed a marked dwarf phenotype accompanied by stunted roots (Figure 3), loss of root stem cells, pale green leaves and early senescence, indicating the important roles of sulfated peptides in plant growth and development. Three known sulfated peptide signals, PSK, PSY and RGF, can almost fully restore root defects of *tpst-1* when added to the culture medium, but can not fully restore phenotypes in the above-ground parts of the plant. This observation suggests that as yet undiscovered sulfated peptides may regulate plant development. A search for novel sulfated peptide signals is now in progress.

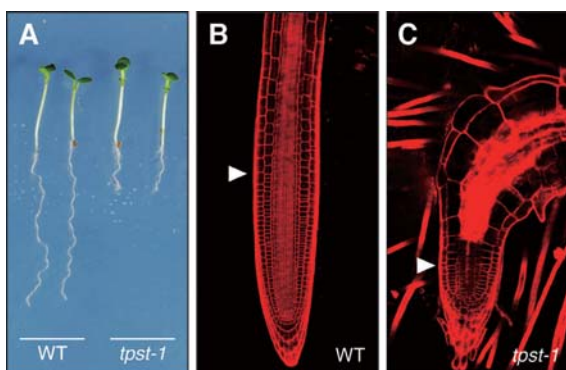


Figure 3. Phenotypes of *Arabidopsis tpst-1* mutant. White arrowheads indicate the root meristem boundary.

2-2 Hydroxyproline arabinosylation

Hyp residues in several secreted peptide signals, such as *Arabidopsis* CLV3 and *Lotus japonicus* CLE-RS2 are further modified with an *O*-linked L-arabinose chain. This modification is physiologically important for these peptide signals. CLE-RS peptides are hydroxyproline *O*-arabinosylated 13-amino-acid glycopeptides that are

responsible for autoregulation of nodulation in leguminous plants.

Biosynthesis of Hyp-bound β -1,2-linked triarabinoside involves two distinct arabinosyltransferases. The first is responsible for the formation of a β -linkage with the hydroxyproline (hydroxyproline arabinosyltransferase), and the second forms a β -1,2-linkage between arabinofuranose residues (arabinosyltransferase). Arabinosyltransferase has already been reported, but there have been no reports on hydroxyproline arabinosyltransferase (HPAT).

Arabidopsis HPAT was recently purified and identified as a Golgi-localized transmembrane protein. Loss-of-function mutations in *HPAT* genes in *Arabidopsis* cause pleiotropic phenotypes that include enhanced hypocotyl elongation, defects in cell wall thickening, early flowering, and early senescence. In addition, a double mutation in *HPAT1* and *HPAT3* significantly impairs the growth of pollen tubes, thereby causing a transmission defect through the male gametophyte (Figure 4). Detailed phenotypic analyses of loss-of-function mutants of *HPAT* genes will provide a more complete picture of how hydroxyproline *O*-arabinosylated glycoproteins and glycopeptides contribute to plant growth and development.

2-3 Chemical synthesis of arabinosylated peptides

Arabinosylation of hydroxyproline (Hyp) is a posttranslational modification often found in secreted peptide signals in plants. We have succeeded in the stereoselective total synthesis of β -1,2-linked tri-arabinosylated CLV3 peptide ([Ara₃]CLV3). Comparison of mono-, di- and tri-arabinosylated CLV3 glycopeptides revealed that the biological activity increased progressively as arabinose chain length increased. Thus, arabinose chain length of CLV3 is important for its biological activity.

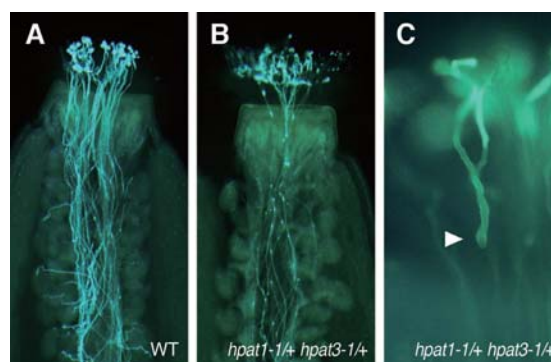


Figure 4. A double mutation in *HPAT1* and *HPAT3* significantly impairs the growth of pollen tubes. Arrowhead indicates aberrant pollen tube.

2-4 Conformation of arabinosylated peptides

NMR spectroscopy and NOE-based structure calculations revealed the structural impact of the arabinose chain on peptide conformation. The arabinose chain of [Ara₃]CLV3 extends toward the C-terminal end of the peptide, and its non-reducing end is positioned proximal to the peptide backbone. Consequently, the arabinose chain causes distinct distortion in the C-terminal half of the peptide in a highly

directional manner. The established synthetic route of [Ara₃]CLV3 will greatly contribute to our understanding of the biology and biochemistry of arabinosylated peptide signals in plants.

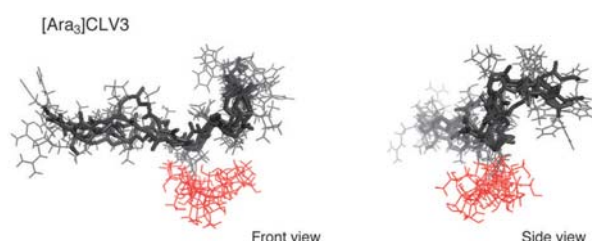


Figure 5. Energy-minimized structure of [Ara₃]CLV3 resulting from a simulated annealing protocol that incorporated NOE-derived distance restraints.

III. Receptors for secreted peptide signals

The receptors or putative receptors for peptide signals identified to date belong to the receptor kinase (RK) or receptor-like protein (RLP) families. Among RKs, the largest subfamily is the leucine-rich repeat RK (LRR-RK) family, which consists of 216 members in *Arabidopsis*. The majority of receptors for small post-translationally modified peptide signals belong to this family. Especially, an increasing number of LRR X and LRR XI members are now being confirmed as receptors for several endogenous small peptide ligands, suggesting that these subgroups are an attractive target for binding analysis with novel peptide signals.

3-1 Receptor expression library

Although both genetic and biochemical methods have been used to identify ligand-receptor pairs in plants, genetic redundancy often interferes with the former approach, and the low levels at which ligand and receptor molecules are often present in tissues can make the latter approach very difficult. As described in the above section, an increasing number of ligand candidates are being identified. If individual receptor kinases could be functionally overexpressed in certain cells at sufficiently high levels and sufficiently high quality for biochemical binding analysis, such a receptor library would facilitate identification of ligand-receptor pairs in plants. To this end, we established a functional and efficient expression system of plant receptor kinases in tobacco BY-2 cells and prepared an expression library of all the potential receptor candidates.

3-2 Structural basis for ligand recognition

Leucine-rich repeat receptor kinases (LRR-RKs) comprise the largest subfamily of the transmembrane receptor kinases in plants. In several LRR-RKs, a loop-out region called an “island domain” that intercepts the extracellular tandem LRRs at a position near the transmembrane domain constitutes the ligand-binding pocket, but the absence of the island domain in numerous LRR-RKs raises questions about which domain specifically recognizes the corresponding ligands in non-island domain-carrying LRR-RKs. We

determined, by photoaffinity labeling followed by chemical and enzymatic digestion, that BAM1, a CLV1/BAM family LRR-RK whose extracellular domain is comprised of 22 consecutive LRRs, directly interacts with the small peptide ligand CLE9 at the LRR6-8 region that is relatively distal from the transmembrane domain (Figure 5). Multiple sequence alignment and homology modeling revealed that the inner concave side of LRR6-8 of the CLV1/BAM family LRR-RKs is slightly deviator from the LRR consensus. Our results indicate that ligand recognition mechanisms of plant LRR-RKs are more complex and diversified than anticipated.

Publication List

[Original papers]

- Endo, S., Shinohara, H., Matsubayashi, Y., and Fukuda, H. (2013). A novel pollen-pistil interaction conferring high-temperature tolerance during reproduction via CLE45 signaling. *Curr. Biol.* 23, 1670-1676.
- Ogawa-Ohnishi, M., Matsushita, W., and Matsubayashi, Y. (2013). Identification of three hydroxyproline O-arabinosyltransferases in *Arabidopsis thaliana*. *Nature Chem. Biol.* 9, 726-730.
- Okamoto, S., Shinohara, H., Mori, T., Matsubayashi, Y.,* and Kawaguchi, M.* (co-corresponding authors) (2013). Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. *Nature Commun.* 4, 2191.
- Shinohara, H., and Matsubayashi, Y. (2013). Chemical synthesis of *Arabidopsis* CLV3 glycopeptide reveals the impact of hydroxyproline arabinosylation on peptide conformation and activity. *Plant Cell Physiol.* 54, 369-374.