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TEACHING TOOLS IN PLANT BIOLOGY™: LECTURE NOTES

Small and Mighty: Peptide Hormones in Plant Biology

INTRODUCTION

A phytohormone (plant hormone) is defined as a naturally occurring plant compound that acts as a signal molecule even at low concentrations. Nine hormone classes are currently recognized in plants: auxins, cytokinins, ethylene, gibberellic acid, abscisic acid, brassinosteroids, salicylic acid, jasmonic acid, and strigolactones (for more information, see the *Teaching Tools in Plant Biology* articles on each of these hormones). Interestingly, 5- to 60- amino acid long peptides also display many characteristics of hormones. In plants, peptide hormones have been found to regulate gene expression and cause changes in a variety of parameters and processes, including cell size and number, fertilization, plant responses to nutrient availability, and defense against pathogens, even at femtomolar (10^{-15} M) concentrations!

Discovery in Animals and Plants

Establishing peptides as hormones involved the work of several Nobel laureates. The idea that peptides can act as signals emerged in the 1900s during the search for a treatment for diabetes. For the discovery and purification of insulin or "isletin," as it was then called because it was secreted from the islets of Langerhans, Banting and Best shared the 1921 Nobel Prize with their colleagues James Collip and John Macleod (www.nobelprize.org). The proteinaceous nature of insulin was determined five years later by John J. Abel. Frederick Sanger received the Nobel Prize in 1951 for determining that insulin comprises two chains, 21 and 30 amino acids long, that are linked through disulfide bridges. It wasn't until the 1980s, however, that the gene sequence of insulin was elucidated, and it was found that both chains were encoded by a single gene. These findings laid the groundwork for defining the criteria used to identify peptide hormones; these compounds are (1) small (<60 amino acids long), (2) secreted, and (3) affect physiological processes.

The first plant peptide was identified in the early 1990s. A group of scientists led by Clarence A. Ryan at the University of Washington found that when adult Colorado beetles fed on leaves of tomato (*Solanum lycopersicum*) plants, proteinase inhibitors rapidly accumulated throughout the plant, making it less palatable for future insect attacks. Gregory Pearce and his team further used this response as a bioassay to test for the causative factor on as many as 30,000 tomato seedlings and then used high-performance liquid chromatography to purify the fraction showing the highest activity. The purified peptide was 18 amino acids long and was named "systemin." It is encoded by a small (~600 bp) gene and processed enzymatically from a ~200 amino acid precursor protein called "prosystemin."

Comparison to Classical Plant Hormones

In many ways, peptide hormones resemble plant hormones, but in one fundamental way they differ. Similar to classical plant hormones, e.g., auxins and cytokinins, the site of synthesis and the site of action of peptide hormones are not necessarily the same. Some peptide hormones travel long distances, for example, from the roots to the shoot where receptor proteins recognize them and initiate appropriate signal cascades to bring about changes in the plant. As an example, in legumes, some CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (CLE) peptides synthesized in nodulated roots are transported to shoots where they trigger feedback regulation, to notify the plant that enough nodules have been produced. Others may only move short distances. For example, another CLE peptide, CLE40, moves locally via the apoplast to neighboring cells where it is perceived by the corresponding receptor. Additionally, members of both groups have no nutritional value. Unlike the classical plant hormones, however, not all peptide hormones are present ubiquitously across all plant species. Peptide families such as the pollen tube attractants LUREs have been identified in *Torenia fournieri* and *Arabidopsis thaliana* but are absent from the model legume *Medicago truncatula*.

Posttranslational modifications (PTMs) can alter peptide activity similar to some classical hormones such as jasmonic acid, on which the addition of a methyl group generates bioactive methyl jasmonate. However, peptides differ from classical hormones in how they are synthesized and in their structures. The classical hormones are products of metabolic pathways, while peptide hormones are encoded directly within genes and processed from polypeptide chains.

It is important to note that not all small plant peptides are peptide hormones involved in signaling. Many small peptides have antimicrobial roles, i.e., they are required to counter attacks by herbivores or bacterial and fungal pathogens. Antimicrobial peptides belong to diverse families like cyclotides, defensins, or NODULE CYSTEINE RICH (NCR) peptides. Some of these peptides disrupt bacterial membranes, while others block translation of bacterial proteins, ultimately killing the organism. Plant peptides can also function as peptidase inhibitors by competitive binding to the active sites of peptidases.

The focus of this teaching tool is peptide hormones with signaling roles. Some examples of peptides with other roles are also described and the accompanying slides are marked with red boxes. A summary of peptide gene families is provided as an appendix in the accompanying slide handouts.

STRUCTURE AND GENERATION OF PEPTIDE HORMONES

Classification and Nomenclature

Peptide hormones are usually derived from larger polypeptides. Typically, the first 16 to 30 amino acids at the amino (or N)

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terminus of the polypeptide constitute the signal sequence that marks a protein for transport to the endoplasmic reticulum/Golgi-dependent secretory pathway. This N-terminal secretion signal is followed by a "variable" stretch of amino acids that differs in sequence and length even between members of the same peptide family. The functional "signaling" peptide is usually closer to the C-terminal end of the polypeptide. Signaling peptides range from 5 to ~30 amino acids in length while "cysteine-rich peptides" can extend up to 60 amino acids. Peptides can then be classified by answering four simple questions. (1) Is the peptide ribosomally synthesized? (2) Does the parent polypeptide have a separate function? (3) Does the peptide have any secondary group modifications? (4) How many cysteines are present in the primary amino acid sequence of the peptide?

The parent polypeptide from which a mature peptide hormone is derived is called a pre-pro-protein. The protein sequence resulting from removal of the N-terminal signal peptide is called a propeptide, which is still inactive. Within the Golgi network or outside the cell, select enzymes called "peptidases" cleave the propeptide at predetermined sites to release the bioactive peptide hormone. In the Golgi, additional modifications can be introduced on the peptide. The processed, secreted peptide mediates its physiological effects non-cell autonomously, i.e., between adjacent cells or within several cell layers.

Peptide Processing

Peptide processing is crucial for converting an inactive peptide to its biologically active version; thus, algorithms have been developed to determine whether a peptide is likely to be cleaved. Online software tools such as "SignalP" can use sequence characteristics to predict whether a peptide will be secreted (N-terminal signal peptide) or whether it encodes a signaling peptide (C-terminal) and can even pinpoint the cleavage site where processing will occur. Experimental evidence has identified a four-amino acid sequence that is recognized by Site-1 (S1P) peptidase enzymes both in humans and plants. It has been shown that an RxLx/RxxL motif (where x indicates any amino acid residue) is just upstream of the cleavage site of some members of the RAPID ALKALINIZATION FACTOR (RALF) family of peptides. The sequences immediately upstream or downstream are recognized by peptidases. Once the peptidase has recognized the cleavage site, the mature peptide is released by hydrolysis of the "scissile" amide (or peptide) bond, which is broken. Peptidases that cleave within the protein are called "endopeptidases," whereas those that cleave only the terminal amino acid are "exopeptidases." Exopeptidases can further be classified as aminopeptidases or carboxypeptidases depending on which end of the protein they cleave. Additionally, the amino acid serving as an electron donor during bond formation or the nucleophile can also be used to classify peptidases, e.g., serine, cysteine, and aspartate peptidases. Alternatively, metallopeptidases can employ metal ions as the nucleophile (www.ebi.ac.uk/merops/). Genetics, chemical genomics, and biochemical studies have been used to demonstrate the importance of peptidases for processing peptides.

What happens if signaling peptides are not correctly cleaved? Scientists tried to answer this question by comparing wild-type Arabidopsis plants with mutants in the *site-1* gene encoding S1P, a subtilisin serine peptidase. When S1P is functional, RALF peptides are correctly processed. When *RALF23* is overexpressed in wild-type plants, the plants are smaller with abnormal root and hypocotyl growth. However, these phenotypes are absent in the *site-1* mutant overexpressing *RALF23*, indicating that the S1P peptidase is required for production of functional RALF23 peptide.

PTMs AND THEIR PRESUMED ROLES

Enzymatic and Nonenzymatic PTMs

Addition of secondary groups can modify the biological activity of a peptide dramatically. These modifications may or may not require enzymes. Nonenzymatic PTMs include the formation of disulfide bridges. Such bridges are often important for a protein's three-dimensional structure and stability. PTMs requiring enzymatic activities include hydroxylation of prolines, addition of arabinosyl groups on hydroxyprolines, and sulfation of tyrosines. Loss of such PTMs can lead to developmental abnormalities in plants, some of which can be recovered by external application of the synthetic peptide carrying the correct PTM. For example, in some plants, if the enzyme that catalyzes the *o*-arabinosylation of certain hydroxyprolines is mutated, the shoot meristem size increases massively compared with the wild type. In tomato, the mutant lacking this function is called *fin* for *fasciated inflorescence*. All floral organs in the *fin* mutant, such as the petals and sepals, are more abundant due to the engorged meristem. In fact, the number of locules (chambers) in the tomato fruit is 6 times higher! These phenotypic abnormalities, especially the increased meristem size, can be recovered by external addition of synthetic arabinosylated CLE peptides.

Importance of PTMs

How do PTMs bring about changes in growth and development? One possibility is that the addition of such chemical "decorations" strengthens or weakens the interaction between the peptide hormone and other proteins such as their cognate receptors. Using a luminescence assay, scientists determined that if the conserved proline on a peptide was hydroxylated, the peptide concentration required for the response to occur was much lower compared with the unmodified peptide, implying a stronger interaction of the hydroxylated peptide with its receptor.

Data so far indicate that PTMs regulate affinity for the receptor by affecting the structural conformation of the peptide and/or by directly interacting with the receptor. For example, in the peptide mutant *clv3*, the meristem size increases eleven times compared with the wild type. However, application of synthetic CLV3 peptide containing one, two, or three arabinose side chains on a hydroxyproline can restore the meristem size to varying degrees. Molecular modeling shows that the bulky arabinose side chains important for its function cause a kink in the peptide backbone, facilitating interaction with the receptor. PTMs like sulfation or hydroxyprolination can also add critical charges or hydrogen bonding opportunities for better interaction of the peptide with the

receptor binding pocket. PTMs might even simply play a protective role; a high number of cysteines makes the peptide bonds unavailable for cleavage by degradative proteases. Disulfide bridges ensure selective degradation of the variable region of the pre-pro-peptide, leaving the active peptide available for binding with its receptor.

It is important to try to understand peptide processing mechanisms because a processing enzyme is a tool that allows us to modulate the degree of activity that peptide hormones have within a plant, which allows their functions to be assessed.

PREDICTION AND IDENTIFICATION OF PEPTIDE HORMONES

An important thrust in peptide research is the identification of the full complement of peptide hormones in a plant. Hundreds of potential peptide hormones may be encoded within each plant genome, but most remain hypothetical since their function and mature biological structure are unknown. The prediction and identification of peptide hormones is fraught with several challenges. First, current genome annotation pipelines are biased against small open reading frames, those most likely to encode peptide hormones because the likelihood of spurious identifications increases exponentially with decreasing length. Second, conservation within a single peptide family is typically limited to only a few important residues reducing the effectiveness of homology search strategies. Third, the rules governing peptidase processing and incorporation of PTMs are poorly defined. To tackle the challenges in identifying peptide hormones, four complementary approaches have been employed.

Bioinformatics: Bioinformatics predicts peptide-coding genes by searching for characteristic sequence patterns. Some patterns are applicable to peptides in general including short coding sequences, the presence of N-terminal signal peptides for secretion, and lack of any transmembrane helices. Other patterns are specific to a given peptide family, such as short characteristic motifs or spacing of conserved cysteine residues. Predicting novel peptide hormones by bioinformatics employs homology searches and Hidden Markov models. Homology searches, such as BLAST, search a gene database for sequences similar to a known sequence of interest. A Hidden Markov model measures the likelihood of each residue/nucleotide at each position along a sequence and is constructed from a sequence alignment of-multiple related sequences. This likelihood matrix is then used to identify similar sequences in a database. Bioinformatics struggles to predict new peptide families, despite being well suited to predict novel peptides of known families.

Transcriptomics: Random short open reading frames often arise by chance and many are not transcribed. RNA sequencing identifies and quantifies transcripts of all expressed genes and can provide experimental support for many peptide-coding genes. Having identified a putative peptide-coding gene by bioinformatics, it is important to use transcriptomics to confirm that the gene is indeed expressed.

Genetics: Reverse genetics is an alternative strategy to validate predicted peptide-coding genes. It begins with a gene of interest and then investigates alleles harboring mutant lesions in that gene. Phenotypes arising from these lesions provide support that the gene of interest is functional. Biochemical genetics, using synthetic peptide libraries built from predicted sequences, is another effective way of identifying functions of peptide hormones. These peptides can be screened for effects on developmental, molecular, and cellular processes. Forward genetic screens involve searching through a library of mutants to identify those giving a phenotype of interest; the causative mutation is then sought. However, the length of most peptide-coding genes and potential functional redundancy among members of a family can limit the ability of both forward and reverse genetics approaches.

Biochemical techniques: Biochemical methods can separate and detect peptides from a complex milieu of cellular macromolecules. Biochemical approaches can be divided into two main strategies. The first, peptidomics, is the large-scale identification and quantification of a complex mixture of peptides by mass spectrometry. Peptidomics can offer parallel identification of multiple peptides, but is challenging due to the low abundance of endogenous peptides and their widely varying physico-chemical characteristics. The second strategy, bioassay screening, uses a specific readout to monitor biological activity such as a calcium spike in cells or enhanced root growth. This involves iterative fractionation steps, with the gradual isolation of the compound(s) responsible for the biological activity, which can subsequently be identified by mass spectrometry. This technique was used to discover the first plant peptide hormone, systemin, and connects an identified, processed peptide structure to a particular biological activity.

Each of these approaches has its pros and cons, but their power is enhanced when they are used together. For example, the success of bioinformatics depends on transcriptomics for accurate genome annotation to provide a complete gene space to search. In turn, reverse genetics requires putative peptide-coding genes to test, which come primarily from bioinformatics searches and to a lesser degree, biochemistry.

MOBILITY AND PERCEPTION OF PEPTIDE HORMONES

Upon secretion from the source cell, peptides enter the apoplastic fluid. Peptides operating non-cell autonomously presumably move from source to target cell by passive diffusion through the apoplast. The resulting concentration gradient emanating from the source tissue can explain positional cues of specific cell types and their ability to dictate cell differentiation of nearby cells. Peptides can also be transported through the xylem or phloem streams to reach their targets. It remains unclear if peptides produced outside of the vasculature can be loaded into xylem or phloem or if all vasculature-mobile peptides must be produced within the vasculature itself. There is a need to develop effective methods to track peptide movement in situ. Currently, indirect approaches exist to study peptide mobility, such as (1) extraction of apoplastic fluid from intact tissue by centrifugation or wicking filter strips, (2) collection of xylem and phloem sap, or (3) heterografting. Collected sample materials from methods 1 and 2 typically are analyzed by mass spectrometry to identify peptides.

Signal Perception

Once a peptide reaches its target cell, it will be perceived through a plasma membrane-localized receptor. A typical receptor kinase

comprises (1) an extracellular ligand binding region (i.e., ectodomain), (2) a transmembrane helix, and (3) an internal kinase domain that transduces the signal. The ectodomain and the internal kinase domain can be encoded by separate proteins requiring cooperation in a complex. Relatively few peptide-receptor pairs are currently known; however, crystal structures of three peptides in Arabidopsis, ROOT GROWTH FACTOR/GOLVEN (AtRGF/AtGLV), INFLORESCENCE DEHISCENT IN ABSCISSION (AtIDA), and TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (AtTDIF), in complex with their receptors and coreceptors have already been solved, and some structural patterns have begun to emerge.

First, peptide receptors identified thus far are almost exclusively members of the leucine-rich repeat class of receptor-like kinases (LRR-RLKs). The LRR-RLKs have an ectodomain comprised of a various number of repeating leucine-rich motifs, forming a concave, extended surface capable of binding peptides. For example, IDA, CLE, and RGF peptides bind along similar clefts on the inner surface of the LRR domain. Second, receptors tend to function in a heterodimer with a coreceptor that helps hold the ligand in place along the LRR inner surface. Coreceptors generally belong to the somatic embryogenesis receptor-like kinase (SERK) family, which consists of five members in Arabidopsis.

Since genes encoding peptide ligands, receptors, and coreceptors exist as part of multimember families, various combinations of interactions are possible. Some evidence suggests that specific receptor/coreceptor combinations target distinct peptide ligands. For example, the HAESA receptor preferentially binds the IDA ligand, while the related HAESA-LIKE2 receptor preferentially binds other IDA-LIKE ligands. This pattern of peptide ligand perception by receptor/coreceptor pairs extends to perception of other ligands including classical hormones. Indeed, the IDA peptide shares its SERK coreceptor with the classical hormone brassinolide. Depending on the coreceptor's ligand and the interacting partner, a different developmental outcome is achieved.

Signal Transduction

Receptors convey signals into the cell to alter cellular traits. Peptides can serve as a molecular glue that holds together a receptor and coreceptor pair. In the case of the AtIDA peptide, both receptor and coreceptor have kinase activity and trans-phosphorylate each other to initiate a downstream MITOGEN-ACTIVATED PROTEIN kinase signaling cascade. This signaling cascade leads to release of a transcriptional repressor, permitting expression of cell wall-modifying enzymes and organ abscission. Interestingly, one member of the CLE family, the TDIF peptide from Arabidopsis, has different effects depending on its cellular context. The TDIF peptide is perceived by its receptor at the surface of either the procambial cells or pericycle cells of vascular tissue. In both cell types, the TDIF-TDR (TDIF RECEPTOR) module signals through the same kinase; however, the targets of this kinase are cell-type dependent, leading to different outcomes. Perception of RALF represents an exception among the peptide hormone receptor/coreceptor pairs identified thus far, in that the identified coreceptors lack an intracellular kinase domain. Instead, the RALF receptor FERONIA additionally interacts with a receptor-like cytoplasmic kinase, and these kinases can trans-phosphorylate each other. Downstream signaling is mediated by a family of enzymes that hydrolyze guanosine triphosphate (GTPases), leading to inhibition of a proton pump and promotion of the respiratory burst oxidase. Acidification of the cell wall is essential for cell wall loosening, while reactive oxygen species (ROS) bursts both promote and inhibit cellular growth. In pollen tubes, both of these effects underlie the ability of RALF to inhibit cell elongation and regulate pollen tube rupture.

Signal Attenuation

Attenuating or "switching off" signaling is essential for a cell to maintain proper sensitivity to external signals. This ensures the cell does not "overreact" to a weak signal or "underreact" to a strong signal. Three different strategies have been documented in plants that attenuate peptide hormone signaling. The first, endocytosis, directs receptor-ligand complexes to the vacuole for degradation. In this way, the cell ensures a continuous supply of "fresh" receptor primed for peptide binding, as well as removal of "spent" peptide, so that the same small pool of peptide does not continue to initiate signaling. The second involves competition between peptides for receptor binding. Peptide antagonists, incapable of inducing downstream signaling, bind to receptors and block the binding of bioactive peptides. As an example, EPIDERMAL PATTERNING FACTOR1 (EPF1) and EPF2 compete with EPIDERMAL PATTERNING FACTOR LIKE9 (EPFL9/STOMAGEN) for binding to the receptor/coreceptor pair, ERECTA-TOO MANY MOUTHS. In the presence of EPFL9/STOMAGEN, neither EPF1 nor EPF2 is capable of initiating downstream signaling processes. In the third strategy, inactive receptors have been identified that function by binding and sequestering peptide away from their bioactive receptor target. In this way, the inactive receptor acts as a decoy to temper the signaling response elicited by the peptide. The ARABIDOPSIS CRINKLY4 receptor has been shown to sequester the CLE40 peptide in such a way.

PHYSIOLOGICAL ROLES OF PEPTIDE HORMONES

Growth and Development

The CLE family of peptide hormones is arguably the most thoroughly investigated of all peptide hormones. The founding member, CLV3, has been shown to negatively regulate stem cell identity of the shoot apical meristem, in a negative feedback loop with adjacent cells. Loss of the CLV3 peptide hormone results in overproliferation of stem cells. The common role among the CLE peptides has been regulation of cell differentiation. Additionally, in legume species CLE peptides have been found to inhibit nodulation although the mechanism by which they do so is not yet clear.

Studies of the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) family of peptides have indicated a common role in regulating cell separation events in various plant tissues. The family was originally identified from a loss of function mutant in Arabidopsis that failed to properly shed sepals and petals after flower pollination. IDA peptides are also important for lateral roots to properly emerge from parent root tissue. Lateral roots initiate from the pericycle layer, near the vasculature of the root, and thus must push their way through the endodermal, cortical, and epidermal cells to emerge from the root.

Both processes, floral organ abscission and lateral root emergence, share a requirement for remodeling and degradation of plant cell walls. Consistent with an involvement of IDAs in cell wall dissolution, mutants lacking IDA or its receptors are affected in expression of cell wall degrading enzymes.

Reproductive Development

An astounding number of peptide hormones have been implicated in orchestrating floral development as well as pollination and fertilization. Incidentally, most of these peptide hormones are from the cysteine-rich class of peptides.

Anther Development and Pollination

The TAPETUM DETERMINANT (TPD) family is responsible for proper cell division and differentiation during pollen development within anther tissue. In Arabidopsis, the absence of the TPD peptide or its putative receptor results in an increased number of microspores. Pollination begins with the arrival of a pollen grain on the stigma of the female parent. Many plant species display self-incompatibility, which means that pollen from the same parent is rejected, forcing outcrossing of the parents and genetic mixing. SCR (S-locus CYSTEINE RICH) peptides, found in certain members of the Brassicaceae family, are central to the identification of self-pollen at the stigma.

Pollen Germination

The pollen grain germinates to produce a pollen tube. Germination has been found to be regulated by several families of peptide hormones, including PHYTOSULFOKINE (PSK), STIGMA1/GRIM REAPER (STIG1/GRI), and LATE ANTHER TOMATO52/POLLEN OLE E 1 ALLERGEN AND EXTENSIN (LAT52/POE). Proper pollen germination requires a sufficient concentration of pollen grains before any will germinate, called the pollen population effect. PSK peptide hormones are the signal responsible for determining sufficient amounts of pollen, through their secretion from pollen grains. To detect the concentration of pollen grains, each grain expresses the PSK receptor to monitor the concentration of PSK peptide in the surrounding milieu. Only at a sufficient density of pollen grains, ample PSK peptide is present to bind pollen surface receptors and initiate signaling and germination.

Peptide hormone regulators of pollen tube growth include RALF, STIG, PSK, and nonspecific LIPID TRANSFER PROTEIN (ns-LTP) family members. PSK also promotes growth of the tube as it travels to the egg. A RALF peptide from tomato has been shown to inhibit growth of the pollen tube, similar to its inhibitory effect on growth seen in other plant tissues. In contrast to the two above peptides, which are expressed in the pollen itself, the STIG family of peptides is expressed in the stigma, from where they too promote pollen tube growth.

Chemotropism

Pollen tubes grow by chemotropism, meaning that their growth is directed by an external signal called a chemoattractant. In the absence of such a chemoattractant to guide the pollen tube to the egg, the pollen tube grows aimlessly. Peptide hormone chemoattractants, which are secreted from synergid cells, producing a concentration gradient emanating from the female tissue, have been identified in several species. Pollen tubes perceive these peptides and grow in the direction of the concentration gradient, leading them straight to the egg cell. The peptide chemoattractants identified thus far are species specific. Some dicot species employ LURE peptides, for example the AtLURE1 peptide of Arabidopsis, which together with its receptors MALE DISCOVERER (MDIS1 and MDIS2) and MDIS- Interacting RLK1 (MIK1/MIK2) is sufficient for pollen tube attraction. By contrast, maize (*Zea mays*) and many other grass species utilize a peptide from the EGG APPARATUS-LIKE (EAL) family, which functions in the same manner as LURE. However, the EAL peptides have additional functions in the regulation of cell differentiation and development in various reproductive tissues.

Fertilization

The EMBRYO SAC (ES) peptide hormone encoded within maize and sorghum regulates the pollen tube burst necessary to release sperm cells. It is expressed and secreted from the female tissue and is perceived by receptors on the surface of the pollen tube tip. Binding of the peptide induces rapid influx of K^+ into the pollen tube and subsequent swelling. By in vitro application of peptide to pollen tubes, bursting can be observed within just a few seconds. Fertilization is achieved upon fusion of the two gametes, sperm and egg. Members of the Early Culture Abundant1 (ECA1) family of peptides trigger fusion of sperm and egg and also are found to regulate other cell functions within the female gametophyte. The founding member of the family, Egg Cell1 (AtEC1) from Arabidopsis, is produced and secreted from the egg cell. Perception of the peptide by the sperm cell induces the sperm to express HAP2 membrane remodeling protein at the cell surface. HAP2 induces membrane fusion between sperm and egg, allowing cell fusion and exchange of gametes.

Plant Abiotic Stress Tolerance

Abscisic acid and ethylene are classical phytohormones that are often classified as “stress” hormones as they are induced when plants face abiotic challenges. Similarly, some peptide hormones are also specifically induced when nutrients, such as nitrogen (N), phosphorus (P), sulfur (S), or iron (Fe), are in short or excess supply, when temperatures are unfavorable (heat/cold stress) or water is limiting (drought or high salinity). For example, CASPARIAN STRIP INTEGRATING FACTOR peptides are induced by excess Fe and together with their receptors GASSHO1/SCHENGEN3 control the permeability of the root vascular stele to water and Fe.

Hundreds of peptide-coding genes transcriptionally respond to abiotic stresses; however, for the sake of simplicity, we take the example of N to illustrate peptide-mediated mechanisms to overcome or tolerate stress. N often is the major nutrient limiting plant growth, as it is required in considerable amounts for the synthesis of essential building blocks such as amino acids, DNA, and RNA. When faced with low N supply, plants are known to employ the following strategies.

Changes in Root System Architecture

Plant root systems not only provide anchorage, but they also help scavenge nutrients and water that cannot be procured by aboveground plant tissues. Depending on the availability of and need for a nutrient, plants are able to adjust their root architecture by modifying various root traits. The CLE-CLV1 signaling module is a checkpoint for optimizing lateral root initiation and emergence in response to N availability. Under prolonged N-deficiency, if a plant produces too many new organs, it will expend all its energy. The *clv1* receptor mutant, even under severe low-N stress conditions, continues to wastefully produce lateral roots. This appears to be a result of overaccumulation of CLE3 and CLE2 peptides that can no longer be sensed by *clv1*. CLE peptides thus help provide information about the overall nutrient status of the plant and help economize N usage. Similarly, overexpression of some CEP peptides induced by low N in roots of *M. truncatula* drastically reduces the number of lateral roots. This indicates that the control of RSA in response to nutrient availability is a complex, finely tuned process mediated by diverse peptide hormones.

Localized Modifications of Gene Expression

In natural environments, nutrients are not homogeneously distributed. It is therefore possible that one section of the root system has no access to N, while another section is enmeshed in an N-rich soil patch. In such a situation, plants employ C-TERMINALLY ENCODED PEPTIDE (CEP) hormones produced in N-starved roots to signal to distant CEPR receptors in the shoot. This stimulates two mobile secondary messengers, CEP DOWNSTREAM1 (CEPD1) and CEPD2, to travel via the phloem to roots in the N-rich region and induce localized expression of nitrate importing transporters. High-affinity nitrate transporters such as NITRATE TRANSPORTER 2.1 in Arabidopsis then mediate N uptake to satisfy whole plant N needs. In legumes, overexpression of CEP1 peptides also inhibits lateral root emergence and enhance nodule development, indicating that CEP1 participates in all three processes of N-stress tolerance.

Interactions with Beneficial Soil Microbes

Under low N conditions, legumes as well as some nonlegumes recruit soil bacteria that convert atmospheric dinitrogen (N_2) to plant-usable ammonia in a process called root nodule symbiosis (RNS). Legumes such as soybean (*Glycine max*) induce the expression of RHIZOBIA-INDUCED CLE peptides (RICs) to control the number of initiated nodules. Conversely, if the plant has enough N in its environment in the form of nitrate, it induces NITRATE-INDUCED CLEs that suppress nodule formation. RICs and their orthologs will be described in more detail below, but it is important to differentiate between these two classes of peptides even though both affect RNS.

Plant-Biotic Interactions

In their natural environment, plants coexist and interact with a plethora of life forms, such as insect herbivores, nematodes, fungi, and bacteria. These interactions largely determine the fitness, health, and survival of a plant. Similar to humans and animals, almost every plant organ has a defined, associated microbial community, called a "microbiome." Microbiomes are often selected by mutual communication between partners and regulated by plant secretions and exudates. Peptide hormones fine-tune these relations between the host plant and biological organism, which can either be beneficial (symbiosis, mutualism) or detrimental (pathogenesis, parasitism) (for more information, see *Teaching Tools in Plant Biology* articles on these topics).

Symbiosis

Legume roots interact with soil bacteria called rhizobia to fix atmospheric N into plant-usable ammonia, in exchange for which host plants provide carbon and develop specialized organs called root nodules that accommodate these beneficial microbes. The number of root nodules that develop per root system is controlled by peptide hormones. Application of synthetic CEP or PSK peptides can enhance nodule numbers, indicating they are positive regulators of nodule organogenesis. However, continuing to produce nodules even if N needs have been sufficiently met will waste plant resources and energy. To prevent this, legumes have a regulatory mechanism in place that inhibits formation of new nodules once an optimal N level has been reached. This phenomenon of autoregulation of nodule number is mediated by root-derived CLE peptides and their shoot-expressed cognate receptors. The MtCLE12/MtCLE13 peptides of *Medicago truncatula* are also known as LjCLE-RS1/LjCLE-RS2 in *Lotus japonicus* and RIC1 and RIC2 in soybean. Receptor kinase mutants that lose the ability to perceive these peptides overproduce nodules and are named *supernumeric nodule*, *hypernodulation aberrant root formation* (*har1*), or *nodule autoregulation receptor kinase*. These tri-arabinosylated CLE peptides can be detected in the shoot xylem vessels and at-least MtCLE12 requires the enzyme hydroxyproline-*O*-arabinosyltransferase (ROOT-DETERMINED NODULATION1) for full activity and binding to the receptor. If pretreated with MtCLE13 or MtCLE12, nodulation is significantly suppressed, indicating that these CLE peptides are negative regulators of nodule formation.

In the legume *M. truncatula*, the absence of a signal peptidase complex inhibits proper bacterial differentiation and therefore impairs N_2 fixation. This is thought to be a result of incomplete maturation of NCR peptides, which are required for "terminal differentiation" of rhizobia before they start fixing N_2 in host nodules (see Appendix in accompanying slide set). Terminal differentiation involves multiplication of the bacterial nuclear genome without cell division, leading to formation of large, non-motile rhizobia (referred to in this terminally differentiated state as "bacteroids"). NCR peptides, however, can act as antimicrobial peptides and are therefore not peptide hormones per se. With over 700 members in *M. truncatula* alone, sublethal concentrations of cysteine-rich NCR peptides have been shown to play a clear role in inducing terminal differentiation of compatible bacteria and also in selecting efficient rhizobial partners.

Another specialized, beneficial interaction between land plants and mycorrhizal fungi helps to improve plant phosphorus (P) uptake. Although no peptide has been clearly implicated in these interactions, a mycorrhizal induced subtilisin-like serine protease

in *L. japonicus* (*LjSbtM1*) is exclusively expressed in root cells containing the nutrient exchange interface called the "arbuscule." This indicates that the peptide processing machinery plays important roles in mycorrhizal interactions.

Host-Pathogen Interactions

Unlike their efforts to accommodate beneficial microbes, plants resist attacks by bacterial, fungal, or oomycete pathogens that cause massive losses to agriculture. To mount an effective defense response, plants need to activate their immune system and initiate rapid signaling cascades upon perception of harmful microbes. The PAMP INDUCED SECRETED PEPTIDE (PIP) family members are induced within an hour of inoculation with the bacterial pathogen *Pseudomonas syringae*. PIPs respond to conserved bacterial elements called PAMPs (pathogen-associated molecular patterns), such as the bacterial flagella required for motility, and to chitin in fungal cell walls. Upon recognition by their cognate receptor RLK7 in Arabidopsis, PIPs activate downstream defense responses, such as increased callose deposition, reduced stomatal aperture, heightened ROS levels, and activation of hormone signaling pathways controlled by salicylic acid, jasmonic acid, or ethylene. This increases their survival rates when challenged by either bacterial or fungal pathogens. Another peptide-receptor pair, PLANT ELICITOR PEPTIDES (PEPs) and PEP-RECEPTOR (PEPR1 and PEPR2), is activated when infection or stress triggers the release of endogenous damage-associated molecular patterns such as cell wall components. Perception of these peptides not only activates localized defenses, such as ROS and calcium bursts, but also activates systemic immune responses.

Microbes with their short life cycles can rapidly evolve proteins that subvert host defense responses. Since peptide hormones are encoded by relatively small genes, fungi, or bacteria can rapidly evolve genes that encode plant peptide mimics. This phenomenon of "molecular mimicry" is exemplified by the RALF family of peptides. RALF peptides, so called because of the rapid alkalinization effect they have in the apoplast, promote root growth. Incidentally, this sudden increase in the apoplast pH is also an early symptom of infection by the fungus *Fusarium oxysporum*. The fungal secreted RALF peptide mimic called F-RALF is recognized by the plant host receptor FERONIA. This leads to alkalinization that is essential for activation of a key kinase required for the infection to spread. In this manner, the fungus hijacks the host signaling machinery to ensure disease spread.

Cysteine-rich plant DEFENSINs (see Appendix) act as effective antimicrobial peptides against pathogens such as the gray mold-causing fungus *Botrytis cinerea*. In radish, the *Raphanus sativus* ANTIFUNGAL PEPTIDE2 peptide disrupts the plasma membrane of the fungal hyphae by binding to the cell wall glucosylceramides, making the plasma membrane more permeable and causing leakage of essential ions.

Plant-Insect Interactions

Parasites also employ molecular mimics that allow them to thrive on their plant hosts. Root-knot nematodes (*Meloidogyne* spp) and cyst nematodes (*Heterodera* spp) are well known parasites of plants. In their juvenile form, nematodes penetrate the roots to reach the vascular tissue where they form "feeding sites" or syncytia and derive their nutrients from these enlarged cells. To develop these syncytia, nematodes produce CLE peptide mimics and secrete them into the host tissue via the stylet. Nematode CLEs can be divided into two classes that either promote cell differentiation of the apical meristems (class A) or inhibit cell differentiation in vascular tissue (class B). This is advantageous to the nematode, which starts swelling as it feeds, ultimately breaking through the root surface to mate and release fertilized eggs. Importantly, perception and activity of parasite-encoded peptides depend on homology to the host-encoded peptides. At least one other family of plant peptides—the CEPs—have been found in root knot nematodes. Indeed, 11 of the 15 amino acid residues of the *M. truncatula* MtCEP1 are identical to the *Meloidogyne* CEP11. Knockdown of the peptide mimics provides further evidence that they are crucial for host infection. Transgenes can be introduced into plants to specifically target and knockdown nematode genes when they feed on the host sap. Using double-stranded RNA interference, suppression of *Meloidogyne IDA-LIKE1* reduced the number of galls formed on roots of infected Arabidopsis.

Plants also encode peptides that can deter insects. In addition to systemin, plants encode wound-inducible CAPE (CAP derived) peptides that inhibit insect growth. Interestingly, CAPE peptides are encoded at the C terminus of a longer polypeptide involved in pathogen defense in Arabidopsis, PATHOGENESIS RELATED1. Pretreatment of tomato leaves with CAPE1 peptides was sufficient to suppress larval growth of cotton leafworm (*Spodoptera litura*).

Cysteine-rich cyclotides are cyclic peptides that form a "cysteine knot" where one disulfide bridge between two cysteines intersects the loop formed by the other two, providing structural stability. Several plant cyclotides show promise as effective biopesticide agents. The best known cyclotide, KalataB1, has both antimicrobial and antiherbivore properties against larvae of *Helicoverpa* (see Appendix).

EVOLUTION OF PEPTIDE HORMONES

Gene families encoding peptide hormones have undergone massive expansions, permitting their rapid evolution. For example, the RALF family in Arabidopsis contains 34 members, while the ns-LTP family in *M. truncatula* contains 134 members. The multiplication of peptide hormone genes has facilitated a plant's ability to "explore" various peptide structures, expand the signaling repertoire, and develop novel families. The value of gene duplications and family expansion to a plant is evident in the fact that many of the duplicated genes are retained. These duplications are often found in evolutionarily stable tandem arrays or segmental arrays of SSP genes that can arise from errors during genetic recombination. Duplicated genes in a cluster provide an excellent platform for neofunctionalization, an evolutionary process by which one gene acquires a novel function; or even the development of new peptide hormone families (see below, SFTI-1). While one gene in a cluster should maintain the original function and expression pattern, the other genes are free to evolve in different directions and to "explore" different functions or

expression patterns in the plant. Therefore, it is plausible that the large repertoire of peptide hormone families seen among the angiosperms arose from a much smaller pool in ancient relatives.

Divergent Evolution

By searching for homologs of the different peptide hormone families among various plant species, it can be seen that certain families are spread across a broader evolutionary range than others. The CLE family appears to be relatively ancient, in that it is found throughout the vascular plant species, including nonseed and nonflowering plants. By contrast, the IDA and RALF families appear to have evolved more recently, as they are limited to the angiosperms. Some families are even genera or species specific. The ES family has been found specifically in maize while the GRIM REAPER (GRI) family is found exclusively in Arabidopsis and closely related species. This specificity implies a recent origin. The GRI peptides are encoded within the pre-pro-protein of the larger and more widely distributed STIG1/GRI peptide hormone family which promotes pollen tube growth. An upstream sequence has developed in these few species that is processed into an 11-residue peptide regulating cell death. The general picture that emerges is one of rapidly evolving genes with plants' continuous innovation upon a shared framework inherited from the early land plants.

Convergent Evolution

Structure

Whereas the above examples illustrate divergent evolution from a common ancestor, there are also examples of convergent evolution. The most clearly documented instance is the Sunflower Trypsin Inhibitor-1 (SFTI-1) peptide. Like the GRI peptide, the SFTI-1 peptide is embedded within a larger, more ancient precursor protein, in this case encoding an albumin. The structure of the SFTI-1 peptide has converged upon that of the Bowman-Birk protease inhibitors. Both peptide structures share a loop between a cysteine-cysteine pair that is responsible for binding and inhibiting the protease active site.

Sequence

Two short and highly conserved sequence motifs are found in PTM-class peptide hormones. They each comprise a mere four residues but are shared between different peptide families. The first four-residue motif, SGPS, is conserved in both IDA and PIP peptide families and is thought to hold a hydroxylated proline. The sequence motif may be responsible for allowing access for a shared hydroxylating enzyme. The second motif, GxGH, where x can be any residue, is shared with PIP and CEP peptide hormone families. This may hint at a common ancestral gene from which the families were derived. Alternatively, it may represent another example of convergent evolution, in which distinct peptides stumble across the same sequence motif to achieve a common goal. Its function is unknown, though the histidine forms the final residue in both sequences and has been implicated in receptor recognition by binding to the pocket of the receptor complex.

CRITICAL QUESTIONS IN THE PEPTIDE HORMONE FIELD

Almost three decades since the discovery of the first plant peptide hormone and several important findings later, many fundamental questions remain unanswered.

Understanding Function and Regulation

Why do conserved peptide hormone families consist of a large number of members in some species? One possibility is that the promoter of each individual gene is responsive to different developmental signals or environmental stimuli. By making the expression of genes tissue or organ specific and/or responsive to incoming pathogens, plants can streamline their response without affecting unrelated pathways. It is also conceivable that peptides within the same family might act together or oppose each other's functions to regulate an outcome. For example, in Arabidopsis, CLE6 and CLE41 are both involved in controlling vascular bundle size but together their effect is additive, indicating that they regulate different downstream genes. More research is required to understand why functional redundancy exists both within and between peptide families.

What degree of "crosstalk" occurs between peptide and classical hormones? Ongoing research has indicated crosstalk with other phytohormones, such as auxin, abscisic acid (ABA), and brassinosteroids. GOLVEN (GLV) peptides can increase abundance of the auxin exporter PIN2 in Arabidopsis root epidermal cell membranes to control auxin gradients, which in turn affect plant gravitropic responses. The *GLV* genes are themselves transcriptionally upregulated by auxin, forming a positive feedback loop. Conversely, a negative feedback loop results when RALF peptides and abscisic acid signaling pathways undergo crosstalk. The RALF peptide receptor FER controls cell elongation in response to abiotic and biotic stresses. FER represses ABA signaling by enhancing the activity of a negative regulator, ABI2. However, ABI2 could also dephosphorylate and inactivate FER.

Peptide processing is also not very well understood. For instance, there are many other potential PTMs for a peptide hormone, such as the addition of a phosphate group (phosphorylation) or an acetyl group (acetylation), which may be waiting to be discovered. Genetic and biochemical screens may help identify novel PTMs of peptide hormones.

Peptide Recognition and Transport

Given the size of peptide hormone families, how are they uniquely recognized by surface receptors? Arabidopsis has almost 600 receptor-like kinases, with unknown ligands. However, over 1000 peptides are encoded in the Arabidopsis genome. This suggests that in plants, a receptor can potentially perceive more than one ligand. Indeed, in *L. japonicus*, the HAR1 receptor kinase has been demonstrated to recognize CLE-ROOT SIGNAL1 and CLE-ROOT SIGNAL2 to control nodule number. Conversely, the peptide RGF1 can be recognized by three different receptors of the LRR-RLK family (RGFR1, RGFR2, and RGFR3). These receptors, however, have distinct expression patterns, implying that they may recognize the same peptide under different developmental circumstances. Many more receptor-ligand pairs await discovery.

Not much is known about the movement of peptide hormones over short and long distances. Is xylem and phloem loading of these peptides mediated by active transport? Short amino acid chains are known to be transported by active peptide transporters; however, none have been identified for peptide hormones per se. Three families of transporters have been identified as potential peptide transporter families: the ATP Binding Cassette Transporter family, Peptide Transporter family, and the Oligopeptide Transporter (OPT) family. When vascular-bundle-expressed AtOPT6 is expressed in frog eggs that are then injected with CLE or CLE-like peptides, electrical currents are induced. Using patch-clamp techniques, voltage across the cell membrane expressing a transporter gene of interest and the surrounding solution are set. Changes due to addition of ions or other substrates can alter the voltage and the resulting current can be recorded. The amplitude and duration of these induced currents are used to characterize whether a substrate is transported with high, low, or no affinity. Physiological activation of AtOPT6 by addition of CLE peptides as measured by the induced current implied that not only can AtOPT6 transport CLE peptides, but it does so with a high affinity. However, AtOPT6 has affinity for many other substrates in vitro; therefore, we cannot yet conclude that this transporter shuttles the CLE peptides inside the plant as well.

APPLICATIONS IN AGRICULTURE AND BIOTECHNOLOGY

Peptides that enhance disease resistance or improve uptake of soil nutrients might provide opportunities to solve agricultural problems. Peptides also have many unique features that can and are being exploited for their usefulness to mankind. The cyclotide Kalata-B1 was isolated from leaves of *Oldenlandia affinis* because it was used by women in central Africa to ease childbirth. Since then, this cyclic peptide has also been shown to have insecticidal and antimicrobial properties. Oral administration of Kalata-B1 could delay symptoms of brain inflammation in mice, offering a promising treatment for multiple sclerosis. Another potential use of cyclic peptides is as delivery agents. Cyclotides such as SFTI-1 can easily pass through cell walls and membranes and can be engineered to deliver a molecule of interest in the bound form to target cells. Not only natural peptides but synthetic peptides with random sequences can also have biological activity. Transgenes engineered with a start codon, a random nucleotide sequence, and a stop codon each coding for a unique short peptide could elicit specific developmental responses when expressed in Arabidopsis.

Some peptide hormones can be easily synthesized in large quantities and often are bioactive at extremely low concentrations. A 1-nM peptide solution prepared from 30 g of a typical peptide would fill five Olympic swimming pools! Using existing technology, the cost to produce enough peptide to cover an acre at biologically relevant concentrations could be as low as a few cents. Watering plants with peptides is possible, although seed coating or application together with fertilizer by banding are also likely strategies. Still, a number of complications can be anticipated. Often a single dose of a peptide is not enough to mediate physiological effects and continuous supply might be necessary, driving up the cost of such treatments. Moreover, drench treatments are not always selective and can potentially be harmful to other soil microorganisms.

CONCLUSION

Peptide hormones have emerged as major regulators of diverse functions in plant growth and development, alongside classical phytohormones. Since peptide hormones are genetically encoded and ribosomally translated, the complexity and number of potential peptide hormones used by a plant far surpasses that of the classical hormones. Although we are only beginning to understand their regulatory functions, their roles in reproductive development, meristematic stem cell size, and symbiotic interactions are already clear. Undoubtedly, new roles for peptide hormones await discovery, as additional families are studied. Peptide hormones may be small in size, but their influence on plant life is indeed mighty!

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