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BIOACTIVE PEPTIDES AS SIGNAL MOLECULES IN PLANT DEFENSE, GROWTH, AND DEVELOPMENT

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ABSTRACT: Until recently, intercellular communication in higher plants was thought to be mediated by the five classes of classical phytohormones *i.e.* auxins, cytokinins, gibberellins, abscisic acid, and ethylene. Hormone action in plants thus appeared to be fundamentally different from that in animals. This view is changing, however, since over recent years brassinosteroids and jasmonates resembling animal steroids and prostaglandins, respectively, have been added to the group of chemical messengers in plants. Furthermore, there is now compelling evidence for the existence of plant (poly)peptide hormones.

The present arsenal of endogenous plant peptide signals includes just four groups of hormones involved in wound signal transduction, in cell proliferation, and in the regulation of salt/water homeostasis, *i.e.* systemins, phytosulfokines, enod40, and natriuretic peptides, but many more are likely to exist. Plants appear to possess the receptors for a plethora of peptide signals. These signals include both endogenous peptides as well as peptides of microbial origin. Furthermore, plant proteases have been identified likely involved in the generation of peptide signals from larger precursor proteins. This article discusses the evidence in support of a general role for bioactive peptides in plant signal transduction with emphasis on the structure and bioactivity of the peptides themselves.

INTRODUCTION

The biochemical machinery necessary for peptide synthesis, secretion, and posttranslational modification is present in every living cell. An enormous structural diversity can be generated by use of this preexisting cellular machinery. Not surprisingly, peptides are commonly used as signal molecules for intercellular communication in prokaryotes, fungi, and animals. Peptide signals in animals include vast numbers of peptide hormones, growth factors and neuropeptides. Are plants any different in this respect? Until very recently they appeared to be. Plants seemed to rely on only five different classes of phytohormones comprising auxins, cytokinins, gibberellins, abscisic acid, and ethylene for the regulation of growth and development [1]. These phytohormones are small diffusible

molecules which were thought to be much better suited to penetrate the rigid cell walls between adjacent cells as compared to large peptide hormones [2]. Research in recent years, however, has indicated that peptides may be widely used as chemical signals in plants as well. In the present work, I intend to summarize the evidence in support of this hypothesis.

Endogenous bioactive peptides, *i.e.* plant-derived peptides that evoke specific cellular responses, provide the most direct evidence for a general role of peptides in the regulation of plant growth and development. Their number, however, is still very limited. Presently, endogenous regulatory peptides in plants include systemin, phytosulfokines, and enod40 [3,4]. Furthermore, there is some indication that plants contain peptides similar in structure and function to natriuretic peptides in animals [5]. Following the initial discovery of systemin as an 18-amino-acid peptide in tomato plants, closely related peptides have been discovered in other *solanaceaeous* species. Systemins are mediators of the defense responses triggered by the attack of herbivorous insects. Phytosulfokines are small sulfated peptides of four or five amino acids which exhibit mitogenic activity. The enod40 group of peptides is involved in cell proliferation as well. The biosynthesis of these peptides, their biological activity, as well as the structural requirements for bioactivity and signal perception will be the first focus of the following discussion.

The endogenous bioactive peptides thus far identified are likely to represent only the tip of the iceberg. Research in recent years has shown that plants have the capacity to generate and to perceive peptide signals providing indirect evidence for a general role of peptides as plant growth regulators. The perception of peptide signals requires receptor proteins for proteinaceous ligands. In higher plants, a large number of receptor-like kinases (RLKs) have been identified possessing extracellular domains which are likely to be involved in protein/protein interaction [6]. Thus, RLKs were hypothesized to be the receptors of (poly)peptide ligands. For two RLKs, involved in meristem and organ development in *Arabidopsis* (CLAVATA1) and in the determination of self-incompatibility in *Brassica* (SRK), the respective peptide ligands have been identified very recently (see below). Likewise, the generation of peptide signals in animals requires proteases that are involved in the maturation of peptide hormones from inactive precursor proteins by limited proteolysis. Proteases of the subtilisin superfamily play a predominant role in this process [7]. Proteases of this family have been identified and may serve a

similar function in plants [8]. They provide further indirect evidence for the common use of peptides as chemical signals in plants.

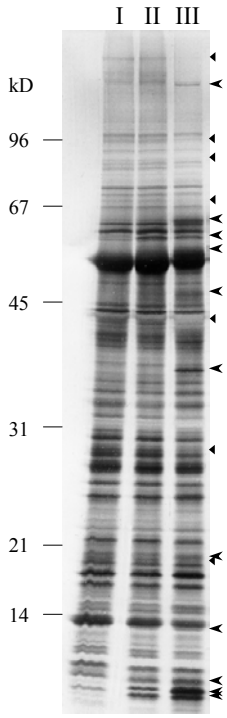
Plants do not only perceive endogenous peptide signals but also react specifically to a large number of exogenous peptides produced by pathogenic microorganisms. Microbial peptides act as elicitors of both general and race-specific defense responses. Numerous structurally diverse peptides from phytopathogenic fungi have been implicated in the latter. In contrast, the attenuation of general resistance responses depends on the recognition of conserved structural features of (poly)peptides common to a wide range of microorganisms. This distinction between „self" and „non-self" is a prerequisite for the development of resistance [9,10]. The nature of peptide elicitors of general and race-specific resistance responses, the structural requirements for their bioactivity, and the molecular basis for recognition by the plant cell will be discussed.

ENDOGENOUS PEPTIDE SIGNALS

Systemin

Tomato plants respond to local injury by herbivorous insects with the induction of a systemic defense response which is characterized by the transcriptional activation of a large number of defense genes and the concomitant accumulation of the respective defense proteins (Fig. (**1**); [11-13]). The search for the signal molecule that allows tomato plants to respond systemically to a local stimulus (*i.e.* wounding) led to the identification of the first plant peptide with signaling function in 1991. The 18-amino-acid peptide was isolated from leaves of tomato plants on the basis of its ability to induce the expression of defense genes, the hallmark of the wound response (Fig. (**1**)). To emphasize its central role and the systemic nature of the wound response the peptide was named systemin [14]. In subsequent years, it was established that systemin is both sufficient and necessary for systemic wound signal transduction. While there is clear evidence for additional signals to exist, a central role for systemin in wound signaling in tomato plants and in other members of the *Solanaceae* [15] is now generally accepted. Both the discovery of systemin as well as its role in wound signaling have been discussed extensively and the reader is referred to recent review articles for further information [4,13,16-19]. In this chapter, I will concentrate on unresol-

ved questions concerning systemin perception as well as its synthesis and degradation. The careful analysis of structural aspects that are relevant for its biological activity have been instrumental in the search for the systemin receptor and will, therefore, be the starting point of the following discussion.



Systemic Wound Response Proteins in Tomato Plants

Defense Proteins
- Serine Proteinase Inhibitor I
- Serine Proteinase Inhibitor II
- Cysteine Proteinase Inhibitor
- Aspartic Proteinase Inhibitor
- Polyphenol Oxidase

Signal Pathway-Associated
- Prosystemin
- Lipoxygenase
- Calmodulin
- Nucloetide Diphosphate Kinase
- AcylCoA-Binding Protein

Proteolysis-Associated
- Leucine Aminopeptidase
- Carboxypeptidase
- Aspartic Proteinase
- Cysteine Proteinase
- Ubiquitin-like Protein

Other Proteins
- Threonine Deaminase

Fig. (1). The wound response in tomato plants. Tomato plants respond to wounding with the transcriptional activation and accumulation of Systemic Wound Response Proteins including defense proteins, proteolysis-associated proteins, signaling-associated proteins, and proteins of yet unknown function in plants defense. The change in gene expression can be monitored on SDS-PAGE gels. In comparison to control plants (I), treatment with systemin (II), or overexpression of the prosystemin cDNA (III) leads to the accumulation of SWRPs (arrowheads) and the downregulation of other, unidentified proteins (triangles). The figure was modified after [13].

Sequence analysis of the defense gene-inducing principle isolated from tomato leaves revealed the amino acid sequence AVQSKPPSKRDPPK-MQTD as the primary structure of systemin (Fig. (**2**)). The most salient

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  1 MGTPSYDIKN KGDDMQEEPK VKLHHEKGGD EKEKIIEKET
 41 PSQDINNKDT ISSYVLRDDT QEIPKMEHEE GGYVKEKIVE
 81 KETISQYIIK IEGDDDAQEK LKVEYEEEEY EKEKIVEKET
121 PSQDINNKGD DAQEKPKVEH EEGDDKETPS QDIIKMEGEG
161 ALEITKVVCE KIIVREDLAV QSKPPSKRDP PKMQTDNNKL
                                Systemin
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Fig. (2). The primary structure of tomato (pro)systemin. The amino acid sequence deduced from the prosystemin cDNA is shown. The systemin precursor comprises 200 amino acids. The 18 amino acids of the systemin oligopeptide (bold, underlined) are located close to the carboxy terminus.

feature of this sequence is the palindromic structure around a central pair of basic residues with two proline doublets at positions 6 and 7, and 12 and 13 [14]. Several studies aimed at the identification of secondary and tertiary structural elements within systemin. At acidic pH, two-dimensional NMR spectroscopy revealed a Z-like-β-sheet structure which is frequently found in DNA-binding proteins [20]. Based on this observation, systemin was suggested to bind to the promoter region of defense genes thereby regulating their expression [20,21]. At neutral pH in aqueous solution, however, proton NMR experiments did not provide evidence for persistent secondary or tertiary structural elements in the systemin polypeptide [22]. Circular dichroism, as a method particularly sensitive to secondary structure, revealed a poly(L-proline) II type, 3_1 helical structure for a substantial part of systemin in aqueous solution [23]. The 3_1 helicity has been observed in oligopeptide ligands of Src homology 3 (SH3) proteins [23]. Hence, the presence of this structural feature in systemin does not support the suggested interaction between systemin and DNA but would rather favor a proteinaceous receptor for systemin. Despite experiments aiming to determine the relevance of the proline residues for bioactivity, it remains unknown whether or not the 3_1 helicity is relevant for systemin activity *in vivo*. Each of the 18 amino acids of systemin was individually replaced by alanine to assess the contribution of single amino acid side chains to the biological activity of systemin. The Pro13Ala substitution resulted in a dramatic loss of defense protein-inducing activity, second only to the Thr17Ala derivative which was completely inactive. Pro13 may thus be structurally important.

Systemin derivatives with Ala substitutions for any of the other proline residues, however, retained most (>10%) of the bioactivity [24].

Progressive deletions from the N- and C-termini indicated that the 18-amino-acid peptide is the minimal structure having full biological activity. Deletions from the N-terminus resulted in a progressive loss of activity, while the deletion of a single amino acid from the C-terminus completely inactivated the peptide. Interestingly, the C-terminally truncated peptide as well as the Thr17Ala derivative of systemin acted as competitive antagonists of systemin activity [24]. Based on these observations it was proposed that systemin may bind to its receptor via the N-terminal part while the C-terminus is essential to activate downstream responses [24].

The analysis of the systemin structure/function relationship identified residues that can be modified without impairing systemin activity. In subsequent studies, some of these residues (Val2, Ser8, and Met15) were modified to generate labeled systemin derivatives as affinity probes for the systemin receptor. A biotinylated systemin derivative (biotinyl-Cys8Ser systemin) was used in initial attempts to isolate the systemin receptor from tomato plasma membranes. The biotinylated peptide could be specifically crosslinked to a 50 kDa protein (SBP50) in plasma membrane preparations from tomato leaves [25]. Binding to SBP50, however, was slow and was saturated only at high concentrations of biotinyl-systemin. Furthermore, competitive displacement of the ligand with alanine-substituted systemin derivatives revealed a lack of correlation between the structural requirements for binding to SBP50 and the biological activity of systemin [25]. Therefore, SBP50 is not likely to be the systemin receptor. In contrast, SBP50 exhibited characteristics of proteases related to yeast kexin and was suggested to be involved in systemin processing thereby facilitating its activity or degradation. [25]. Further attempts to identify a high-affinity binding site for systemin in tomato plasma membrane preparations using either biotinylated, or radiolabeled systemin derivatives were not successful (Doares, Schaller, and Ryan; unpublished).

Progress was made possible by use of a different source for membrane preparations. It had been shown by Felix and Boller [26] that cell suspension cultures of a wild tomato species (*Lycopersicon peruvianum*) give a characteristic response to systemin. After addition of systemin an alkalinization of the culture medium was observed paralleled by an efflux of K^+. Systemin also caused an increase in the activities of 1-

aminocyclopropane-1-carboxylate (ACC) synthase and phenylalanine ammonia-lyase, *i.e.* two enzymes with established functions in plant defense [26]. Medium alkalinization in response to systemin treatment provided a convenient assay for systemin activity. The alkalinization response was dose-dependent and saturated at about 1 nM systemin. The structural requirements within systemin were the same for medium alkalinization and for defense gene induction [26,27]. Therefore, the two responses appeared to be mediated by the same perception system and cultured cells of *L. peruvianum* were subsequently used in the search for the systemin receptor.

In microsomal membrane preparations from *L. peruvianum* cells, Meindl *et al.* [28] characterized a high-affinity, saturatable binding site for a systemin derivative extended at the C-terminus by ^{125}I-iodotyrosine. Using ^{125}I-Tyr2,Ala15-systemin as the ligand, a similar binding site was observed by Scheer and Ryan [29] on the surface of *L. peruvianum* cells. The binding sites described by the two groups exhibited very similar characteristics and are likely to reside in the same protein. Both groups found antagonists of systemin activity to be able to compete for binding of the respective radioligands. Furthermore, the biological activities of alanine-substituted systemin derivatives were correlated to their ability to compete with the binding of the radioligands [29]. While the C-terminal part of systemin is essential for bioactivity, a corresponding systemin fragment was not able to displace the radioligand from its binding site. Meindl *et al.* [28] concluded that systemin perception requires a two-step mechanism involving binding to the receptor via its N-terminal part and activation of cellular responses by the C-terminus of systemin. Thus, the systemin-binding site in *L. peruvianum* cells exhibited characteristics that had been predicted for the systemin receptor from the analysis of the systemin structure/activity relationship [24]. Photoaffinity labeling localized the binding site to a 160-kDa protein. This protein likely represents the functional systemin receptor in *L. peruvianum* cells and, hence, was named systemin receptor 160 (SR160) [29].

The abundance of SR160 at the cell surface was found to increase in response to treatment with methyl jasmonate [29]. Upregulation at the transcriptional level by wounding and jasmonates, which are downstream signaling molecules in the wound signal transduction pathway [30,31], has also been described for other proteins of the signaling pathway including lipoxygenase [32], allene oxide synthase [33,34], prosystemin [13,35], calmodulin [36], and the catalytic and regulatory subunits of a

polygalacturonase [37]. The increase in the abundance of the systemin receptor and of other components of the wound signal transduction pathway due to their *de novo* biosynthesis may enhance the capacity of tomato plants to respond to continuous attack by herbivores with a further increase in defense gene expression.

It is expected that the characterization of SR160 will provide profound insights into systemin perception and the transduction of the wound signal into cellular responses. Presently, this can only be inferred from the changes observed after systemin treatment of cultured cells or differentiated plants. The earliest systemin-triggered changes in tomato leaf mesophyll cells are an increase in the cytosolic free calcium concentration within 1 - 2 minutes [38] and a transient depolarization of the plasma membrane potential [39]. Depolarization of the plasma membrane in response to systemin has also been observed in cultured *L. peruvianum* cells [26] and was shown to depend on the influx of Ca^{2+} as well as the activity of a protein kinase [40]. By use of inhibitors of the plasma membrane H^{+}-ATPase [40] and of various ionophores (Frasson and Schaller, unpublished) it could be shown that depolarization of the plasma membrane potential is sufficient to induce the expression of defense genes. However, when membrane depolarization was suppressed by application of fusicoccin which activates the plasma membrane H^{+}-ATPase, defense gene expression was inhibited [40]. These data indicate that the depolarization of the plasma membrane potential and the influx of Ca^{2+} are necessary elements in the signal transduction pathway toward the activation of defense gene expression [4]. Either one or both of these events in concert with the activity of a protein kinase [41,42] lead to the activation of phospholipase A2 [43] and the subsequent release of linolenic acid from membrane lipids [43-45]. Linolenic acid, via the octadecanoid pathway, is converted to jasmonic acid which ultimately triggers defense gene induction [31,46]. For further details on this signaling pathway, which resembles eicosanoid signaling in the inflammatory response of animal macrophages, the reader is referred to the comprehensive review article by Ryan and Pearce [19] and to a recent paper from the same laboratory [43].

In addition to the problem of how the systemin signal is perceived and transduced in target tissues, another complex of open questions revolves around the factors governing systemin synthesis and degradation. Systemin is synthesized as 200-amino-acid precursor protein, prosystemin (Fig. (**2**); [35]). In analogy to animal systems, it had been assumed that

processing of prosystemin to release systemin is necessary for the activation of the peptide signal [27,47]. Recent data show that this may not be the case. The prosystemin polypeptide was overexpressed in both prokaryotic [48] and eukaryotic [49] hosts. When the recombinant protein was tested for biological activity, it turned out to be active in inducing medium alkalinization in *L. peruvianum* cell cultures as well as defense gene expression in tomato plants [49,50]. The activity was shown to reside exclusively in the C-terminal, *i.e.* systemin, part of the prosystemin structure [50], and the perception systems used by prosystemin and by systemin are likely to be identical [49,50]. Systemin has originally been isolated as the actual defense gene-inducing factor and it has been shown to be the minimal structure that retains full biological activity [14,24]. Thus, prosystemin is processed to generate systemin *in vivo*, and mature systemin is not likely to be a random degradation product of prosystemin. While the data show that proteolytic processing is not necessary for the activation of systemin, it may still be required to facilitate systemin release and/or systemic translocation. The proteases involved in systemin maturation still remain to be identified, however.

The expression of several proteases including exopeptidases (leucine aminopeptidase, carboxypeptidase) and endoproteinases (aspartic proteinase, cysteine proteinase) is induced systemically upon wounding of tomato or potato plants [13,51-57], and for some of these proteases a role in the regulation of systemin activity has been discussed [12,54,56]. However, whenever this was investigated, induction by wounding was found to be rather slow, resembling the induction of defense genes rather than that of signal pathway components [32,35-37,54,56]. Therefore, these proteases may rather contribute directly to deterring the insect predator [58] or else, they may be responsible for rapid protein turnover facilitating C and N salvaging in damaged leaves and providing an adequate amino acid pool for the synthesis of abundant defense proteins in systemically induced leaves [56,59].

Systemin exerts its biological activity at extremely low concentrations (fmol/plant). Therefore, in analogy to animal systems, proteases have to be postulated that inactivate systemin and clear the system from residual active hormone. Experimental data indeed support the existence of such enzymes. Felix and Boller observed that the transient nature of systemin-triggered alkalinization response in *L. peruvianum* cells is due to proteolytic inactivation of systemin rather than desensitization of the perception system [26]. Inactivation of systemin was, in fact, observed in

cell-free growth medium of *L. peruvianum* cultures [26], and the site of proteolytic cleavage was identified as the Lys14/Met15-bond of systemin [27]. The alkalinization of the culture medium in response to a systemin derivative in which this peptide bond had been stabilized by *N*-methylation was no longer transient. Furthermore, in tomato plants, the *N*-methylated systemin derivative exhibited an enhanced defense gene-inducing activity as compared to native systemin [27], indicating that this processing event contributes to systemin inactivation *in vivo*. While the protease responsible for processing of systemin at the Lys14/Met15-bond has not been isolated yet, such an activity was detected in plasma membrane preparations from tomato leaves [25,27], and SBP50 which shares several characteristics with the yeast kex2 protease is a possible candidate [25]. Obviously, many questions remain open with respect to the identity of the proteolytic systems involved in systemin maturation and inactivation, as well as their tissue specific and subcellular localization and they will attract the attention of future studies.

Phytosulfokines

It is well known that suspension-cultured plant cells require a critical initial cell density for growth. In cultures below that threshold density the cells fail to divide. In 1969, Stuart and Street demonstrated that the growth of *Acer pseudoplatanus* cells in low-density culture could be restored by the addition of 'conditioned' medium [60]. Conditioned medium was derived from high-density cultures after separation of the cells by dialysis. Hence, a low-molecular-weight 'nursing' factor must be released by high-density cultures, and Stuart and Street initiated work to establish the chemical nature of this factor [60]. For decades, however, all attempts to purify this nursing factor failed, likely because a fast and sensitive assay system was not available. A highly sensitive bioassay was eventually developed by Matsubayashi and Sakagami [61]. In this assay system, conditioned medium and fractions thereof were tested for mitogenic activity on mechanically dispersed *Asparagus* mesophyll cells in 24-well microplates. The mitogenic activity was purified and found to reside in two factors subsequently named phytosulfokine-α and -β (PSK-α and PSK-β). Amino acid sequence analysis and mass spectrometry identified the phytosulfokines as a sulfated pentapeptide (Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln) and its C-terminally truncated tetrapeptide deriva-

tive (Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr), respectively (Fig. (**3**)). Synthetic PSKs exhibited the same mitogenic activity as the purified native compounds, thus confirming the structures [61].

a)
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  1 MVNPGRTARA LCLLCLALLL LGQDTHSRKL LLQEKHSHGV
41 GNGTTTTQEP SRENGGSTGS NNNGQLQFDS AKWEEFHTDY
81 IYTQDVKNP
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b)

PSK-α PSK-β

Fig. (3). The structure of (prepro)-phytosulfokines. a) The amino acid sequence of prepro-phytosulfokines deduced from the sequence of the cDNA is shown. The signal peptide for secretion (dotted line) and the position of phytosulfokines within the precursor (bold, underlined) are indicated. b) Structures of the sulfated penta- (PSK-α) and tertrapeptides (PSK-β).

The work on phytosulfokines culminated last year in the cloning of the cDNA for the PSK precursor protein from rice (Fig. (**3**); [62]). The rice cDNA has the capacity to encode a 89-amino-acid prepro-phytosulfokine indicating that PSKs have to be released from their precursor by limited proteolysis, a feature they share with systemin and with animal peptide hormones in general. By virtue of a 22-amino-acid signal peptide at the amino terminus, prepro-phytosulfokine was shown to be targeted to the secretory pathway of cultured rice cells resulting in the accumulation of correctly processed PSK in the culture medium [62]. The PSK precursor was found to be expressed in all analyzed tissues of rice seedlings but, consistent with its mitogenic activity *in-vitro*, expression was highest in root and shoot apices [62]. The rate of cell division was correlated to the expression level of prepro-phytosulfokine in transgenic rice cultures thus confirming the role of PSKs in mitosis [62]. The *PSK* gene was detected in *Oryza sativa*, *Asparagus officinalis*, *Arabidopsis thaliana*, *Daucus carota*, and *Zinnia elegans* and PSK-α was found to be present in cell culture media of all these species suggesting conservation of phytosulfokines between monocot and dicot plants [61-64].

Matsubayashi and Sakagami also initiated work towards the identification of the phytosulfokine receptor. In order to generate labeled PSK derivatives as affinity probes for the PSK receptor, functional groups had to be identified within PSKs that can be modified without impairing their bioactivity. The structure/activity relationships of PSKs were analyzed using the same bioassay of PSK activity that had already been used during PSK purification [61,65]. PSK-α exhibited half-maximal activity at 4 nM and was shown to be more active than PSK-β. The active core was shown to reside within the N-terminal tripeptide of PSK-α, which still retained 20 % of the full PSK-α activity. In contrast, the N-terminally truncated derivative was essentially inactive. Both sulfate groups were found to be essential for biological activity. The mono(Tyr1)- and mono(Tyr3)-sulfated peptides retained 0.6 % and 4 % of PSK-α activity, respectively, while the unsulfated peptide was found to be inactive [65]. Both the isoleucine and the threonine residues of PSK-α were shown to be functionally relevant since replacement with either valine or serine, respectively, resulted in a 20-fold reduction of bioactivity [65].

A radioligand was synthesized by introducing ^{35}S into the tyrosine sulfate to generate [^{35}S]PSK-α. Specific binding sites for [^{35}S]PSK-α were detected on intact rice suspension cells and in plasma membrane-enriched fractions [63]. The ability of PSK derivatives to displace the radioligand from its binding sites was correlated to their mitogenic activity in the bioassay [63]. Characterization of the plasma membrane-located binding site required the synthesis of a second radioligand ([^{3}H]PSK-α) with higher specific activity by catalytic tritiation of a tetradehydroisoleucine-containing PSK-α analog [66]. Using [^{3}H]PSK-α, saturatable, reversible binding was demonstrated to plasma membrane preparations and high (K_d of 1.4 nM)- and low (K_d of 27 nM)-affinity binding sites were characterized. The observed correlation between the bioactivity of PSK analogs and their ability to compete with the radioligand for binding, as well as the affinity of binding which corresponds to the threshold of bioactivity in the bioassay indicate that the high-affinity binding site within rice plasma membranes may represent the functional PSK receptor.

Whereas systemin is a peptide signal involved in the regulation of plant defense responses [14,67], phytosulfokines appear to be the first true plant peptide growth regulators. They clearly display mitogenic

activity which is distinct but depends on the activity of other plant hormones like auxins and cytokinins [68]. Furthermore, under certain circumstances, their activity may extend to the regulation of cell differentiation [64]. Further work in this area holds the promise of exciting new discoveries related to the perception of the phytosulfokine signal and the cellular machinery involved in signal transduction.

Enod40

Soil-borne bacteria of the family *Rhizobiaceae* and leguminous plants form a symbiotic relationship during which a new organ, the root nodule, is developed. Within these root nodules the bacteria fix atmospheric dinitrogen and the product of nitrogen fixation, ammonia, is exported to the plant [69,70]. Root nodules develop from primordia which are established at specific sites in the root cortex shortly after *Rhizobium* infection. The peptide enod40 is believed to play a critical role in inducing the de-differentiation and the mitotic division of root cortical cells, *i.e.* the initial steps in nodule development. This however, is not entirely undisputed [3,4,69-72].

During early stages of nodule development, early nodulin (*ENOD*) genes are expressed in the plant root and they are postulated to mediate nodule morphogenesis. The spatial and temporal patterns of expression in the root pericycle and cortical cells prior to the onset of mitotic cell divisions suggested the *ENOD40* gene to be responsible for the initiation of nodule formation [73,74]. As a matter of fact, root cortical cell division was induced in *Medicago trunculata* plants overexpressing *ENOD40* [75] and, furthermore, the induction of *ENOD40* gene expression was shown to be required for nodule development [76]. In transgenic *M. trunculata* overexpressing *ENOD40,* the extent of nodule formation was correlated to the expression level of *ENOD40* [76].

While a role for the *ENOD*40 gene in nodule initiation is well documented, it is less obvious what the gene product is that triggers the cellular responses. The transcripts of *ENOD*40 genes do not contain long open reading frames (ORFs) and it was initially assumed that the mRNA itself is the active principle [77]. A sequence comparison of all cloned *ENOD40* genes, however, revealed the presence of two conserved regions. The 5'-proximal conserved region contains a short ORF with the capacity to encode the enod40 peptide of 10 – 13 amino acids (Fig. (**4**)).

a) *Gm*ENOD40a ME-LC**W**QTSI**HGS**
*Gm*ENOD40b ME-LC**W**LTTI**HGS**
*Pv*ENOD40 MK-FC**W**QASI**HGS**
*Lj*ENOD40 MR-FC**W**QKSI**HGS**
*Sr*ENOD40 MK-LC**W**QKSI**HGS**

b) *Ps*ENOD40 MKFLC**W**QKSI**HGS**
*Vs*ENOD40 MKLLC**W**QKSI**HGS**
*Ms*ENOD40 MKLLC**W**QKSI**HGS**
*Mt*ENOD40 MKLLC**W**EKSI**HGS**
*Tr*ENOD40 MKLLC**W**QKSI**HGS**

c) *Nt*ENOD40 MW---**W**DEAI**HGS**
*Os*ENOD40 ME-DE**W**LEHA**HGS**
*Ob*ENOD40 ME-DE**W**LEHA**HGS**
*Zm*ENOD40 ME-DA**W**LEHL**HGS**

Fig. (4). The primary structures of enod40 peptides. Enod40 peptides are compared from legumes with determinate nodules (a), legumes with indeterminate nodules (b), and non-legumes (c). Gaps (-) were introduce to optimize the alignment. The invariant residues are shown in bold face. The figure was modified after [79].

The 3'-distal part of the mRNA, in spite of the conserved region, does not contain a protein-coding ORF. Stimulated by this unexpected finding, several independent methods were employed to demonstrate that the enod40 peptide is actually expressed *in vivo*. Using immunological methods, the peptide was shown to be present in nodules, but not in roots, of soybeans as well as in the medium of tobacco protoplasts expressing the *GmENOD40* transgene but not in wild-type protoplasts [78]. Furthermore, when a translational fusion was made between the ORF of soybean enod40 and green fluorescent protein (GFP) and expressed in tobacco protoplasts, GFP fluorescence in these protoplasts was similar in intensity to the GFP fluorescence expressed with its own translational start site [78]. The same technique was used to demonstrate that the AUG start codon of the conserved ORF is the only AUG that functions as an efficient translational start site in tobacco *ENOD40* [74]. These studies allow the conclusion that enod40 is the primary translation product. This is in contrast to both systemin and phytosulfokines which are synthesized as larger precursor proteins.

When the peptide-coding part of the *ENOD40* gene was transiently expressed in *M. trunculata*, an induction of cortical cell division was observed indicating that indeed the peptide is the inducing factor [75]. Surprisingly however, expression of the conserved 3'-untranslated region (3'UTR) was found to stimulate the identical cellular responses [75,78]. A rationale to explain this finding has been put forward according to which the 3'UTR may regulate the translation of the ORF. It is assumed that the endogenous *ENOD40* mRNA is expressed but is not translatable as a result of the binding of an inhibitory protein to the 3'UTR. This protein is titrated by transient expression of extra 3'UTR, thus allowing translation of the endogenous enod40 [3].

The data strongly support a role for the enod40 peptide in nodule initiation which may reside in its mitogenic activity. Notwithstanding, there is clear evidence that *ENOD40* function extends beyond the initiation of nodule formation. In alfalfa, antisense inhibition of *ENOD40* expression arrested callus growth, while overexpression of *ENOD40* gave rise to embryogenic tumors [77]. Furthermore, *ENOD40* genes were found in non-legumes like tobacco [78] and even monocot plants like maize and rice [79]. In rice plants, *ENOD40* expression was found to be confined to the parenchyma cells surrounding the protoxylem during early stages of lateral vascular bundle formation and a function in the differentiation of the vascular bundles was suggested [79].

While evidence for the enod40 peptide being an endogenous plant growth regulator is accumulating, definite proof is still missing. This is mainly due to the lack of a convenient assay system. As seen above, such an assay system was instrumental for the purification and characterization of both systemin and phytosulfokines. Furthermore, it allowed the generation of labeled peptide derivatives for the characterization of receptor sites and it will ultimately lead to the isolation of the receptor proteins. Undoubtedly, the development of a suitable assay for enod40 activity would greatly advance this field of research.

Natriuretic Peptides

Natriuretic peptides (NPs) are a group of peptide hormones in animals that are critically involved in salt and water homeostasis (for review see [5]). NPs include atrial NP (ANP), brain NP (BNB), C-type NP (CNP), and urodilatin all of which are synthesized as larger precursor proteins. In

the initial processing steps, the 24-amino-acid signal peptide and the two C-terminal arginine residues of preproANP are cleaved to yield proANP. ANP, which corresponds to the 28 C-terminal amino acids of proANP is believed to be the main biologically active ANP. Active ANP is circular in structure due to the formation of an intramolecular disulfide bridge [5]. The cellular perception of ANP involves binding to two of three different receptor proteins with cytoplasmic guanylate cyclase domains, the formation of cyclic guanosine-3',5'-monophosphate (cGMP), and the subsequent regulation of cation channel activity [5]. Evidence is accumulating that a NP signaling system, surprisingly conserved in structure and function, is operating in plants as well.

The first line of evidence is provided by a series of studies revealing specific effects of synthetic rat ANP (rANP) *in planta*. In *Tradescantia*, rANP induced stomatal opening in a concentration-dependent manner [80]. The effect was shown to depend on the circular secondary structure of rANP: Linearization of rANP by disulfide reduction abolished the activity of the peptide [81]. This finding was interpreted as an indication for a highly specific, receptor-mediated process. As a matter of fact, spcific binding of ^{125}I-rANP to microsomal and plasma membrane preparations of *Tradescantia* was demonstrated *in vitro* [80,82], and to tissue sections *in situ* [82]. Further experiments indicated a possible conservation of the rANP-triggered signal transduction machinery between vertebrates and plants. The involvement of cGMP was suggested by the finding that rANP-induced stomatal opening was suppressed by inhibitors of guanylate cyclase, while a membrane-permeable cGMP analog was able to mimic the rANP effect [81]. Furthermore, rANP was found to induce radial water movements out of the xylem in *Tradescantia multiflora* shoots and this response was similarly dependent on cGMP [83].

Despite this evidence, NPs presently cannot be considered plant peptide hormones. The establishment as a new peptide hormone requires the identification and the structure elucidation of the plant-derived molecule as well as its functional characterization. So far, an endogenous plant NP (PNP) has not been identified. Several steps toward this goal have been taken, however. Immunological evidence was obtained for the presence of NPs throughout the plant kingdom using antibodies directed against different parts of proANP [84]. PNP has been partially purified from ivy leaves by immunoaffinity chromatography using immobilized antisera directed against human and canine ANP [85]. While the resulting

protein preparation still contained a number of proteins, immunoreactive polypeptides were present that corresponded in mass to rANP [86]. Immunopurified ivy PNP was found to be biologically active, causing a rapid and transient increase of cGMP concentrations and cation influx in the stele tissue of maize roots [86,87]. Like rANP, ivy PNP caused stomates to open and it was found to be more active on a molar basis as compared to rANP [85].

Considering the apparent functional and structural (immunological crossreactivity) conservation between plant and vertebrate NPs, it should be possible to identify *Arabidopsis* sequences related to preproANP in databases upon completion of the *Arabidopsis* genome project which is expected within the current year. While still unpublished, such a sequence may have already been identified [5]. The molecular characterization of the cDNA-encoded polypeptide will eliminate all doubts as to whether or not NPs rank among plant peptide hormones.

INDIRECT EVIDENCE FOR ENDOGENOUS PEPTIDE SIGNALS

Receptor-like Protein Kinases

Indirect evidence for a general role of peptide hormones for intercellular signaling in plants stems from the observation that plants possess putative receptor proteins for (poly)peptide ligands known as receptor-like protein kinases (RLKs). While in all cases the direct biochemical interaction with a putative peptide ligand remains to be shown, the existence of RLKs involved in cell differentiation [88], morphogenesis [89], embryogenesis [90], meristem development [91], self-incompatibility [92-97], pathogen infection [98-102] and hormonal responses [103-105] points to a possible involvement of peptide signals in these processes. RLKs share a C-terminal, cytoplasmic serine/threonine kinase domain, a transmembrane domain and an N-terminal extracellular domain which is thought to interact with the peptide ligand (Fig. (**5**); [2,106-109]. Based on the structure of their extracellular domains, RLKs can be assigned to seven different classes. Most RLKs belong to the leucine-rich repeat (LRR) and the S-domain classes, respectively [6,108,109]. Exceptions include kinases with extracellular domains resembling epidermal growth factor (EGF) repeats [110,111], tumor necrosis factor receptors (TNFRs) [88], lectins [112], pathogenesis-related proteins [100], or kinases with novel

extracellular domains [113]; Fig. (**5**)). In analogy to animal systems, RLKs are expected to interact with (poly)peptide ligands. This seems to hold true for CLAVATA1 (CLV1) [91] and the S-locus receptor kinase (SRK) [92], the only two plant RLKs for which the endogenous ligands have been identified. Therefore, the discussion in this paragraph will be restricted to CLV1 and SRK and their respective ligands.

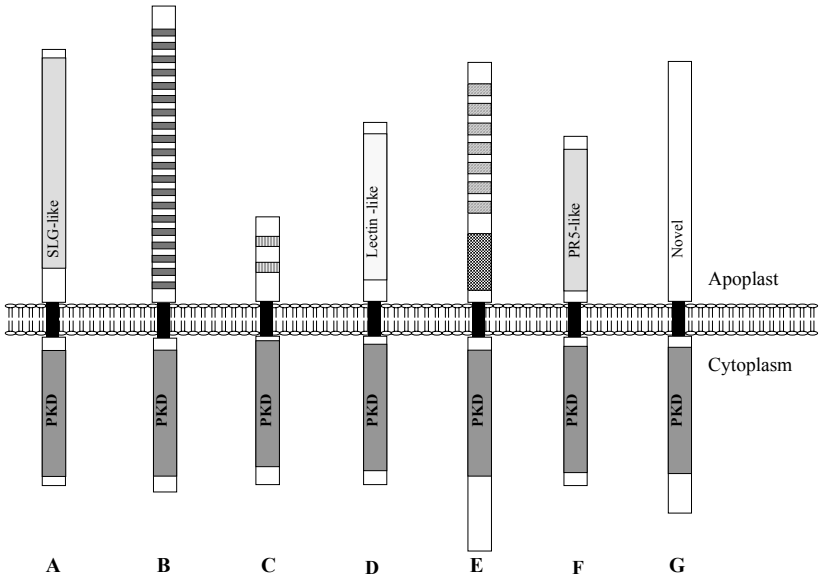

Fig. (5). Modular structure of plant receptor-like kinases *(RLKs).* Schematic diagrams are shown of (A) SRK, a *Brassica* S-locus receptor kinase with sequence similarity in the extracellular domain to the S-locus glycoprotein (SLG); (B) Erecta, an *Arabidopsis* RLK with 20 LRRs; (C) WAK1, an *Arabidopsis* RLK with two epidermal growth factor-like repeats; (D) lecRK, an *Arabidopsis* RLK, with an extracellular domain resembling lectins; (E) CRINKLY4, a maize RLK with a region similar to tumor necrosis factor receptor and seven novel repeats; (F) PR5K, a RLK the extracellular domain of which resembles the pathogenesis-related protein PR5, and (G), a novel RLK from *Catharanthus roseus* (references are given the text). While the putative ligand-binding domains in the apoplast are highly divergent, RLKs have a conserved cytoplasmic protein kinase domain (PKD). The figure was modified after [109].

CLAVATA1

CLV1 belongs to the class of LRR-RLKs. By 1998, more than forty plant kinases had been identified in this group [6]. The extracellular LRR motif

has been described as a versatile peptide-binding domain. Hence, LRR-RLKs are believed to bind (poly)peptide ligands like animal receptor kinases do. Within a scaffold of positionally conserved leucines, variable residues confer specificity for different peptide ligands to the LRR motif [107]. Hence, the sequence of the extracellular ligand-binding domain does not provide any clues as to the nature of the respective peptide ligands, and the ligands for the LRR-RLKs have remained elusive. Genetic tools may now have allowed the identification of the ligand for CLV1.

All organs of the plant shoot are derived from the shoot apical meristem as meristematic cells divide continually and start to differentiate to form new organs. Meristem maintenance requires a balance between the proliferation and the differentiation of meristematic cells. In *Arabidopsis thaliana*, three mutants *clv(clavata)1*, *clv2*, and *clv3* have been described in which this delicate balance is disturbed. In these mutants, excessive meristem proliferation results in the formation of a club (latin: *clava*)-like structure. Genetic analyses revealed that the gene products of the two unlinked loci *CLV1* and *CLV3* act in closely associated steps, or else form a complex (*e.g.* a receptor/ligand complex), in the same signaling pathway [114-116]. Therefore, the identification of CLV1 as a LRR-RLK suggested CLV3 as a possible ligand of CLV1.

The recent cloning of two tagged alleles of *CLV3* provided strong evidence in support of this hypothesis [117]. *CLV3* was shown to encode an extracellular, 96-amino-acid protein. Expression of CLV3 in the uppermost cell layer of the shoot apical meristem was sufficient to control cell proliferation and differentiation across the entire meristem. Apparently, CLV3 produced in one region of the meristem acts on the CLV1 RLK located in another region of the meristem, *i.e.* CLV3 acts in a non-cell-autonomous manner [117]. These results are consistent with CLV3 being the ligand of CLV1 or, alternatively, a molecule involved in CLV1 ligand formation (*e.g.* a precursor).

While a direct biochemical interaction between CLV1 and CLV3 has not been demonstrated, there is strong evidence for an interaction *in vivo* [118]. It was shown that CLV1 exists in two protein complexes of either 185 or 450 kD. The 185 kD complex represents the inactive form of the receptor complex consisting either of a CLV1 homodimer or possibly of a CLV1/CLV2 heterodimer [118,119]. In presence of functional CLV3, the 185 kD complex recruits additional protein components (a protein phosphatase and a small GTP-binding protein) and is converted into the

450 kD form which represents the activated receptor complex. Apparently, binding of CLV3 leads to activation of CLV1, phosphorylation, and association with other signaling factors [118,120].

S-locus Protein Kinase

Most higher plants have hermaphroditic flowers, containing both male (anthers) and female (stigma, style, and carpel) organs. While this arrangement facilitates the transfer of pollen from one flower to the other by pollinating insects, it also promotes self-pollination. Self-pollination, however, leads to inbreeding and, consequently, to a reduction in gene-flow. Thus, plants have evolved different strategies to prevent self-fertilization, including the spatial separation of male and female flower parts as well as a separation in time of the maturation of male and female sex organs. Furthermore, many plants are able to identify and reject their own pollen, a phenomenon that has been described as self-incompatibility (SI) [121,122].

SI is controlled genetically by the highly polymorphic S locus [123]. In *Solanaceae*, the S locus encodes an allele-specific ribonuclease expressed in female flower tissues which is thought to provide the biochemical basis for the rejection of pollen carrying the same allele [124-126]. In *Brassica* (cabbage), the biochemical basis of pollen rejection is not known yet, but again, SI is controlled by the S locus complex, a highly polymorphic cluster of genes [127,128]. When a pollen grain is deposited on the surface of the stigma containing the same S allele as the pollen, an incompatible reaction leading to pollen rejection is triggered by action of the S locus gene products. Apparently, the S locus gene product expressed in the stigma recognizes the S locus gene products present in the pollen. Two of the S locus genes, those for the S locus glycoprotein (SLG) [129] and the S-locus receptor kinase (SRK) [92,93,95], are required for the phenotypic expression of SI [130]. They are co-expressed in female tissues and absent from male reproductive tissues [131], and have been suggested to interact functionally as the female determinant of self-incompatibility [131]. While SLG appears to have a stabilizing function, SRK itself is viewed as the receptor for the male determinant of self-incompatibility in pollen (for review see [121]). In a recent paper, Schopfer et al. describe a secreted pollen protein called

SCR (S locus cysteine-rich protein) and propose it to be the ligand of SRK [132].

The *SCR* gene was identified as a single copy gene located between *SRK* and *SLG* at the polymorphic S locus. Loss-of-function and gain-of-function studies showed that the *SCR* gene product is both necessary and sufficient to determine the specificity of SI [132]. *SCR* gene expression was found to be restricted to anthers. Three *SCR* genes were sequenced. They were shown to code for cysteine-rich, small (8.4 to 8.6 kD), basic (isoelectric point of 8.1 to 8.4), secreted proteins. Apart from the signal sequence at the N-terminus and 8 conserved cysteine residues, sequence conservation at the SCR amino acid level was found to be very limited. The cysteines of SCR were proposed to engage in disulfide bridges, resulting in a protein fold with highly divergent, exposed surface loops. The extensive sequence divergence and the pattern of expression are consistent with SCR being the male determinant of SI [132]. While a direct interaction of SCR and SRK resulting in the activation of the receptor and downstream signaling remains to be shown, the data strongly support the hypothesis that SCR is the ligand of SRK. Hence, SCR appears to be a new plant peptide hormone involved in the determination of self-incompatibility.

Processing Proteases

Plants do not only possess the tools for the perception of peptide signals, they also have enzymes potentially involved in the processing of peptide prohormones. The existence of such enzymes provides additional indirect evidence for a general role of peptide hormones in plant signal transduction processes.

In animals, most (poly)peptide hormones, growth factors, and neuropeptides are generated from larger, biologically inactive precursor proteins. The close examination of precursor primary structures indicated that the active peptides are flanked by pairs of basic amino acids. Therefore, the maturation of precursors to release the active peptides was postulated to involve limited endoproteolytic processing at sites marked by pairs of basic residues followed by the exoproteolytic trimming of the peptide termini [133]. The search for proteases with the required substrate specificity resulted in the initial discovery of the yeast kex2 protease (kexin), which is necessary for the maturation of the α-mating factor

pheromone, followed by the identification of seven related proteases in mammals called proprotein convertases (PCs). PCs are critically involved in the processing of polypeptide precursors of hormones, growth factors, neuropeptides, receptor proteins, bacterial toxins, and viral glycoproteins. The function of PCs in proprotein processing has been reviewed extensiveley [7,134-139].

Mammalian PCs, just like kexin, cleave their substrates carboxy-terminal of paired basic residues and they share a conserved catalytic domain resembling that of bacterial subtilisins. The catalytically important residues Asp, His, and Ser are arranged in the catalytic triad in a way that is typical for subtilisins but distinct from the arrangement found within the (chymo)trypsin clan of serine proteases. The subtilisins and (chymo)trypsins have thus served as a prime example of convergent evolution [140,141].

Until recently, proteases of the subtilisin clan of serine proteases (subtilases) were thought to be restricted to prokaryotes. The discovery of the PCs, *i.e.* mammalian subtilases, greatly stimulated the interest in these enzymes. As of 1997, 200 subtilases were known and their number is steadily growing [141]. They have been grouped into six distinct families, the subtilisin, thermitase, proteinase K, lantibiotic peptidase, pyrolysin, and kexin families (Fig. (**6**)). Four of these families are restricted to micro-organisms while only the pyrolysins and kexins are found in both micro-organisms and higher eukaryotes and these are the two families that are relevant to the following discussion of plant enzymes possibly involved in the generation of peptide signals.

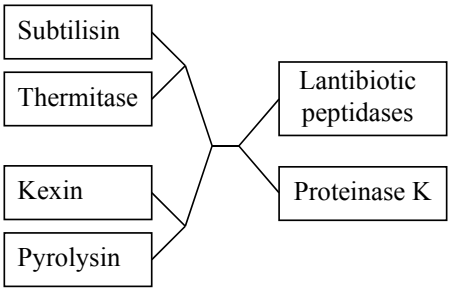

Fig. (6). Families of subtilases within the clan of subtilisin-like serine proteases. A general layout of the relationship between the six subtilase families is shown. The dendrogram is based on a sequence alignment of the catalytic domains only. The figure was modified after [141].

Several lines of evidence point to the existence of plant proteases related to mammalian PCs in both structure and function. In transgenic plants, the existence of a kexin-like activity was demonstrated. Tobacco plants were engineered to overexpress the precursor of the KP6 killer toxin. The KP6 preprotoxin is encoded by a double-stranded RNA virus which is present in some natural isolates of *Ustilago maydis*, a fungal pathogen of maize. In *U. maydis*, the processing of the KP6 protoxin to release the active toxin requires a kexin-like protease within the secretory pathway [142]. In transgenic tobacco plants overexpressing the prepro-toxin, correct processing of the precursor and secretion of the toxin was observed indicating the presence of a kexin-like activity in the secretory pathway of tobacco plants [143,144]. The protease was later shown to be a Golgi-resident enzyme and the kexin-like substrate specificity was confirmed [145].

A protein (SBP50) was identified in preparations of tomato leaf membranes that interacts specifically with the wound signal systemin. Competition experiments with a series of Ala-substituted systemin derivatives identified those amino acids within the systemin primary structure relevant for the interaction with SBP50 [25]. They constitute a sequence motif typically found in furin, a PC with an extended sequence requirement for substrate recognition [146,147]. A possible function of SBP50 as a PC-like protease was supported by the observation of an immunological relationship between SBP50 and a PC from *Drosophila*. Also, processing of systemin on the carboxy side of the central dibasic (Lys9-Arg10) pair indicated the presence of a PC-like activity in tomato plasma membrane preparations [25,27].

While the data suggest the existence of a kexin-like protease(s) in plants, ultimate proof remains to be provided. The situation is reminiscent of that in animals where the existence of proprotein convertases had been postulated already in the early 1960s. Nonetheless, the hunt for these proteases lasted for three decades and more. The PCs evaded all attempts of biochemical characterization and purification. The breakthrough came in 1984 with the identification of kexin by genetic complementation of a yeast mutant. The discovery of kexin paved the way for the identification of mammalian PCs by molecular biological techniques exploiting the sequence conservation within the catalytic domains [148]. The same rationale was used in an attempt to identify PCs in plants. The most highly conserved regions surrounding the catalytically important residues were identified by sequence comparison of kexin and mammalian PCs.

Oligonucleotides were derived from these regions and employed as primers in the polymerase chain reaction (PCR) to amplify related sequences from the tomato genome. The gene family of subtilases was found to comprise a minimum of 15 members in tomato plants and its complexity thus exceeds that of mammalian PCs [8]. In contrast to mammalian PCs, however, tomato subtilases belong to the pyrolysin rather than the kexin family of subtilases. The observation that no kexins were isolated while the PCR primers used in these experiments were actually based on sequences conserved among kexins may indicate that kexins do not exist in tomato. This notion is corroborated by the fact that the *Arabidopsis* genome project, while being close to completion, did not yield any sequences related to kexin-like subtilases.

Pyrolysins are more closely related to bacterial subtilisins than kexin and they were thought to share with subtilisins their broad substrate specificity resulting in a degradative function rather than a role as processing proteases. The recent discovery of two mammalian pyrolysins indicates that this is not necessarily so. The site-1 protease (S1P) from hamster is a pyrolysin involved in the regulation of lipid composition of animal cells [149]. It participates in the activation of a transcription factor (nuclear sterol regulatory element binding protein, nSREBP) by cleaving one (site 1) of two processing sites in the nSREBP precursor. S1P cuts between Leu and Ser of the site-1 processing site Arg-Ser-Val-Leu-Ser. Recognition requires the Arg and Leu residues while Ser and Val could be replaced with Ala without reducing cleavage efficiency [150]. A second pyrolysin called subtilisin/kexin-isozyme 1 (SKI1) was cloned from man, rat and mouse. SKI1 exhibited a substrate specificity similar to that of S1P - cleaving pro-brain-derived neurotrophic factor (pBDNF) between Thr and Ser of the sequence Arg-Gly-Leu-Thr-Ser - but different from the PC specificity [151]. Apparently, processing proteases in mammals include members from both, the kexin and the pyrolysin family of subtilases. Considering the likely absence of kexins from higher plants, their respective functions would have to be carried by pyrolysins alone. As a matter of fact, there is increasing evidence for plant pyrolysins playing a role both in protein processing as well as in protein degradation.

The first pyrolysin to be cloned from a higher plant was cucumisin from *Cucumis melo*, an extracellular protease highly abundant in melon fruit [152]. Cucumisin was shown to have a broad substrate specificity in that it cleaves a variety of small peptide substrates and eight peptide bonds within the oxidized insulin B chain [153-155]. A similar, broad

specificity was also observed for related subtilases from *Helianthus annuus*, *Maclura pomifera*, and *Taraxacum officinale* [156-158]. Therefore, these proteases are likely to serve degradative functions during fruit ripening and leaf senescence, respectively. Several cDNAs encoding pyrolysins have been cloned from *Alnus* [159], *Arabidopsis* [159,160], and *Lilium* [161], but the respective enzymes have not been characterized and their functions remain unclear.

The so far most comprehensive analysis of plant subtilases has been done in tomato. Fifteen genes were identified in the haploid tomato genome which fall into 5 distinct subfamilies including the P69, tmp, *Le*SBT1, *Le*SBT2, and *Le*SBT3/4 subfamilies (Fig. (**7**); [8]. Members of the P69 subfamily were initially identified as inducible components of the plant defense response triggered by pathogen infection [162-164]. Later, P69A and P69D were suggested to play a role in plant development, while P69B and P69C were shown to be expressed following pathogen infection [165] and treatment with salicylic acid or the fungal toxin fusicoccin [165,166]. For P69E and P69F, a highly specific expression has been described in the root tissue and in hydathodes, respectively [167]. Unfortunately, the substrate specificity of the P69 subtilases has not been characterized and - with the notable exception of LRP - a tomato cell wall protein of unknown function, none of the *in vivo* substrates has been identified [168]. Therefore, a function for the P69s as either degradative or processing proteases remains to be established.

The tomato protease tmp is highly similar to LIM9 from *Lilium logiflorum* [161,169]. This protease has been identified as an extracellular protein which is differentially expressed in anthers during late stages of microsporogenesis [161]. The highly specific pattern of expression points to a very restricted role of LIM9 in pollen development, and possible functions in the remodeling of the extracellular matrix and tapetal cell apoptosis have been discussed [161]. Unfortunately, as for the P69s, the specificity *in vitro* and the substrates *in vivo* remain to be identified. The latter is also true for enzymes of the *Le*SBT1, *Le*SBT2, and *Le*SBT3/4 subfamilies. One of these subtilases however, *Le*SBT1, has been overexpressed in the baculovirus/insect cell system and the recombinant enzyme characterized biochemically [170]. *Le*SBT1 was shown to be an extracellular protease that is secreted in form of an inactive zymogen. Zymogen activation requires the sequential processing of the signal peptide for targeting to the secretory pathway, the prodomain, and a 21-amino-acid auto-inhibitory peptide at the amino terminus [170]. In

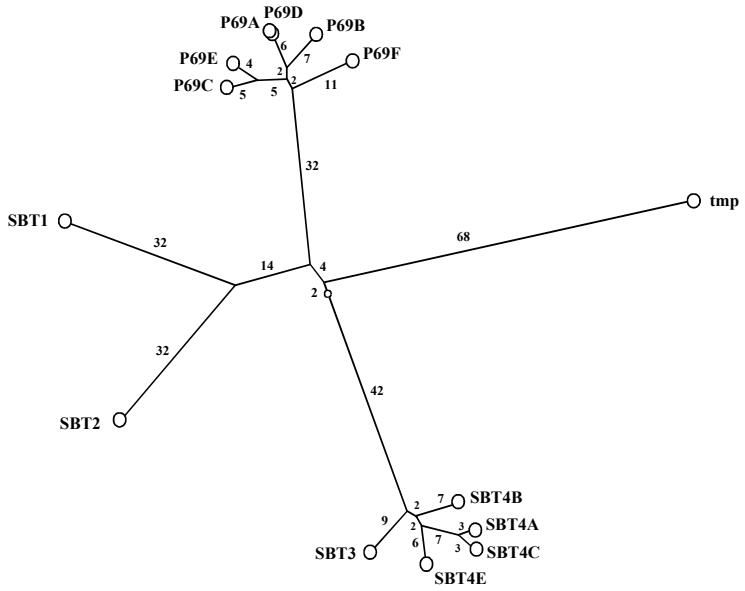

Fig. (7). Phylogenetic relationship of tomato subtilases. An unrooted phylogenetic tree is shown based on the amino acid sequences deduced from tomato subtilase genes and cDNAs. Numbers indicate PAM distances (accepted point mutations per 100 residues) between sequences. The figure was modified after (8).

contrast to other plant subtilases, *Le*SBT1 exhibited a narrow substrate specificity cleaving polypeptide substrates preferentially but not exclusively carboxy-terminal of Gln residues. These properties make *Le*SBT1 a likely candidate for a proprotein processing protease potentially involved in the generation of a peptide signal, as opposed to an enzyme with merely degradative function.

Further evidence for a role of plant pyrolysins in signaling processes was provided by the analysis of the *sdd1* mutant in *Arabidopsis*, which is affected in the density of leaf stomata. The *SDD1* gene was isolated by positional cloning and found to encode a subtilase. Loss of *SDD1* function resulted in an increase in stomate density suggesting a role for the protease in pattern development (T. Altmann; Max Planck Institute of Molecular Plant Physiology, Golm, Germany; pers. communication). The major challenge for future work will be the identification of the *in vivo* substrates of SDD1 and *Le*SBTs. These substrates may well include the precursors of endogenous bioactive peptides with signaling function in plant growth and development.

EXOGENOUS PEPTIDE SIGNALS

Plants, being sessile organisms, have to be able to adapt to the ever-changing environment with appropriate biochemical responses. A prerequisite for adaptation is the ability to perceive these changes which include biotic as well as abiotic stress factors. Microbial infection is a particularly threatening form of biotic stress. Consequently, plants have evolved to sense the presence of a pathogen and to react to infection with appropriate defense responses including the development of resistance. Hence, a surveillance system must be present which - similar to the phenomenon of self-incompatibility (discussed above) - allows the distinction between "self" and "non-self" [9,10]. Two types of resistance are known and are referred to as general and race-specific resistance, respectively. For the induction of a general resistance response, a structure has to be recognized by the plant cell that is absent from plant cells but common to a wide range of microorganisms [9,171]. In contrast, race-specific resistance depends on the presence of a specific protein in a certain race only of a microbial species (*i.e.* the avirulence (*avr*) gene product) and a corresponding protein in the resistant plant species (*i.e.* the resistance gene product). This type of interaction is described by the gene-for-gene concept [172-176]. In general resistance and in race-specific resistance as well, it is a molecule produced by the microorgnism (or generated by an enzyme produced by the microorganism) that is recognized by the plant cell as being foreign, followed by the induction of a defense response. Such molecules have been referred to as elicitors [10, 177]. Elicitors can be (poly)peptides, carbohydrates, lipids, small secondary products, or a combination thereof. The structural diversity of elicitors is immense and beyond the scope and intention of this review. Thus, the following discussion will be restricted to microbial (poly)peptide elicitors of plant pathogen resistance and will focus on a few examples that are most instructive in demonstrating the underlying principles.

General peptide elicitors

General Peptide Elicitors in Bacteria

Unlike vertebrates, plants do not have an immune system but they nevertheless are able to detect the presence of a pathogen and to respond

with a broad set of defense responses including localized cell death (*i.e.* the hypersensitive response, HR) and induced resistance against a broad array of pathogens (*i.e.* systemic acquired resistance, SAR). The first bacterial elicitor of HR and SAR to be characterized was harpin, a heat-stable, glycine-rich protein from *Erwinia amylovora* the causal agent of fire blight in apple and pear [178]. Subsequently, several harpins have been characterized in many different phyto-pathogenic bacteria ([179,180] and references therein). Harpins are extracellular proteins secreted via the Sec-independent type-III secretion system [181]. General characteristics of harpins include high glycine- and low cysteine-content, heat stability, low mobility during SDS-PAGE, and the ability of full-length and truncated polypeptides to elicit the HR ([182], and references therein). Harpins induce defense responses and resistance in a variety of non-host plants [180,182,183]. Hence, they are general elicitors in the sense of the above definition, *i.e.* elicitor activity does not depend on the presence in the infected plant (or cell culture) of a corresponding resistance gene. However, plants also respond to mutants lacking secreted harpins and to bacteria that lack the type-III secretion system [171,181]. Consequently, additional and more general structures must exist that are recognized by the plant cell. Very recently, the bacterial flagellum has been identified as such an elicitor-active structure [171].

The bacterial flagellum consists of a rotary motor anchored in the cell surface and a long, helical filament composed of multiple subunits of a single protein, flagellin. Within the flagellin protein, it is the most conserved region close to the N-terminus that is recognized by a specific chemo-perception system of the plant cell resulting in the activation of defense responses including an extracellular alkalinization, the oxidative burst, the HR, and the induction of pathogenesis-related gene expression [171,184]. As a convenient assay system to further characterize the response, elicitor-induced pH changes of the growth medium of tomato cell suspension cultures were monitored. In this cell culture system, flagellin was found to cause a rapid and transient alkalinization of the growth medium. A 22-amino-acid peptide (flg22) corresponding to the conserved N-terminal region retained full flagellin activity and causes half-maximal alkalinization at 30 pM. Competitive antagonists of flg22 also inhibited the response to flagellin or crude bacterial extracts from *Erwinia carotovora*, *E. chrysanthemi*, *Pseudomonas syringae*, *P. aeruginosa*, *P. fluorescens* and *E. coli* indicating that the flg22 epitope is the major if not the only determinant of recognition by the plant cell.

Interestingly, this epitope is not conserved in the flagellins from *Agrobacterium tumefaciens* and *Rhizobium meliloti*, two plant-associated bacteria. Accordingly, peptides corresponding to the respective regions of these flagellins did not exhibit elicitor activity in both the cell culture bioassay and in *Arabidopsis* plants. Apparently, the modification of the respective region within the flagellin protein, while retaining full flagellin functionality, enables *Agrobacteria* and *Rhizobia* to evade the plant chemo-perception system [171,184].

Treatment of *Arabidopsis* seedlings with flg22 resulted in growth arrest allowing for a convenient screen for natural and mutagen-induced genetic variation in this response. Three genetic loci designated *FLS1*, *FLS2*, and *FLS3* were identified and *FLS2* was identified by positional cloning [184]. Recently, the interesting finding was reported that *FLS2* encodes a putative LRR-RLK. *FLS2* thus resembles *Xa21* the rice gene conferring resistance to *Xanthomonas oryzae*, and CLV1 (discussed above), the LRR-RLK involved in the regulation of meristem proliferation [185]). It therefore seems likely that FLS2 is a functional receptor of flagellin or fragments thereof.

General Peptide Elicitors in Fungi

General resistance against *Phytophthora*, a widespread phyto-pathogenic oomycete, is elicited by a family of small (10 kDa) secreted proteins called elicitins [10,186]. *Phytophthora* elicitins, while highly similar, have been grouped in acidic α-elicitins (*e.g.* capsicein, parasiticein, α-megaspermin) and basic β-elicitins (*e.g.* cryptogein, cinnamonin, β-megaspermin). The β-elicitins, and cryptogein in particular, are more potent elicitors of general defense responses (including HR and SAR) than the α-elicitins [187-189]. Consequently, most studies concentrated on the highly active cryptogein. The threedimensional structure of cryptogein has been elucidated by both X-ray crystallography and by NMR in solution yielding nearly identical results. The overall structure has a novel fold comprising three disulfide bridges, six α-helices, and a beak-like motif composed of an antiparallel two-stranded β-sheet and an Ω-loop [190,191]. In tobacco plasma membranes, a single class of high affinity (K_d = 2 nM) binding sites was observed for [^{125}I]cryptogein [192]. A later study using [^{125}I]derivatives of four different elicitins revealed that binding relies on those amino acid residues that are conserved among

elicitins and that all elicitins bind to a common site in tobacco plasma membranes which is believed to be the elicitin receptor [188]. Binding to the receptor is thought to stimulate cellular responses including the influx of Ca^{2+}, extracellular alkalinization, acidification of the cytosol, depolarization of the plasma membrane potential, the oxidative burst, protein phosphorylation and changes in gene expression ([188,193-196], and references therein). While the characteristics of binding to tobacco plasma membranes were very similar for all elicitins, the activation of cellular responses correlated with the *in-vivo* activity of α-, and β-elicitins [188].

As discussed for bacterial elicitors of general defense responses, additional determinants of general resistance must exist in fungi, since elicitins are found in the entire genus *Phytophthora* and some *Pythium* species but are notably absent from many other phytopathogenic fungi [10]. These additional elicitors include glucans, chitin and chitosan oligosaccharides derived from the fungal cell wall, ergosterol, *i.e.* the main sterol in most higher fungi (reviewed in [197]), but also oligopeptide elicitors derived from fungal glycoproteins [198]. Such an elicitor and its interaction with cultured parsley cells have been studied in considerable detail. In parsley cell cultures, a surface glycoprotein from the soybean pathogen *Phytophthora megasperma* elicits typical non-host, general resistance responses including the influx of Ca^{2+}, the alkalinization of the apoplast, the depolarization of the plasma membrane potential, the oxidative burst, protein phosphorylation, defense gene activation, and phytoalexin production [198-201]. The elicitor activity was found to reside solely in the protein moiety of the 42-kDa fungal glycoprotein and could be confined to the 11-amino-acid core of a 13–amino-acid peptide (Pep13) within the C-terminal hydrophilic region of this protein. Systematic replacement with alanine identified two amino acids (Trp2 and Pro5) within Pep13 (V<u>W</u>NQ<u>P</u>VRGFKVYE) that are critical for elicitor activity [202]. Rapid, high-affinity binding of radiolabeled Pep13 to parsley plasma membranes was demonstrated. Binding of structural Pep13 analogues correlated well with their respective elicitor activities [202]. The binding site, which is thought to be the elictor receptor, was shown to reside in a ≅ 100-kDa membrane protein and was partially purified from parsley microsomal membranes [203,204]. Activation of the receptor by elicitor binding stimulates a plasma

membrane Ca^{2+}-permeable ion channel resulting in increased cytosolic Ca^{2+} which is necessary for subsequent cellular responses [205].

Notwithstanding, the structural diversity among peptide elicitors of general pathogen resistance and the obvious involvement of different receptor proteins, the initial cellular responses observed after receptor activation are very similar. The first responses include ion fluxes across the plasma membrane (Ca^{2+}/H^{+}-influx, K^{+}/Cl^{-}- efflux), resulting in a depolarization of the plasma membrane potential and an alkalinization of the apoplast. Hence, the responses mediated by general peptide elicitors closely resemble those triggered by the endogenous defense signal systemin [4,26,38-40,206]. In contrast, race-specific peptide elicitors (discussed below) cause a hyperpolarization of the plasma membrane potential and the acidification of the apoplast rather than extracellular alkalinization [175,207,208].

Race-specific Peptide Elicitors

As opposed to non-host resistance discussed in the previous two paragraphs, race-specific elicitors are involved in defense reactions that can be described genetically in terms of the "gene-for-gene" concept. This concept was developed in the 1940s by Flor [172,209] who observed that in the interaction of flax with the flax rust fungus *Melampsori lini* the development of resistance depends on the presence of two dominant genes, the *avr* (*avirulence*) gene in the fungus and the corresponding *R* (*resistance*) gene in the plant. As a biochemical basis for the gene-for-gene concept, a ligand-receptor model of disease resistance was proposed. According to this model, resistance develops as a consequence of the activation of a receptor (*i.e.* the *R* gene product) by interaction with its ligand, the race-specific elicitor. The elicitor may be produced by the action of an *avr* gene-encoded enzyme or else, the *avr* gene product itself has elicitor activity. In the latter case, the *avr* gene product qualifies as a bioactive peptide in the sense that it elicits specific cellular responses. The cloning of many *avr* genes and, more recently, the cloning of matching plant resistance genes provided good evidence in support of this model. It cannot be the aim of this review to cover this area of research comprehensively. This has been done in recent review articles [176,210-216].

Two examples have been chosen for discussion, *i.e.* the interactions of tomato with *Pseudomonas syringae* pv. tomato and with *Cladosporium fulvum*, a bacterial and a fungal pathogen, respectively. These two plant pathogen interactions are among the few that have been characterized not only in genetic terms but also at the molecular and biochemical levels and they are thus well suited for a discussion of the fundamental principles.

Race-specific Peptide Elicitors in Bacteria

Most plant pathogens propagate in the extracellular space of the host. Hence, R proteins were expected to be cell surface receptors of extracellular signals. While this appears to be true for Xa21 (the product of the rice *R* gene for *Xanthomonas oryzae* resistance [98]), most *R* genes for bacterial pathogens rather encode cytoplasmic proteins [212,217]. This finding was difficult to reconcile with the receptor/ligand model of plant disease resistance. Upward of 40 *avr* genes have been cloned from bacteria. Most of them encode hydrophilic proteins that lack signal sequences for secretion [218,219]. Avr proteins do not induce the HR when injected into leaves of plants possessing the corresponding *R* gene. Only living bacteria carrying the *avr* gene are able to induce the resistance response. Apparently, avirulence depends on additional factors [219]. Additional genes required for avirulence are located in the *hrp* (hypersensitive response and pathogenicity) cluster. Several of the *hrp* genes code for components of the contact-dependent type III secretion system. In some mammalian pathogens (*e.g.* strains of *Yersinia*, *Salmonella*, *Shigella*), type III protein translocation complexes function in the translocation of bacterial proteins into the cytoplasm of target cells of the host [219]. Therefore, bacterial avirulence factors are now believed to be delivered directly into the host cells by the type III secretion apparatus [220-226]. The data imply that these race-specific elicitors are perceived intracellularly and they are in good agreement with the surprising finding that plant *R* genes for bacterial pathogens – *i.e.* putative receptor proteins for the *avr* gene products – code for cytosolic proteins as well. While this is also true for *Pto*, the tomato *R* gene that confers resistance to *Pseudomonas syringae* carrying the corresponding avirulence gene *avrPto* [216,227], *Pto* differs from other *R* genes for bacterial pathogens in that it does not encode a protein comprising a leucine rich repeat (LRR) domain and a nucleotide binding site, but rather an intracellular

serine-threonine protein kinase lacking any obvious receptor domain [227,228]. Nevertheless, the *Pto-avrPto* system is the only one for which a direct interaction of the respective R and avr proteins has been demonstrated. Pto and avrPto were found to interact specifically in the yeast two-hybrid system, an assay which tests for interaction of two proteins by reconstitution of a functional transcription factor. Furthermore, mutations that disrupt the interaction resulted in a loss of avrPto recognition within the plant cell [229,230]. Thus, formation of the Pto-avrPto complex is necessary for the resistance response but it is not sufficient, since the activation of cellular responses requires Prf, a second serine-threonine protein kinase [228,231-233].

Like most other bacterial *avr* genes, *avrPto* was identified by selecting for clones in a bacterial DNA library that confer an avirulent phenotype to a *Pseudomonas syringae* strain that is normally virulent on tomato cultivars carrying the *Pto* resistance gene [234]. *AvrPto* encodes mRNAs of 0.7 and 0.75 kb whose translation product is a hydrophilic 164 amino acid protein of 18.3 kDa. AvrPto bears no similarity to other proteins in the GenBank and EMBL databases [235]. Likewise, the deduced protein products of other bacterial *avr* genes range from 18 to 100 kDa in size and lack substantial sequence similarity to proteins of known biochemical activity or motifs indicative of specific functional domains [219]. Hence, the biochemical function of these *avr* gene products remains unknown. It is believed that bacterial avr proteins play a role in pathogenicity in the compatible plant/pathogen interaction. Once recognized by the R protein as a component of the plant surveillance system, however, they become avirulence factors [212]. The *avrBs2* gene of *Xanthomonas campestris* pv. *vesicatoria* provides an example in support of this hypothesis. Deletion of this *avr* gene causes a reduction in pathogenicity confirming a role as virulence factor on a compatible host [236,237]. AvrBs2 bears resemblance to *Agrobacterium tumefaciens* agrocinopine synthases and may play a role in the *Xanthomonas* adaptation to the host extracellular space, or it may provide the bacterium with a carbon and nitrogen source during colonization of the host plant [212,237].

Race-specific Peptide Elicitors in Fungi

The interaction of tomato with the leaf mould fungus *Cladosporium fulvum* is a typical gene-for-gene relationship. The development of

resistance depends on the presence of a matching *avr* gene for each *Cf* resistance gene [210,215,238]. *Avirulence* gene products are race-specific elicitors. They have been detected in the apoplastic fluid of *C. fulvum*-infected tomato plants [239]. AVR4 and AVR9 proteins were isolated from apoplastic fluids based on their ability to elicit a hypersensitive reaction on tomato plants carrying the corresponding *Cf-4* and *Cf-9* resistance genes, respectively. Both AVR4 and AVR9 are synthesized as prepro-proteins that are processed upon secretion to yield mature proteins of 86-88, and 28 amino acids, respectively [240-242]. The production of mature AVR9 involves both fungal and plant proteases [243]. AVR4 and AVR9 are characterized by 8 and 6 cysteine residues, respectively, that are engaged in the formation of disulfide bridges. Disulfide bridge formation and the rigidity of the resulting structure are crucial for the function of the proteins as avirulence factors [244]. For AVR9, the threedimensional structure as well as the structural requirements for elicitor activity have been determined. The polypeptide forms a so-called cystine knot in solution in which the Cys3-Cys6 disulfide bond penetrates a ring formed by the Cys1-Cys4, and Cys2-Cys5 disulfide bonds and the intervening polypeptide chain [245,246]. This structural motif is common among inhibitory and toxic polypeptides [247]. The rigid structure is thought to provide resistance against degradation by plant and fungal proteases present in the extracellular space [238,244]. Systematic substitution with alanine and the synthesis of mutant peptides revealed the hydrophobic residues present in both solvent-exposed surface loops as relevant for elicitor activity [248,249].

The AVR9 peptide was labeled with iodine-125 at the N-terminal tyrosine residue, and ^{125}I-AVR9 was used to identify a high-affinity (K_d = 0.07 nM) binding site (HABS) in tomato plasma membrane fractions [250]. According to the receptor/ligand model of gene-for-gene interactions, one would expect the HABS to be identical with Cf-9, the resistance gene product. Surprisingly, however, the binding site was shown to be present in both resistant and susceptible tomato cultivars, as well as in other *Solanaceae* [250] showing that Cf-9 is not the AVR9 receptor *per se*. Nevertheless, the HABS appears to be required to initiate the resistance response, since a positive correlation exists between the binding affinity and the necrosis-inducing activity of mutant AVR9 peptides [251]. Possibly, Cf-9 does not recognize AVR9 itself as its ligand but rather the AVR9/HABS-complex, and the interaction of all three components may be necessary to elicit the defense reaction.

Alternatively, the HABS may not be involved in the defense reaction but may rather represent the target of AVR9 as a virulence factor in the compatible plant/pathogen interaction. In the latter case, a second binding site for AVR9 must exist which may by formed by Cf-9 and a second signal-transducing component yet to be identified [238,251]. This model resembles the perception of CLV3 which is thought to be the endogenous peptide signal in the regulation of meristem proliferation/differentiation (*cf*. above, [238]). The perception of CLV3 appears to involve the formation of a heterodimeric complex including CLV1 (a LRR-RLK) and CLV2. CLV2 resembles Cf-9 in that it is a transmembrane protein comprising an extracellular LRR domain and a very short cytoplasmic domain [119]. Like CLV2, Cf-9 has an extracellular LRR domain but lacks a cytoplasmic domain with any obvious signaling function [252]. A similar structure was determined for the *R* gene products Cf-2, Cf-4, and Cf-5 [215,253-255], and the specificity for the avr protein was shown to reside in the extracellular LRR domains of Cf proteins [256]. Therefore, the LRR appears to provide the recognition element for the race-specific elicitors but an additional factor is obviously required for the activation of cellular responses. This factor may be the HABS, or else, a CLV1-like LRR-RLK. Biochemical data will be essential to either support or reject this model of AVR9 perception.

GENERAL CONCLUSIONS

The examination of general and race-specific elicitors of plant defense reactions revealed that plant cells are able to perceive a large number of structurally diverse peptide signals. It seems likely that during evolution the machinery to perceive and transduce exogenous peptide signals was not generated *de novo* but was rather recruited from pre-existing cellular signaling components. Thus, the recognition of exogenous peptide elicitors by the plant cell may indicate the presence of endogenous peptide signals and the corresponding signal perception/transduction machinery. The structural similarity between CLV1, a LRR-RLK involved in meristem maintenance, and FLS2 or Xa21, *i.e.* the putative receptors of exogenous peptide elicitors, provides support for this hypothesis. Likewise, structural similarity was observed between the resistance gene product Cf-9 and CLV2. Both proteins have been suggested to be part of a receptor complex and contain LRRs likely to

provide the ligand binding sites. Hence, LRRs appear to be versatile perception modules for extracellular and intracellular elicitors as well as for endogenous peptide signals.

In some cases, structural similarity is not restricted to the perception system for exogenous and endogenous peptides but rather extends to the signals themselves. The AVR9 peptide elicitor and SCR, the male determinant of self-incompatibility in *Brassica*, for example, are both small cysteine-rich proteins in which disulfide bridges provide a rigid structural scaffold for exposed surface loops. A comparison of systemin, an endogenous peptide signal for plant defense, with peptide elicitors of general resistance shows that even the initial cellular responses are highly similar. These observations, in addition to the presence *in planta* of receptor-like kinases and processing proteases as indirect evidence for a general role of peptide signals in intercellular communication in plants, all seem to indicate that the plant peptide signals identified thus far may just be the tip of the iceberg. Obviously, we are just beginning to unravel the complexity of peptide signaling in higher plants. The years to come will likely see the identification of new peptide signals as regulators of plant growth and development and promise exciting new discoveries in the elucidation of both signal perception and cellular responses.

ABBREVIATIONS

avr	=	avirulence
HABS	=	high-affinity binding site
hrp	=	hypersensitive response and pathogenicity
LRR	=	leucine-rich repeat
HR	=	hypersensitive response
PC	=	proprotein convertase
R	=	resistance
RLK	=	receptor-like kinase
SAR	=	systemic acquired resistance
SBT	=	subtilase
SCR	=	S locus cysteine-rich protein
SI	=	self-incompatibility
SLG	=	S locus glycoprotein
SRK	=	S locus receptor-like kinase
SWRP	=	systemic wound response protein

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