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review

Plant signalling peptides

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Key words: CLAVATA3, ENOD40, insulin-like protein, natriuretic peptide, phytosulfokine, signalling peptides, systemin, RALF.

Abstract

Biochemical and genetic studies have identified peptides that play crucial roles in plant growth and development, including defence mechanisms in response to wounding by pests, the control of cell division and expansion, and pollen self-incompatibility. The first two signalling peptides to be described in plants were tomato systemin and phytosulfokine (PSK). There is also biochemical evidence that natriuretic peptide-like molecules, immunologically-related to those found in animals, may exist in plants. Another example of signalling peptide is ENOD40, a product of a gene, which became active early in the root nodulation process following *Rhizobium* infection of legumes. Other predicted bioactive peptides or oligopeptides have been identified by means of genetic, rather then biochemical methods. The *Arabidopsis* CLAVATA3 protein is required for the correct organization of the shoot apical meristem and the pollen S determinant *S*-locus cysteine-rich protein (SCR also called *S*-locus protein 11, SP11).

The plant signalling peptides discovered so far are involved in various processes and play an important role in communication between cells or organs, respectively. This review will focus on these peptides and their role in intercellular signalling.

Introduction

In multicellular biological systems, cell–cell interactions are indispensable for coordinating cell growth and differentiation in various organs. In animal systems these intercellular communications are mainly mediated by chemical signals such as steroids, peptides and other small bioactive compounds. Peptides are probably the most commonly used signal molecules in animal systems. This is most likely to be a result of several factors: the ease with which structural variation can be introduced; the availability of a common secretion mechanism for peptides; and also the fact that the activity of peptide signalling molecules can be controlled by common processing and modification mechanisms. Dozens of signalling peptides have been identified and most are recognized by specific receptors anchored in the plasmalema of the recipient cells (Bisseling 1999).

Hormone is a term once applied in both botanical and zoological contexts. For animal physiologists it denotes any molecule, usually of small molecular mass, secreted outside cells and carried to specific target cells/organs by whose response they bring about a specific and adaptive physiological response. Hormones tend to be either water-soluble peptides and proteins, or steroids. In animals, polypeptide hormones classically fall into two major categories: endocrine hormones (Douglass *et al.* 1984) and membrane-anchored growth factors (Massague and Pandiella 1993). The endocrine polypeptide hormones, and some growth factors, are synthesized as preprohormones in the secretory

pathway of specialized cells and are sequestered in vesicles until released into extracellular compartments in response to appropriate physiological cues (Douglass *et al.* 1984). Processing of polypeptide hormones from larger prohormone precursors in vesicles is mediated by members of the endopeptidase family. The hormones are then transported to target cells, either nearby or long distances away, where they are recognized by specific receptors to initiate signalling pathways. Some well-known examples are: growth hormone, adrenocorticotropic hormone, oxytocin, vasopressin, calcitonin, parathormone, glucagons, insulin and endorphins (Douglass *et al.* 1984, Massague and Pandiella 1993). Polypeptide hormones are usually small, being derived from precursors that are 100-500 aa in length (Douglass *et al.* 1984). Some of these polypeptides are produced as a single copy from a precursor, such as insulin, but some precursors harbor several copies of the hormone, such as proencephalin, while still other precursors are processed to produce multiple hormones having different physiological roles, such as pro-opiomelanocortin (Massague and Pandiella 1993). The variability of structural conformations is critical for the high specificity of receptor binding that exists among hundreds of polypeptide hormones (Ryan *et al.* 2002).

A different scenario for polypeptide synthesis and release is found with membrane-anchored precursors of growth factors. Examples include TGF-α (Transforming Growth Factor, 50 aa derived from a 160 aa precursor), EGF (Epidermal Growth Factor, 53 aa derived from a 1207 aa precursor), and TNF-α (Tumor Necrosis Factor, 157 aa derived from a 233 aa precursor) (Massague and Pandiella 1993). The precursors of most members of this class of polypeptides are generally between 150 to 650 aa in length, although some are larger. They are synthesized through the secretory pathway; the proproteins, however, are not processed in vesicles but are anchored in vesicle membranes. The vesicles fuse with the plasma membrane of the cell, exposing the polypeptide to the extracellular space. Upon appropriate cues, membrane-associated proteases are activated that release the factors, which can be recognized by their receptors (Douglass *et al.* 1984, Massague and Pandiella 1993).

Plants are using small (phyto)hormones such as: auxins, cytokinins, gibberellins, abscisic acid, ethylene (Kende and Zeevaart 1997) and brassinosteroids (Bishop and Koncz 2002) for cell-to-cell communication. They work at low concentrations (excluding nutritious activity), and regulate growth and development of plants by modifying a gene expression and/or metabolic processes. Besides these "classical" hormones plants may also use oligo- and polypeptides as signalling molecules, which may play similar role to peptide hormones from animal and yeasts.

Systemin

Systemin, a polypeptide isolated from plant tissues (tomato leaves), is synthesized as a result of mechanic damage to plant organs or injury caused by an insect. It is able to move fast in plant and to induce genes encoding proteinase inhibitor I and II - one of the elements of a cascade leading to immunological reaction (Pearce *et al.* 1991).

Systemin regulates the activation of more than 20 defensive genes. During systemic reaction, the stimulation of MAP kinase (Mitogen Activated Protein Kinase) is followed by synthesis of trypsine inhibitors (Meindl *et al.* 1998, Pearce *et al.* 2001b). As a result of direct action of systemin on uninjured cells, the changes in cytoplasmic concentration of Ca^{2+} ions are observed. These changes cause the activation of phospholipase A (PLA_2), which catalyses the degradation of membrane ingredients leading to releasing of a free linolenic acid from membranes and converting it to jasmonic acid (Ryan 2000). Simultaneous increase in cytoplasmic Ca^{2+} concentration leads to modulation of plasma membrane H^+-ATPase and in consequence to membrane depolarization and changing of ion fluxes (Moyen *et al.* 1998, Ryan and Pearce 1998). In plants defensive mechanisms polyphenol oxidase, calmodulin and H_2O_2 are also involved. Accumulation of these compounds is observed after wounding or treatment with systemin or metylejasmonate (Constabel and Ryan 1998, Schaller 1999, Bergey and Ryan 1999, Ryan 2000, Orozco-Cardenas *et al.* 2001). Increased polygalacturonase activity under influence of systemin and as a result of leaf injury is also observed (Bergey *et al.*

1999). In plants and animals there are large analogies in signal transduction triggered either by wounding or pathogen. In case of plants, the signal molecule conveying this information is systemin, whereas jasmonic acid is responsible for direct physiological response (Ryan 2000). In animals, this function is played by cytokines and prostaglandins (Bergey *et al.* 1996, Barciszewski and Legocki 1997, Stratmann *et al.* 2000).

Systemin is a polypeptide consisting of 18-amino acids with the sequence
AVQSKPPSKRDPPKMQTD,
which is active when present at fentomoles per plant (Bergey *et al.* 1996). The cDNA and gene structures reveal that this polypeptide is present in proteolytic cleavages from the C-terminal region of a 200-amino acid precursor called prosystemin (Table). The gene coding of the prosystemin is composed of 11 exons containing five repeated regions in central region (McGurl and Ryan 1992). It was also shown that as a result of prosystemin proteolysis both molecules of systemin and longer polypeptides could appear. Both prosystemin and all products of its proteolysis (containing an 18-amino acids systemin fragment) reveal biological activity (Dombrowski *et al.* 1999). It was affirmed however, that extracts from tomato leaves lack the potential enzymes that could separate systemin from prosystemin.

Li and Howe (2001) isolated another prosystemin (prosys B) from tomato leaves, which varied from the earlier one in positions of a few amino acids and resulted from an alternative splicing.

It was shown recently that the polypeptides similar to systemin also function in other species of Solanaceous family beside tomato (potato, black nightshade and bell pepper) (Constabel *et al.* 1998). The identity of amino acids sequences of systemin-like polypeptides in these species ranges from 73 to 88 % (Constabel *et al.* 1998). Recently, two polypeptides have been found in tobacco and, because of their biological activity, are called systemin I (Tob Sys I) and II (Tob Sys II) (Pearce *et al.* 2001a). As in the case of tomato these two kinds of systemin in tobacco are also built of 18 amino acids. However, there are some chemical differences between these two types of systemin from both plants. The molecules of Tob Sys I and II are linked to pentose sugars, removal of which deprives them of their bi-

Table. Properties of known plant signalling peptides.

Peptide name	Function	Precursor	Signal sequence	Size of peptide	Bidnding protein or receptor(s)
Systemin	defensive responses	164-165, 200 aa (depend on species)	No	18 aa	LLR receptor kinase (160 kDa)
PSK-α	cell proliferation	89 aa	22 aa	4-5 aa	LLR receptor kinase (120 kDa)
CLAVATA3	shoot meristem organization	96 aa	18 aa	78 aa	CLV1 and CLV2 LLR receptor kinase complex (185 kDa)
SCR/SP11	self-incompatibility	74-77 aa	Yes (no detailed data)	50-59 aa	SRK receptor kinase (110 kDa)
ENOD40	root nodulation	No	No	10-13, 24, 27aa (depend on species)	bind to Nodulin100 subunit of SuSy
RALF	inhibition root growth and seed germination	115 aa	25 aa	45 aa	binding protein (120 kDa and 25 kDa)
IrPNP	regulation of ion transport and transpiration	Yes (no detailed data)	?	?	?
Leginsulin (insulin-like peptides)	?	Yes (no detailed data)	?	32 aa	Bg protein

abbreviations: aa – amino acids, No – lack, LLR – leucine-rich repeats, ? – unknown

ological activity. Moreover, tobacco systemin contains hydroxylated prolines. Both systemin molecules are the products of a single gene encoding 165 amino acids prepropeptide, which has a signal sequence on its N-terminus (Pearce *et al.* 2001a). Besides the gene encoding this prepropeptide, another gene was also found in tobacco (Pearce *et al.* 2001a). It encodes a 164 amino acid peptide that contains 18 amino acids systemin sequence. Because it was identical to Tob Sys I (except for having Ala instead of Thr at 3 position) it was called Tob Sys Ia (Pearce *et al.* 2001a). Besides some differences in chemical structure, systemin from tomato and from tobacco are similar in terms of their functional properties (Lindsey *et al.* 2002).

The prosystemin is synthesized within cells surrounding vascular bundles of leaves, stalks and shoots. It was also established that systemin could move relatively fast through phloem from injured places to other plant organs (Narvaez-Vasquez *et al.* 1995), where it bound to the receptor proteins responsible for physiological response (Bergey *et al.* 1996). In studies conducted on cell suspension from tomato, it was found that the protein receptors (of 160 kDa molecular weight) were located on a surface of a plasma membrane and that they bound systemin. It was shown that plant systemin-binding protein was similar to animal cytokine and growth factor receptors (Stratmann *et al.* 2000). Scheer and Ryan (2002) found that it was a member of a family of the leucine-rich repeats (LRR) receptor kinases. It had an extracellular (N-terminal) systemin-binding domain, a transmembrane domain, and a cytoplasmic (C-terminal) kinase domain (Meindl *et al.* 1998, Scheer and Ryan 1999, 2002). Furthermore, extracellular domain of this receptor exhibits a protease activity. On this basis it is assumed that when the systemin fulfills its physiological function, it undergoes degradation by its own receptor (Leon *et al.* 2001).

Phytosulfokines

For many years it was well known that the relative growth rates of suspension-cultured cells depended strictly on the initial cell density (Stuart and Street 1969). Even if sufficient amount of auxin and cytokinin is present in the medium, the initial cell density in the growth medium has to be higher than 10,000 cell/ml. It was shown that cells in low-density cultures rapidly proliferated after media obtained from rapid dividing, high-density cells cultures were added to that culture. It was assumed on this basis that dividing cells secreted a specific factor into their environment. This factor maintains the mitotic activity of whole cell culture (Matsubayashi and Sakagami 1996).

Matsubayashi and Sakagami (1996) were the first who isolated two active factors, termed phytosulfokines (PSK-α and PSK-ß) from conditioned medium derived from mesophyll cell culture of asparagus (*Asparagus officinalis* L.). They promoted cell division at nanomolar concentrations (10^{-8}–10^{-9} M) even as low initial cell densities as 320 cells/ml (Matsubayashi and Sakagami 1998). The presence of PSK-α in suspension-cultured asparagus mesophyll cells was detected immunologically in the first 48 hr after initiating the cultures and 48 hr before the first cell division was observed (Matsubayashi *et al.* 1999b).

PSK-α is probably necessary for plant cell proliferation *in vivo* as well as *in vitro* (Yamakawa *et al.* 1999, Yang *at al.* 2000). It promotes somatic embryogenesis of carrot cells (Kobayashi *et al.* 1999, Hanai *et al.* 2000b) and a tracheary element differentiation of *Zinnia* mesophyll cells (Matsubayashi *et al.* 1999b) in the presence of adequate concentrations of auxins and cytokinins. (Matsubayashi *et al.* 1999a). Therefore, it was proposed that mature PSK was secreted from individual cells in response to changes in the levels of auxin and cytokinin (Yang *et al.* 2001) and that it played a role as an autocrine-type growth factor regulating cellular dedifferentiation and proliferation in plants. PSK-α promotive effect on chlorophyll synthesis in etiolated cotyledons of cucumber as well as on a growth and a chlorophyll content of *Arabidopsis* seedlings in conditions of a high nighttime temperature was also shown (Yang *et al.* 2001). PSK-α stimulates adventitious root formation in cucumber hypocotyls and adventitious bud formation in *Antirrhinum majus* (Yamakawa *et al.* 1998).

During recent years peptides identical with PSK-α have been purified from media derived from at least 12 plant cell lines including dicots (Matsubayashi

et al. 1999b, Hanai *et al.* 2000b) and monocots (Matsubayashi *et al.* 1997), suggesting that they are widespread in higher plants (Yang *et al.* 1999a,b).

PSK-α is a disulfated pentapeptide [H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH] and PSK-ß is a C-terminal-truncated tetrapeptide [H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH], which probably is derived as degradation product of PSK-α, having lost the C-terminal Gln, probably by carboxypeptidase cleavage (Matsubayashi and Sakagami 1996). Deletion of the sulphate groups of Tyr^1 and Tyr^3 resulted in nearly full loss of PSK-" activity, indicating that the modification of both amino acids was very important for biological activity. Synthetic analog of PSK-α without the first and the second C-terminal amino acids retained 8 % and 20 % of the activity, compared with native PSK-α, respectively. In contrast, an N-terminal truncated analog exhibited little or no activity, suggesting that the N-terminal sulphated threepetide is the active core (Matsubayashi and Sakagami 1996). As mentioned above, also in animal sulphated tyrosines are structures found in peptides. The examples of compounds of that kind are neuropeptides. Removal of these residues from these oligopeptides leads to loss of their biological activity (Hanai *et al.* 2000a). It seems that PSKs are the only example of post-translational sulphated tyrosine residues in plants.

PSK-α derived from a larger precursor polypeptide and its amino acid sequence is perfectly conserved among various species (Yang *et al.* 1999b). Synthesis of PSK-α seems to be similar to animal polypeptide hormones and growth factors. The rice PSK gene (*OsPSK*) is a single-copy gene consisting of one large intron and two exons (Yang *et al.* 2000). Analysis of this sequence indicates that it codes PSK-α precursor - preprophytosulfokine (PP-PSK) containing 725 base pairs. The gene is encoding an 89-amino acid polypeptide (9.8 kDa) with 22-amino acid hydrophobic N-terminal signal peptide (Table), commonly found in animal hormone precursors. The N-terminal signal peptide is supposed to mediate translocation across membranes of the endoplasmic reticulum during prohormone synthesis and to allow its secretion into the extracellular space. The PSK sequence occurs only once in this sequence and is located at the C-terminus (Yang *et al.* 1999b). PSK gene is expressed in the leaf, apical meristem, hypocotyl, and root of seedlings, as well as in cultured cells (Matsubayashi *et al.* 1999a).

Four genes, *AtPSK1–AtPSK4*, encoding precursors of PSK-α have been identified in *Arabidopsis* (Yang *et al.* 2000, 2001). Analysis of *AtPSK2* and *AtPSK3* cDNAs, shows that both of these genes consist of one intron and two exons. Both *AtPSK2* and *AtPSK3* encode PSK-α precursors (Yang *et al.* 2001). Each protein has a probable secretion signal at the N-terminus and a single PSK sequence close to the C-terminus. Both precursors contain dibasic processing sites flanking PSK, analogous to animal and yeast prohormones. Although the PSK domain including the sequence of PSK-α and three amino acids preceding it are perfectly conserved, the precursors have limited similarity among *Arabidopsis* and rice (*Oryza sativa*) (Yang *et al.* 1999b, 2000, 2001). This suggests a new level of diversity among polypeptides that are processed into the same signalling molecule in plants, a scenario not found in animals and yeast (Yang *et al.* 1999b, Yang *et al.* 2001).

PSK is derived from post-translational modification. The presence of *O*-sulphated tyrosine in PSK implies that tyrosyl-protein sulphotransferase

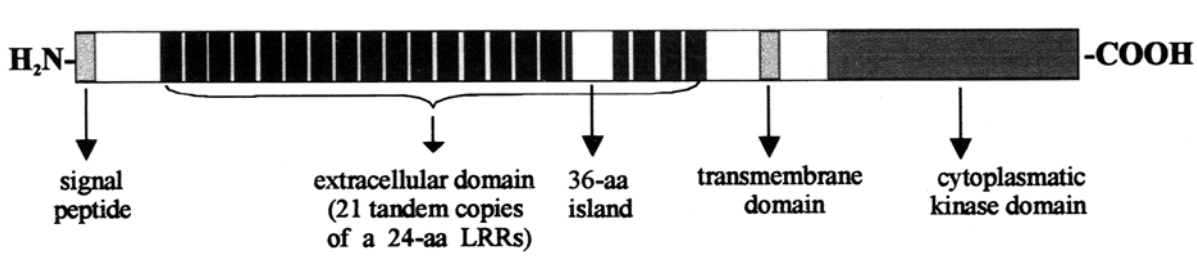

Fig. 1. Scheme of the 120-kDa receptor kinase of PSK
[aa-amino acid, LRR - leucine rich repeats] (modified from Matsubayashi *et al.* 2002)

(TPST) is involved in PSK biosynthesis (Hanai *et al.* 2000a). It is assumed that, similarly as in case of animal peptide hormones PSK-α is set free as a result of proteolytic degradation of the precursor. The enzymes taking part in this reaction can be present in a cell wall or in an extracellular space (Hanai *et al.* 2000a).

The fact that PSKs are active in suspension cultures has facilitated the search for binding proteins that might serve as a PSK receptor. Evidence for the existence of high-affinity binding sites for PSK in rice plasma membrane was provided. The observed binding of [^{35}S] and [^{3}H] labeled PSK was competitive and reversible, and localized to the outer surface of the cell membrane (Matsubayashi *et al.* 1997, Matsubayashi and Sakagami 1999). Ligand saturation analysis using [^{3}H]-PSK revealed the existence of both high- and low-affinity binding sites with K_ds of 1.4 nM and 27.0 nM, respectively. It was calculated that each rice cell might possess more than 10^4 sites able to bind PSK-α. Because auxin and cytokinin have no influence on this process, it is considered that PSK-α co-operates with these hormones in the process of cell division by its own receptors or/and by signal transduction pathway.

Using ^{125}I-labeled PSK analog, Matsubayashi and Sakagami (2000) showed that putative receptor proteins for PSK were 120 kDa and 160 kDa glycosylated membrane proteins. Recently Matsubayashi *et al.* (2002) purified a 120-kDa membrane protein, specifically interacting with PSK, from carrot microsomal fractions. The corresponding cDNA encoded a 1021-amino acid receptor kinase (112 kDa) that had features found in several hormone receptors in plants and animals. It contained an NH_2-terminal hydrophobic signal sequence, extracellular leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic kinase domain. Seventeen potential N-linked glycosylation sites were also found. The major extracellular domain of this protein contained 21 tandem copies of a 24–amino acid LRR (Matsubayashi *et al.* 2002). It was suggested that this string of LRRs played a key role in protein-protein interactions. In addition, a 36–amino acid island was detected in the 18th LRR (Fig. 1). An island domain has also been found among the extracellular LRRs of the brassinosteroid receptor BRI1 and has been shown to be critical for its function (Li and Chory 1997). The cytoplasmic region of the predicted amino acid sequence contains all 12 subdomains found in almost all eukaryotic serine-threonine kinases. The kinase region of this protein shares substantial sequence identity with those of the known plant hormone receptors BRI1 and CLV1 (Li and Chory 1997, Clark *et al.* 1997). Southern blot analysis of carrot genomic DNA with the full-length cDNA of this protein indicated that a single gene encodes this protein. Northern blot analysis, showing the expression pattern of the corresponding gene, detected a single class of mRNA. This mRNA accumulated ubiquitously in leaf, apical meristem, hypocotyl, and root of carrot seedlings, although its expression level was far lower than that in cultured carrot cells Matsubayashi *et al.* (2002).

CLAVATA3

In *Arabidopsis thaliana* the presence of genes taking part in cellular path of transduction signal controlling SAM (Shoot Apical Meristem) cell proliferation and differentiation was discovered (Clark 1997, 2001). To these genes belong: *CLV1-3* (CLAVATA) and *WUS* (WUSCHEL)(Clark *et al.* 1995, Laux *et al.* 1996). *CLV1* encoded transmembrane receptor Ser/Thr kinase consisting of a leader sequence, a putative extracellular domain of 21 leucine-rich repeats (which often appear in receptors domain binding signal peptides both in plants and in animals) and 15 putative N-linked glycosylation sites (Clark *et al.* 1997). *CLV2* encoded a receptor similar to *CLV1* but devoid of cytoplasmic domain. *In vivo*, inactive complex CLV1- CLV2 is formed (185 kDa weight), which is linked with disulphide bridge. *CLV1* and *CLV2* are expressed in the central layer of the SAM (Fletcher *et al.* 1999). *CLV3* contain the sequence of small extra-cellular polypeptide (78 amino acids) with an amino-terminal putative signal sequence (Fletcher *et al.* 1999). So far CLV3 is not homologue to any known plant or animal protein. It is synthesized in SAM region on L_1 and L_2 tunica layer of nondifferentiated cells, especially near the apical surface (Fletcher *et al.* 1999). Cock and McCormick (2001) found that a 13-amino acid conserved domain of CLV3 existed in the C terminus of five maize endo-

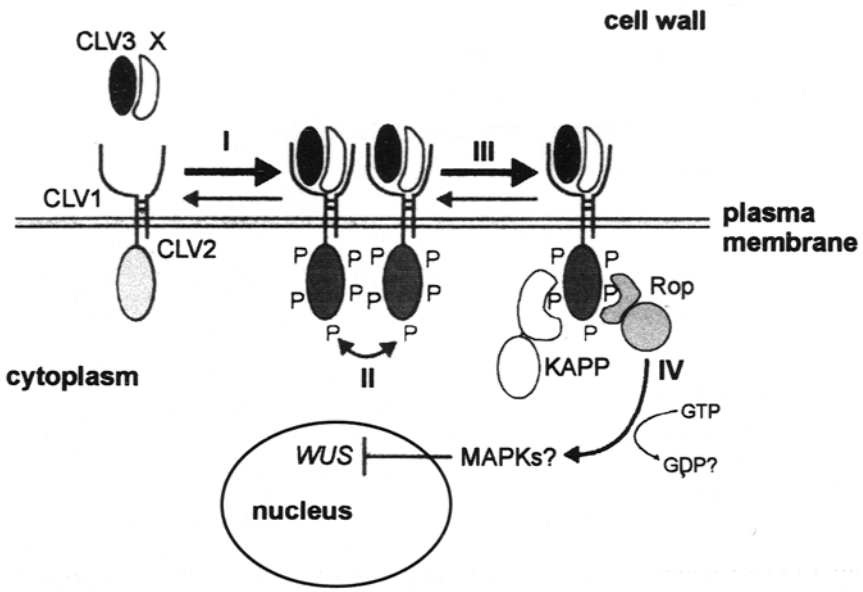

Fig. 2. A model of CLAVATA signal transduction (modified from Clarc 2001): **I** – formation of the complex CLV1-CLV2-CLV3, **II** – activation of the intracellular protein kinase domain of CLV1 and autophosphorylation, **III** - formation of the complex CLV-KAPP – Rop, **IV** – transport of signal through MAPK kinases which restrain WUS gene expression.

sperm-specific polypeptides (ZmEsrs) and DeYoung and Clark (2001) showed this domain in the C terminus of 21 *Arabidopsis* CLV3-like polypeptides, which began with either a dibasic (20 polypeptides) or a single basic residue (6 polypeptides). However, *WUS* encoded putative transcription factor, which promoted and/or restrained the cell divisions. It is interesting that this gene expression always takes place in a few cells located in the central part of the L_3 layer. Although *WUS* gene expression takes place only in these cells, its product is transported to others cells of the SAM, which undergo division and then differentiation (Clark 2001).

The signal transduction pathway in SAM, leading either to stimulation or inhibition of cell proliferation, is still not well known. Especially important is the balance between regulation of cell proliferation and organ formation in the shoot and flower meristems of *Arabidopsis*. It is considered that CLV1 associates with CLV2 through disulfide bonds and forms 185 kDa complex (Clark 2001). CLV3 protein interacts with receptors CLV1-CLV2. Binding of CLV3 leads to formation of the complex (CLV1-CLV2- CLV3), to activation of protein kinase domain, and to the autophosphorylation of CLV1 (Fletcher 2002). The phosphorylation of the intracellular domains of CLV1 activates, whereas its dephosphorylation terminates specific signalling pathway. It was affirmed that two additional proteins, KAPP (Kinase--Associated Protein Phosphatase) and Rho GTPase--related protein are involved in CLV1-CLV2-CLV3 action (Fig. 2). This 450-kDa complex may control *WUS* gene expression through MAP kinases (Mitogen--Activated Protein kinase) (Fletcher 2002). *WUS* promotes cell division in the meristems and acts antagonistically to the *CLV* genes (Laux *et al.* 1996). The positive and negative feedback interactions between *CLV* genes and WUS maintain the balance required for stem cell proliferation, both temporally and spatially (Schoof *et al.* 2000). Rop GTPase and KAPP phosphatase take part in modulation of CLV1 phosphorylation status and because of that antagonistically regulate cell proliferation in SAM (Fletcher *et al.* 1999, Clark 2001) (Fig. 2.).

SCR/SP11

For a few years genetic mechanism of sporophytic self-incompatibility (SI), in which genes expressed in the pollen and in the stigma determine whether pollen-stigma interaction is compatible or incompatible, is well understood in *Brassicacae*. SI is controlled by a single multiallelic locus (S-locus) (Brugiere *et al.* 2000). This S-locus is highly polymorphic and more than 60 different S alleles have been identified in brassicas. Molecular analyses revealed a complex structure for the S locus, which contains several physically closely linked genes (Gaude and Cabrillac 2001). Three genes have been involved in recognition of self-pollen by stigma. Two of them *SLG* (for S locus glycoprotein) and

SRK (for S locus receptor kinase) are expressed in stigma, whereas the third *SCR* gene (for S locus cysteine rich) is only expressed in pollen and in the tapetum (Schopfer *et al.* 1999, Lindsey *et al.* 2002).

The *SLG* gene encodes a glycoprotein secreted into the cell wall of stigmatic papillae (Nasrallah *et al.* 1985). This gene is usually intronless (except a few alleles, which contain one intron, where two proteins are encoded: a secreted and a membrane-anchored form) (Tantikanjana *et al.* 1993). The *SRK* gene consists of seven exons and encodes a membrane-protein that consists of: an extracellular domain, which acts as a ligand-binding region, a single transmembrane region and a cytoplasmic domain exhibiting a serine-treonine kinase activity (Giranton *et al.* 2000).

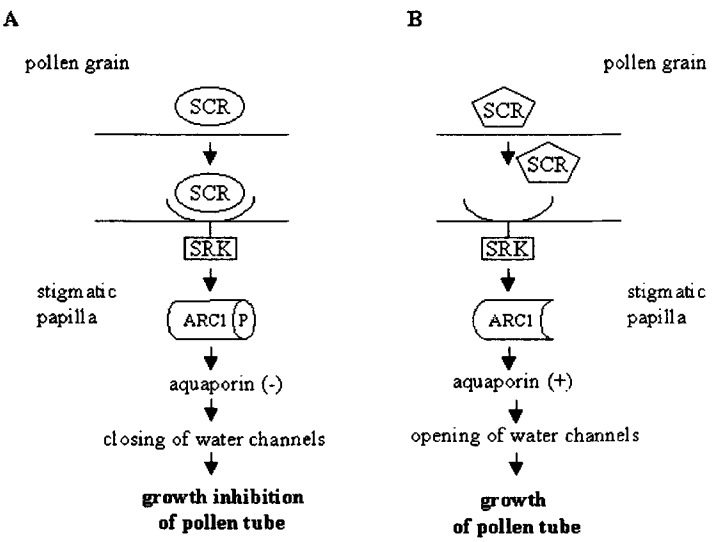

Fig. 3. A model of signal perception and response in the SI response in *Brassica*: **A** – self pollen, **B** – non-self pollen.

The *SCR* genes show structural polymorphism and encode small hydrophilic cysteine-rich polypeptides (<10 kDa), similar to pollen coat protein (PCP), containing 74-77 amino acids with the putative signal peptide cleavage site (Schopfer *et al.* 1999, Matsubayashi *et al.* 2001). Mature SCR proteins (named also SP11) consist of 50-59 amino acids and might diffuse between developing pollen and migrate through the cell wall from the pollen coat to the plasmalemma of the interaction stigmatic cell. These proteins bind the ectodomain of SRK, but binding is possible between specific alleles (Gaude and Cabrillac 2001). Therefore, SRK and SCR appear to act as a receptor-ligand pair during pollen self recognition (Lindsey *et al.* 2002). Gaude and Cabrillac (2001) suggested that interaction of SCR with SRK leads to a conformational change of the SRK kinase domain that allows activation of SRK receptor kinase, which in turn is involved in initiation of a signal transduction cascade, leading to the SI response. ARC1 protein, which interacts with the cytoplasmic domain of SRK is one of the components of this pathway (Kachroo *et al.* 2002). The study of Ikeda *et al.* (1997) showed that a specific aquaporin (water channel) could be an end point of SCR-induced signalling pathway (Fig. 3). SCR also may interact with SLG, but not as strongly as with SRK. The biological significance of SCR-SLG interaction however, is unclear (Franklin-Tong 2002). There are two possibilities: SLG might modulate the SRK-SCR interaction response or SLG is involved to pollen adhesion to the stigma *via* its interaction with proteins present on the outside surface of a pollen grain (Brugiere *et al.* 2000).

ENOD40

Some soil bacteria (mainly belonging to genera *Rhizobium*) are able to induce nodule formation on the roots of legumes. The interaction between these bacteria and host plant starts with signal exchange and recognition of the symbiotic partners (Miklashevichs *et al.* 2001). Secreted by plant roots specific flavonoids stimulate bacteria, which in turn start to synthesize lipochitooligosacharides (LCO), called Nod factors (Lerouge *et al.* 1990), responsible for activation in roots *Enod* (Early nodulin) genes (Ryan 1996, Barciszewski and Legocki 1997, Downie and Walker 1999, Miklashevichs *et al.* 2001). ENOD40 is one of the earliest nodulins, which is expressed by *Rhizobium* in the legumes (Crespi *et al.* 1994, van de Sande *et al.* 1996).

The *ENOD40* gene was found in several leguminous species. Its homologues were also present in same non-leguminous plants – tobacco and rice

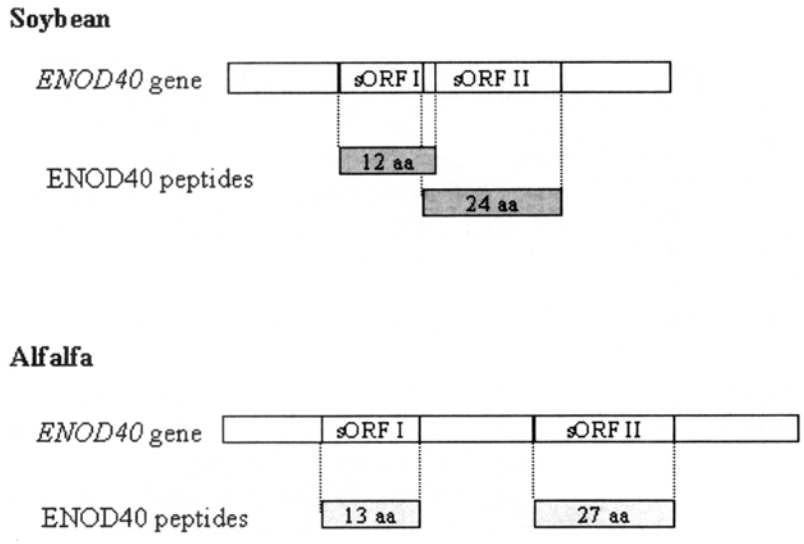

Fig. 4. Localization of sORFs on *ENOD40* genes and encoding peptides from alfalfa and soybean (modified from Ryan *et al.* 2002)

(van de Sande *et al.* 1996, Kouchi *et al.* 1999). Genomes of different legumes contain more than one copy of that gene (for example soybean, alfalfa and French bean) (Kouchi and Hata 1993, Papadopoulou *et al.* 1996, Fang and Hirch 1998). All these *ENOD40* genes contain two highly conserved short open reading frames (sORF); called boxes I and II (Fig. 4). In alfalfa *ORF I* at the 5' end encodes 13 aa and *ORF II* in central part of gene encodes 27 aa peptides (Albrecht *et al.* 1999, Sousa *et al.* 2001). In soybean second sORF of 24 aa overlaps the box I 12 aa sORF (Ryan *et al.* 2002) (Fig. 4). At the 3' end is a region encoding a conserved untranslated RNA domain with highly stable secondary structures, which may be involved in initiating plant cell division (Crespi 1994, Fang and Hirh 1998). However, ENOD40 has no N-terminal transit sequence and therefore it seems unlikely that it is synthesized in the Golgi system or in the cytosol (Ryan *et al.* 2002).

ENOD40s are produced as short peptides composed of 13 (alfalfa, pea, vetch), 12 (soybean, lupine) or 10 (tobacco) amino acids (van de Sande *et al.* 1996) (Table). These short peptides are biologically active without their posttranslational modification. However, other biologically active plant peptides (*e.g.* systemin) are produced by the cleavage from their precursors (Sousa *et al.* 2001, and see Table). Before initiation of cell division *ENOD40* is first expressed in the pericycle adjacent to the protoxylem poles in legume roots. It is also expressed in the dividing cortical cells, and that in consequence leads to initiation of nodule development (Charon *et al.* 1997, Downie and Walker 1999, Lindsey *et al.* 2002). It seems that ENOD40 does not induce a process of cell division by itself, but rather its action depends on other factors involved in cell cycle activation. Mylona *et al.* (1995) suggested, that it could change the cytokinin/auxin ratio in cortical cells.

Recently, Röhrig *et al.* (2002), using wheat germ *in vitro* translation system, showed that two small peptides of 12 aa (A) and 24 aa (B) were directly synthesized on soybean *ENOD40* mRNA. Both peptides specifically bind to the same 93-kDa protein, nodulin 100, which is a subunit of sucrose synthase (SuSy), the enzyme that catalyzes the reversible conversion of sucrose and UDP into UDP-glucose and fructose. It is postulated, that binding of peptides A and/or B to SuSy may either regulate enzyme activity or may direct SuSy to specific intracellular sites (Röhrin *et al.* 2002). On the basis of their data Röhrin *et al.* (2002) suggested that ENOD40 peptides were involved in the control of sucrose utilization in nitrogen-fixed nodules.

ENOD40 gene is expressed not only during root nodule morphogenesis, but also during lateral root development (Papadopoulou *et al.* 1996). The gene homologous to the *ENOD40* in rice may play a role in vascular differentiation (Kouchi *et al.* 1999). Expression of gene similar to *ENOD40* in tobacco protoplasts increases their sensitivity to auxin. Moreover, *ENOD40s* are induced during early steps of mycorrhizal interactions (Fang and Hirsh 1998). This suggests that some levels of signal transduction in both symbiotic interaction: mycorrhizae and nodule formation are common (van Rhijn *et al.* 1997). This hypothesis was indepen-

dently strongly supported by Endre *et al.* (2002) and Stracke *et al.* (2002).

Stracke *et al.* (2002) cloned orhologous *SYMRK* (symbiosis receptor-like kinase) genes from bird's-foot trefoil (*Lotus corniculatus*) and pea (*Pisum sativum*), which were required for both fungal and bacterial recognition. The determined cDNA sequence codes a protein of 923 aa, with a signal peptide, 3 extracellular LRR domains, a transmembrane and an intracellular protein kinase domain (Stracke *et al.* 2002). The *NORK* (nodulation receptor kinase) gene cloned by Endre *et al.* (2002) from *Medicago sativa* is a coding protein nearly identical to SYMRK. The genes identified by both groups seem to belong to the LRR receptor subfamily. Nevertheless, the mechanism by which bacterial Nod factors are recognized by SYMRK and NORK receptor kinases in legume plants remains unknown. There are two possibilities: Nod factors might directly or indirectly bind to extracellular domains of these RLKs. Because LRR sequence motifs have a general function in protein recognition it is more probable that both SYMRK and NORK receptors are only indirectly involved in recognizing Nod factors. It is postulated that primary recognition could be performed by secreted extracellular molecules, which are recognized by the LLR receptors after binding of lipo-chitooligosaccharidian to chemical structure Nod factors. This function may be played by lectins, well known for their involvement in carbohydrate-recognition mechanisms (Spaink 2002).

Natriuretic peptides

Natriuretic peptides (NPs) or natriuretic factors (NFs) belong to a large hormonal protein family (Martin *et al.* 1990, Vesely *et al.* 1993) that was identified in vertebrate (Gering *et al.* 1996, Takei 2001), various invertebrate species (Takei 2001), in *Paramecium sp.* (Takei 2001) and plants (Vesely and Giordano 1991, Billington *et al.* 1997, Yang *et al.* 1999c, Gering 1999, Vesley *et al.* 2001). In mammalians, several kinds of NPs were classified, based on the place of synthesis and the NPs function: ANP (Atrial Natriuretic Peptide), VNP (Ventricular Natriuretic Peptide), BNP (Brain Natriuretic Peptide), CNP (type C Natriuretic Peptide) and RNP (Renal Natriuretic Peptide, urodilatin) (Gering 1999, Takei 2001). In mammalians and humans ANP is released into blood circulation from the right atrium of the heart and its main task is to support a proper flow of the blood stream. Other NPs, expressed in different parts of kidneys, control water and ion homeostasis (Goetz 1991, Koller and Goeddel 1992, Anand-Srivastava and Trachte 1993, Vesely *et al.* 1994, Patil *et al.* 1997).

During several previous years it was shown that animal NPs could influence a variety of processes in plants. Synthetic rANP (rat ANP) of concentration lower than 1 μM induced opening of stomata pore in *Tradescantia sp.* (Gering *et al.* 1996). This polypeptide loses its biological activity after breaking disulphide bridge between 7th and 23rd amino acid residue, and this could suggest that a specific secondary structure of protein decides on its binding to a receptor (Pharmawati *et al.* 1998a). The presence of specific rANP binding sites in cell membrane from leaves of *Tradescantia sp.* and shoots was proved by *in situ* method by Suwastika *et al.* (2000). The stimulating influence of rANP on opening stomatal guard cells was reversibly inhibited as a result of preceding treatment of stomatal guard cells with guanylate cyclase inhibitor - LY 8353, while 8-Br-cGMP (cell permeant cyclic cGMP analogue) reacted in the similar way as rANP. These results suggested that in plants (like in animals), NP reaction depended on the stimulation of guanylate cyclase activity (Pharmawati *et al.* 1998a). The increase in cytoplasmatic cGMP concentration could have a significant influence on a rate flow of membrane water and ions, what was confirmed experimentally. By applying the ^{2}H-NMR (^{2}H-Nuclear Magnetic Resonance) method it was revealed that rANP promoted radial water movements from the xylem of *Tradescantia* shoots. Similar mechanism was revealed for 8-Br-cGMP, while LY 83583 and $HgCl_2$ (water channels inhibitor) restrained this process. On this basis it was assumed that NPs could control transport of water *via* the regulation of guanylate cyclase and water channels activity (Suwastika and Gehring 1998).

The antibodies against different fragments of animal ANP react with proteins present in plant extracts. It has been established by applying various

methods that in plants many types of NPs (which molecular weight ranges from 3 to 10 kDa) may occur. This corresponds to the size of C-end fragments and whole (pro-NP) of animal NP (Pharmawati *et al.* 1998b).

In extracts from leaves and shoots of *Dracena goldseffiana* the presence of rANP (1-98, 31-67, 99-126) homologues was detected (Vesely and Giordano 1991). The quantity of this polypeptide in the studied plant was similar to this occurring in heart atrium and ventricle of rat and ranged from 124 to 129 ng/g of plant tissue (Vesely and Giordano 1991). Furthermore, by means of high-performance gel permeation chromatography (HPGPC) ANP-like peptide and its prohormone were detected in leaves and stems of *Metasequoia glyptostroboides* (Yang *et al.* 1999c). The profile elution of an equivalent to the animal ANP, from a fraction derived from *Metasequoia* stems, showed that it is a little larger, compared to the pure, synthetic rANP. These facts suggest a possibility that ANP may be present in this plant as a prohormone, like in an animal kidney. It is considered that homologous protein system was already present at the early stage of the land plants evolution to allow trees to reach heights greater than of 9 meters and made easier the transport of water and mineral salts in stems (Yang *et al.* 1999c).

A protein isolated and purified from ivy (*Hedera helix*) extracts, equivalent to human ANP (1-28) was called irPNP. Its molecular weight is similar to rANP and like this polypeptide it stimulates opening of stomatal guard cells. This effect was observed at concentrations of IrPNP 100 times lower when compared to rANP (Billington *et al.* 1997). IrPNP increased endogenic cGMP level in stele tissue isolated from maize roots (*Zea mays*) as fast as rANP did (within 30 seconds). The maximum content of this cyclic nucleotide was observed 10 minutes after irPNP treatment, and after another 5 minutes cGMP concentration returned to the initial level. cGMP also delayed the inflow of the K^+ ions but this effect was not correlated with proper changing of the H^+ ions. Increase in the cGMP level was accompanied with potassium inflow into the root cells (Pharmawati *et al.* 1998b). However, after applying synthetic animal ANP, kinetine and LY 83853 no physiological effect was observed. This result suggests that cell membranes in plants contain a specific NPs receptor, having activity of guanylate cyclase, as it in case of animal receptors NPs (Chinkers *et al.* 1989, Pharmawati *et al.* 1998b, Koller and Goeddel 1992).

Pharmawati *et al.* (1999) observed that in maize root vascular tissues irPNP caused an immediate net H^+ influx and delayed net K^+ and Na^+ uptake. Delayed net K^+ influx was also observed in response to 8-Br-cGMP, however, it was not accompanied by significant changes in net H^+ fluxes. Pharmawati *et al.* (2001) also confirmed that ANP and irPNP considerably increased the amount of cGMP in protoplasts of stomata cells. IrPNP did not induce the increase in cGMP in the presence of EGTA, which decreased the intracellular Ca^{2+} level (Pharmawati *et al.* 2001).

Synthetic peptide identical to C-end (aa 99-126) of rANP also changed osmotic potential in protoplasts obtained from mesophyll cells, in a manner depending on the concentration and the time. It was shown that regulation of osmotic potential in plant cell depended on NP, but was independent from cGMP. This data confirms the suggestion, that plant proteins that are homologous to C-end of vertebrate proteins take part in maintenance of water and ion balance (Maryani *et al.* 2001).

Evidence confirming the presence of the protein hormonal system in plants is a recent report by Vesely *et al.* (2001). By Southern blot hybridization method, they showed the presence of the ANP gene sequence in ivy (*Hedera helix*) roots, stems and leaves. Northern blot analysis of the whole RNA isolated from leaves, roots and stems showed presence in stems of a single ANP transcript – mRNA of 850 bp, homologous to mRNA prohormone rANP. Data presented by the authors indicates that despite the filogenetic distance between plants and animals there is a similar hormonal system (Vesley *et al.* 2001).

Discussed data gives the evidence supporting the existence in plants equivalents of animal natriuretic peptides. In both types of organisms, the mechanism of signal molecule activity could depend on regulation of water and ion balance. We need further studies to better understand the role of NPs in plant. Theses studies will show in which cells and

tissues the pro-peptide forms of these compounds are synthesized, how their maturation proceeds, as well as they should also explain what is the structure of their receptors and how they work.

RALF

During isolation of systemin from tobacco leaves another short protein was isolated. Because this 5-kDa polypeptide induces a rapid alkalinization of the culture medium of tobacco and tomato suspension cells it was named RALF (Rapid Alkalization Factor) (Pearce *et al.* 2001a). It has been shown that a clone isolated from a tobacco leaf cDNA library coded a 115 amino acids pre-proprotein, which contained a 25-amino-acid signal peptide (Table 1) at its N-terminus and 45-amino-acid RALF sequence at its C-terminus. Within the RALF two disulfide bridges were identified between Cys-18 and –28 and between Cys-41 and -47, which were necessary for biological activity. RALF pre-pro-proteins are present in various tissues (xylem fibre, cambial region, callus) and organs (leaves, roots, hypocotyls, fruits) from 16 species of plants representing 9 families. It has been shown that RALF genes are highly conserved. The sequence identity of tobacco RALF pre-proprotein from leaves with respective protein from tomato is 87 %, *Arabidopsis* 80 %, potato 74 % and *Medicago* 71 %. RALF polypeptide not only induced a rapid alkalinization, but also induced MAP kinase activity (Pearce *et al.* 2001b). Moreover, tomato synthetic homologue of the polypeptide inhibited root growth and development as well as germination of tomato and *Arabidopsis* seeds (Pearce *et al.* 2001b). RALF gene products are found in different organs and cells of various species, suggesting that RALF might have a more general role in plants (Ryan *et al.* 2002). Currently conducted studies should explain the molecular mechanism of RALF, as well as the structure of its receptor.

Insulin-like peptides

Insulin is the protein hormone, secreted by beta-cells of vertebrate pancreas in response to high blood glucose levels. It is involved in maintenance of a proper glucose level in blood. This hormone and two structurally related polypeptides IGFI and IGFII (Insulin Growth Factor I and II) act *via* membrane receptor, protein kinase. Its activation leads to stimulation of tyrosine kinase activity intrinsic to the receptor and phosphorylation of tyrosine residues on both the receptor itself and other target cellular proteins (Komatsu and Hirano 1991).

So far there is no direct evidence that insulin or its homologues are present in plants. Goodman and Davis (1993) affirmed that insulin markedly accelerates (five times) cotyledons growth of sunflower (*Heliaticus annuus*), watermelon (*Cirullus sp.*) and cucumber (*Cucumis sativus*). This was the first evidence that insulin and IGF promoted the rate of post-germinative development in plants. The presence of protein kinases in plant tissues and well known role of insulin in fat and carbohydrate metabolism, persuaded scientist to explore probable influence of insulin and IGF on the activity of enzymes involved in conversion of fat to carbohydrates in fat storing seeds. They detected an increase in the enzyme activity, which occurs during converting fatty acids from triglycerides to carbohydrates. The activity of acyl CoA dehydrogenase, citrate synthase, malate dehydrogenase increased twice at insulin concentration of 0.1 $U \cdot dm^{-3}$ in all three plant species, when compared to control. During germination of oil storing seeds there is a rapid, synchronous increase in the activity of glyoxysomal enzymes and in the number of glyoxysomes. An increase in both glyoxysomal enzymes activity (*e.g.* isocitrate lyase, malate synthase), as well as catalase and glycolate oxidase activity (enzymes which do not take part in convertion of fatty acids from triglycerides to carbohydrates) was found. Comparable effect was observed in case of seeds treated with IGFI and IGFII (Goodman and Davis 1993).

In recent years many publications appeared, pointing at the presence of insulin-like substances in some plants, whose action is linked to improvement of glucose metabolism. Water extracts from such plants as: *Medicago sativa*, *Eucalyptus globulus*, *Agrinomia eupatoria*, *Coriandrum sativum*, *Viscum album*, *Zygophyllum gaetulum*, *Sambucus nigra*, *Aloe vera* and *Globularia alypum* stimulate insulin secretion from pancreas β cells, growing *in vitro* (Gray and Flatt 1997, Gray and Flatt 1998a, Gray and Flatt, 1998b, Gray and Flatt 1999a, Gray

and Flatt 1999b, Skim *et al.* 1999, Gray *et al.* 2000, Okyar *et al.* 2001). The water extract from mentioned plants did not lose its activity after treating with high temperature. However, this activity was lost (depending on plant species) after treating the water extracts with acid or alkali (0.1 mol·dm^{-3} HCL or NaOH).

In soybean seeds (*Glycyne sp.*) the presence of a cysteine-rich glycoprotein – Bg (Basic 7S globulin) (Watanabe *et al.* 1994) was found. The protein homologues to Bg were discovered also in other *Leguminosae* plants (*e.g.* azuki bean, lupin, mung bean, cowpea) and cultured carrot (*Dancus carota*) cells (Komatsu and Hirano 1991). The Bg was able to bind insulin, IGFI and IGFII and had a protein kinase activity, which corresponded to about two thirds of the tyrosine kinase activity of the rat insulin receptor. This suggested that this type of protein could be involved in mechanism regulated by insulin in many plant species (Komatsu and Hirano 1991). However, there is no evidence that Bg-like proteins function as insulin receptors or IGF receptors in plants. The structural characteristics of Bg (Komatsu and Hirano 1991) were compared to those of the insulin and IGFs receptors. No homology in amino acid sequence was found among these proteins (Ullrich *et al.* 1985, 1986). Bg showed structural similarities to the insulin receptor in glycosylation, the presence of a cysteine rich domain and subunits (27 kDa and 16 kDa) linked together by disulphide bridge. Bg is localized in the middle lamella of cell walls and the cell membrane (Nishizawa *et al.* 1993); it suggests that Bg may have insulin receptor-like function. Watanabe *et al.* (1994) succeeded in isolating the protein (4 kDa) from germinating seeds embryo, which could bind with Bg protein and took part in binding insulin to this peptide. It was called leginsulin and consisted of 32 amino acids. It had a stimulating effect on a phosphorylation activity of Bg, which may suggest that it was involved in cellular path of signal transduction. Analysis performed using a mass spectrometry revealed that this protein was post-translationally cleaved (glycine residue from C-terminal was removed) similarly as in the case of numerous protein hormones in animals. Leginsuline's cDNA was cloned, sequenced and although its sequence was not similar to cDNA clones encoding insulin or insulin-like factors, it seems that leginsuline is putative plant protein hormone (Watanabe *et al.* 1994). Moreover, it was found to be highly similar (65 %) to the PA1b peptide of pea seed 2S albumin PA1 (Higgins *et al.*1989). The structure and biosynthesis of this PA1b peptide was described but the function and site of accumulation is not known.

Conclusions

The field of plant signalling peptides is set to expand (Gering 1999, Lindsey 2001, Linsey *et al.* 2002, Matsubayashi *et al.* 2001, Ryan and Pierce 2001, Ryan *et al.* 2002). As the genomics and proteomics tools are applied to the functional analysis of plant peptides, we can expect new classes of small polypeptides involved in signal transduction. For example, the fully sequenced *Arabidopsis* genome includes a large number of short open reading frames for possible peptide signal molecules (The Arabidopsis Genome Initiative, 2000).

The classification of the above described polypeptides into: hormone, growth factor or signalling peptides is difficult on the basis of currently available data. Several plant polypeptides such as PSK-α, systemin, CLV3, SCR, RALF, irPNP and leginsulin are synthesized as pre-proproteins (Table), apparently through the secretory pathway like animal hormones and growth factors (Lindsey 2001). Conserved dibasic pairs of amino acids that are candidates for processing proteinases sites are found in all tomato-related prosystemins (Constabel *et al.* 1998), in all nine of the *Arabidopsis* RALF precursor proteins (Pearce *et al.* 2001b), in three out of four PSK-α precursor proteins (Yang *et al.* 2001), and in 20 out of 26 CLV3 precursor proteins (DeYoung and Clark 2001). At least in case of systemin, the dibasic amino acids may be sites of degradation. Whether the same dibasic sites in other signalling polypeptides lead to their production or to degradation remains to be determined (Lindsey 2001, Rayan *et al.* 2002). Only the precursors of tomato prosystemin and of ENOD40 do not have signal sequences (Table 1) and are apparently synthesized in the cytosol on free ribosomes, a scenario not found with animal polypeptide hormone precursors.

PSK-α, systemin and CLV3 are secreted outside the cell where they interact directly with specific receptors. SCR appears to be unique in that it is synthesized in the tapetum, transported to the pollen, and then to the stigma receptors. To date, the receptors for plant signalling peptide appear to be either leucine-rich repeat receptors (PSK-α, systemin and CLV1 receptors) or cysteine-rich receptor kinases (SCR receptor) (Lindsey 2001, Linsey *et al.* 2002, Matsubayashi *et al.* 2001, Ryan *et al.* 2002). Until now, we did not know so much about RALF, ENOD40, irPNP and leginsulin receptors. Studies concerning molecular structure of these receptors are currently under investigation.

In final conclusion it is worth mentioning that *Arabidopsis* genome has at least 340 receptor-like kinase (RLK) genes. The largest recognizable class of transmembrane sensors in this plant is Ser/Thr kinases, including 174 LRR-receptor kinases (The Arabidopsis Genome Initiative, 2000). Between them, only a few are known for their biological functions (Torii 2000, Matsubayashi *et al.* 2002). Therefore, we may predict that in the near future new types of different signalling peptides will be found.

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