REVIEW PAPER

An update on cell surface proteins containing extensin-motifs

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Abstract

In recent years it has become clear that there are several molecular links that interconnect the plant cell surface continuum, which is highly important in many biological processes such as plant growth, development, and interaction with the environment. The plant cell surface continuum can be defined as the space that contains and interlinks the cell wall, plasma membrane and cytoskeleton compartments. In this review, we provide an updated view of cell surface proteins that include modular domains with an extensin (EXT)-motif followed by a cytoplasmic kinase-like domain, known as PERKs (for proline-rich extensin-like receptor kinases); with an EXT-motif and an actin binding domain, known as formins; and with extracellular hybrid-EXTs. We focus our attention on the EXT-motifs with the short sequence Ser-Pro$_{(3-5)}$, which is found in several different protein contexts within the same extracellular space, highlighting a putative conserved structural and functional role. A closer understanding of the dynamic regulation of plant cell surface continuum and its relationship with the downstream signalling cascade is a crucial forthcoming challenge.

Key words: Cell surface, extensin, extensin motif, formin, PERK, plant cell wall.

Introduction

The integrity of an extracellular matrix mainly depends on the correct self-assembly of its individual components. Mutants with defective glycosaminoglycan, proteoglycans, or hydroxyproline (Hyp)-rich collagens are often lethal in animals (Buehler, 2006). Likewise, some defects in the cell wall or extracellular matrix of plants (Cannon *et al.*, 2008; Velasquez *et al.*, 2011) and related green-algae (Keskiaho *et al.*, 2007) are related to hydroxyproline-rich glycoproteins (HRGPs) like extensins (EXTs). The current fast evolving view of the plant cell wall suggests that it is a dynamic and responsive structure, which exists as part of a continuum with the

plasma membrane. Plant cell expansion, involving cell wall loosening, deposition of new materials, and subsequent rigidification, must be tightly regulated to allow the maintenance of cell wall integrity and coordination during plant development. Complex sensing of environmental cues as well as cell wall abnormalities requires sensitive signalling components between the cell wall side and the inside of the cell, crucial for proper development (Ringli, 2010*b*). To this end, plant cells have developed a rich diversity of complex proteins with diverse extracellular domains: wall associated domains present in WAKs (wall associated kinases) (Kohorn and Kohorn,

2012), malectin-like (ML) domains in *Catharanthus roseus* receptor-like kinase1 (CrRLK1L) (Lindner *et al.*, 2012; Wolf and Höfte, 2014), lectin in LecRLK (Vaid *et al.*, 2013), leucine-rich repeats (LRR) in LRR-RLK (Shpak, 2013), the EXT-motif (Velasquez *et al.*, 2012), and so on, which connect to intracellular domains such as kinases or bind to microtubules, actin, Ca^{+2} modules, etc. These complex modular proteins are able to perform actions on wall sensing, signalling, and cytoskeleton reshaping capabilities. In this review, we will cover exclusively cell surface proteins with EXT-motifs and cytoplasmic kinase domains (PERKs) or actin-microtubule binding domains (formins), together with extracelullar hybrid-EXTs including leucine-rich-repetitive-EXT (LRR-EXT) and hybrid arabinogalactan protein (AGP)-EXT (HAE) (Fig. 1A). Our motivation is to provide an updated view of these less characterized surface proteins that contain EXT-motifs.

The EXT-motif can be defined as a short sequence of Ser-Pro$_{(3-5)}$ present two or more times along the protein chain (Showalter *et al.*, 2010). In the *Arabidopsis thaliana* genome there are 42 encoded proteins (out of 177) that contain Ser-Pro$_{(3-5)}$ motifs, considered EXT proteins since most of them

also contain the YVY crosslinking motif next to the Ser-Pro$_{(3-5)}$ (Showalter *et al.*, 2010). EXT-crosslinking is catalysed by apoplastic peroxidases (Schnabelrauch *et al.*, 1996; Jackson *et al.*, 2001); classical EXT molecules form a dendritic glycoprotein network in the cell wall (Cannon *et al.*, 2008) that possibly interacts with the positively charged pectin network (Valentin *et al.*, 2010). However, PERKs and formins also have the EXT-motifs repeated few times but they lack the YVY crosslinking sequence. In this review, we will not include proline-rich (PR) motifs like PPVx(K/T) (x corresponds to any amino acid), KKPCPP (Fowler *et al.*, 1999) found in 15 known PRPs (Showalter *et al.*, 2010) within the EXT-motif, and a small group of five proteins that contain few Ser-Pro$_{(3-5)}$ repeats without the crosslinking motifs together with a putative sequence for glycosylphosphatidilinositol (GPI) anchor addition (Showalter *et al.*, 2010).

The relevance of an EXT-based glycoprotein network in biological processes such as polarized cell expansion in growing root hairs (e.g. EXT6–7,10–13) (Velasquez *et al.*, 2011), in pollen tubes (e.g. EXT18) (Choudhary *et al.*, 2015) and in embryo development (e.g. EXT3) (Hall and Cannon, 2002; Cannon *et al.*, 2008) has already been reported. Together with
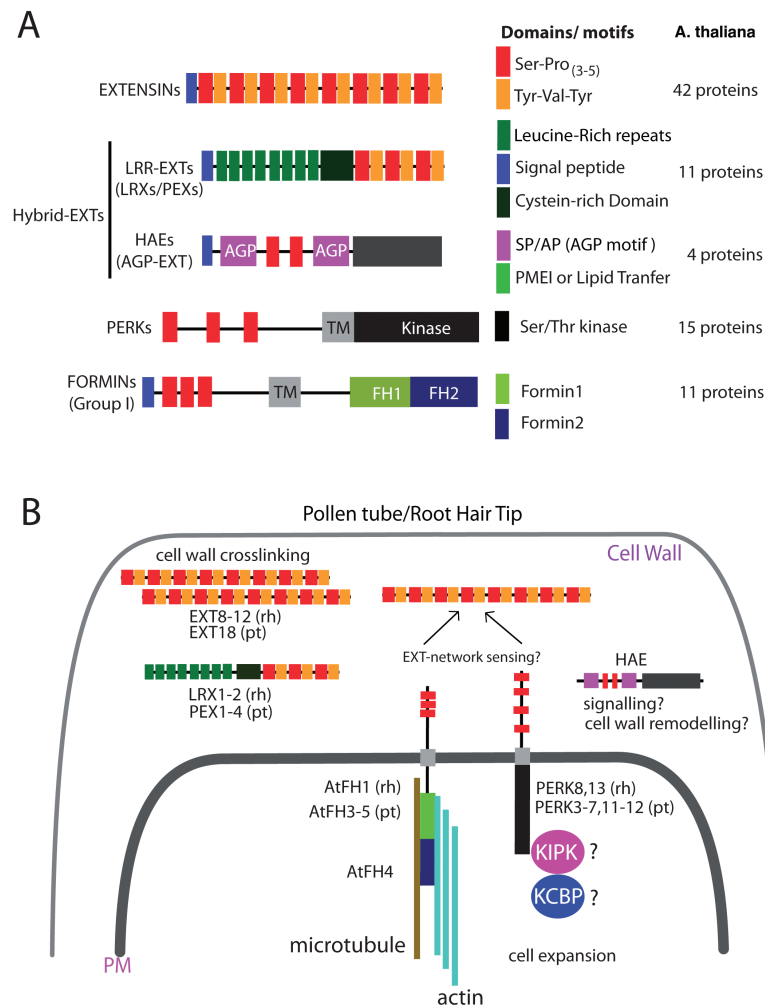

Fig. 1. Cell surface proteins containing EXT-motifs. (A) Extensin-like motifs are present in several proteins: classical Extensins (EXTs), hybrid Extensins (LRR-EXTs and HAE), formins, and PERKs. Number of encoded proteins in Arabidopsis are also indicated for each group. (B) Proposed functions of these proteins in polarized growing cells. (rh = root hair; pt = pollen tube). TM, transmembrane domain

the integrin-like proteins possibly involved in focal adhesion (Knepper *et al.*, 2011), Class I formins with an extracellular EXT-motif are good candidates to mediate the physical connection of cytoskeleton–plasma membrane–cell wall components. In addition, PERK proteins are interesting candidates to sense the integrity of the EXT-based network, triggering downstream signalling responses.

'Classical' EXT-motifs are usually *O*-glycosylated

In the recent years, the biosynthesis, structure, and function of 'classical' EXTs in plant cells has been exhaustively reviewed (Showalter *et al.*, 2010; Lamport *et al.*, 2011; Velasquez *et al.*, 2012; Hijazi *et al.*, 2014), so here we focus only on the hybrid EXT proteins. Briefly, 'classical' EXTs are highly repetitive glycoproteins defined by their conserved hydrophilic short rigid blocks of contiguous *O*-glycosylated Ser-Pro$_{(3–5)}$ motifs and hydrophobic YVY motifs involved in EXT-crosslinking (Schnabelrauch *et al.*, 1996; Held *et al.*, 2004; Cannon *et al.*, 2008). The Pro residues of the Ser-Pro$_{(3–5)}$ EXT-motif, at least in 'classical' EXTs, are usually hydroxylated by prolyl 4-hydroxylases to hydroxyproline (Hyp) and then *O*-glycosylated with sugar chains of up to four linear arabinose (Ara) residues on each Hyp (Velasquez *et al.*, 2011, 2015b; Ogawa-Ohnishi *et al.*, 2013). It has been proposed that the primary sequence of a given glycoprotein is a good predictor of the *O*-glycosylation type in a large group of HRGPs containing EXT-motifs. This is known as the *Hyp contiguity hypothesis* that predicts Hyp-*O*-arabinosylation of contiguous Hyp residues (≥2) and arabinogalactosylation of clustered noncontiguous Hyp residues (Shpak *et al.*, 1999, 2001). By using repetitive synthetic peptides of a different number of contiguous proline residues [Ser-Pro$_{(2–4)}$] all of them were modified with up to four arabinoses, unless some of them were underhydroxylated (Shpak *et al.*, 2001; Estévez *et al.*, 2006).

The fine structure of the Hyp-*O*-arabinosides is β-L-Ara*f*-(1,2)-β-L-Ara*f*-(1,2)-β-L-Ara*f*-(1,3)-α-L-*t*Ara*f*. In the last few years, it has been demonstrated that three groups of arabinosyltransferases (AraTs) were involved in the addition of the first three L-Ara residues. First, HPAT1–HPAT3 (for hydroxyproline *O*-arabinosyltransferases), which belong to the GT8 CAZy (carbohydrate enzymes) family (Ogawa-Ohnishi *et al.*, 2013), add the first arabinose *O*-linked to Hyp. Then, the AraTs RRA1–RRA3 (reduced residual arabinose) of the GT77 family (Egelund *et al.*, 2007; Velasquez *et al.*, 2011) would transfer the second arabinose unit. Finally, the XEG113 enzyme from the GT77 family (Gille *et al.*, 2009) possibly adds the third arabinose residue with the same stereochemistry. At the same time, a novel peptidyl-Ser-galactosyltransferase, originally named SGT1 (or SERGT1), which belongs to the GT96 family, adds a single α-Gal*p* residue to each Ser residue in the Ser-Pro$_{(3–5)}$ motifs of EXT (Saito *et al.*, 2014). It has also been proposed that *O*-glycosylation contributes to stabilizing the EXTs in an extended conformation (Stafstrom and Staehelin, 1986). Recently, it was hypothesized

that both *O*-glycans (Ser-*O*-galactosylation as well as Hyp-*O*-arabinosylation) would also trigger conformational changes on the theoretical triple helix assemblage of polyproline EXT backbones, suggesting that they have a direct impact on the degree of Tyr-crosslinking, at least in the 'classical' EXT group (Velasquez *et al.*, 2011, 2015a; Hijazi *et al.*, 2014). It remains to be experimentally established whether these Ser-Pro$_{(3–5)}$ motifs are also modified with *O*-glycans in LRR-EXT, HAE, PERK, and formin surface proteins. It is possible that these proteins are also *O*-arabinogalactosylated in other regions, as in the putative AGP motifs of HAE proteins.

Hybrid-EXTs: LRR-EXT and HAE

The LRR motif is generally 20–29 residues long and contains a conserved 11-aminoacid segment with the consensus sequence LxxLxLxxN/CxL (where x can be any amino acid and L positions can also be occupied by valine, isoleucine, and phenylalanine), which is usually repeated several times (Fig. 1A). This LRR domain is widely present in proteins associated with pathogen resistance, often containing a nucleotide-binding domain (commonly referred to as NB-LRR proteins) (Jones and Dangl, 2006), as well as in the large group of receptor-like kinases (RLKs) (e.g. BRI1, CLV1, FLS2, TMM, etc.). Several diverse ligands bind to the LRR domain of specific RLKs (Tena *et al.*, 2011); for example, the growth hormone brassinolide binds to BRI1 (Kinoshita *et al.*, 2005), while the small peptide CLV3 is the ligand of CLV1 (Trotochaud *et al.*, 1999); the bacterial flagellin is for FSL2 (Boller and Felix, 2009) and stomagen, EPF1 and EPF2 are ligands of TMM (Lee *et al.*, 2015). In addition, several RLKs with LRR domains are also multimerized, highlighting the role of the LRR domain in protein–protein interactions. However, for LRR-EXT, it is still unknown whether the LRR domain binds to any extracellular ligand and also whether they form dimers. In addition to the LRR domain, LRR-EXT proteins have a cysteine-rich domain consisting of five Cys residues regularly spaced by 10–18 variable amino acids and two well conserved Asp and Gln residues (Cx$_{10–18}$Cx$_{10–14}$NCx$_{6–7}$Qx$_4$Cx$_{9–11}$C) (Baumberger *et al.*, 2003b). The location of each Cys is strongly conserved between LRR-EXTs suggesting that disulfide bonds are important in the final conformation and/or function of LRR-EXT proteins.

There are 11 LRR-EXTs encoded in the Arabidopsis genome. They are classified into two main groups based on their expression patterns and sequence similarities: seven LRX genes (for leucine-rich repeat extensin, LRX1–LRX7) that are mostly expressed in vegetative tissues and four PEXs (for pollen extensin, *PEX1–PEX4*) highly expressed in reproductive tissues (Stratford *et al.*, 2001; Ringli, 2005). Some of them are present in polarized growing cells such as root hairs (LRX1 and LRX2) (Baumberger *et al.*, 2003b; Ringli, 2010a) or pollen tubes (PEX1–PEX4) (Table 1 and Fig. 1A,B). It has been reported that the triple mutant *lrx3 lrx4 lrx5* showed developmental growth defects in roots and epidermal cells as well as significant changes in cell wall composition, and possibly, in its structure (Draeger *et al.*, 2015). One of the

Table 1. *Overview of LRR-EXT proteins (LRXs and PEXs) with emphasis on those present in polarized expanding cells (pollen tubes or root hairs)*

Gene code/ Protein	Tissue expression[a]/ (subcellular localization)	Assumed function/ mutant phenotype	References
At3g19020/AtPEX1	pollen	–/–	(Rubinstein *et al.*, 1995*a*; Baumberger *et al.*, 2003*a*)
At1g49490/AtPEX2	pollen	–/–	(Baumberger *et al.*, 2003*a*)
At2g15880/AtPEX3	pollen	–/–	(Baumberger *et al.*, 2003*a*)
At4g33970/AtPEX4	pollen	–/–	(Baumberger *et al.*, 2003*a*)
ZmPEX1	pollen	–/–	(Rubinstein *et al.*, 1995*a*,1995*b*)
At1g12040/AtLRX1	root hair	Root hair growth/*lrx1* mutant showed shorter aberrant root hairs. Double mutant *lrx1 lrx2* showed an enhanced phenotype with ruptured root hairs	(Baumberger *et al.*, 2001, 2003*b*; Hall and Cannon, 2002; Ringli, 2010*a*)
At1g62440/AtLRX2	root hair	Redundantly to LRX1 control roots hairs	(Baumberger *et al.*, 2003*b*)
At4g13340/AtLRX3	root, leaves, stem, flowers	Redundantly control roots and epidermal cells/triple mutant *lrx3 lrx4 lrx5* showed shorter roots, crater-like structures in leaf epidermis and chemical cell wall changes in leaves and stems	(Baumberger *et al.*, 2003*a*; Draeger *et al.*, 2015)
At3g24480/AtLRX4	root, leaves, stem, flowers	Redundantly control roots and epidermal cells/–	(Draeger *et al.*, 2015) (Baumberger *et al.*, 2003*a*)
At4g18670/AtLRX5	root, leaves, stem, flowers	Redundantly control roots and epidermal cells/–	(Baumberger *et al.*, 2003*a*; Draeger *et al.*, 2015)
At3g22800/AtLRX6	root	–/–	(Baumberger *et al.*, 2003*a*)
At5g25550/AtLRX7	pollen	–/–	(Baumberger *et al.*, 2003*a*)

[a] Based on reporter, RT-PCR, Northern blot analysis or Genevestigator (Hruz *et al.*, 2008).
At= *Arabidopsis thaliana*; Zm= *Zea mays*.

best studied LRR-EXT protein is LRX1 (Baumberger *et al.*, 2003*b*; Ringli, 2010*a*). The *lrx1* mutant showed aberrant root hairs (Baumberger *et al.*, 2001) and LRX1 is partially redundant with its closest homologue LXR2 (Baumberger *et al.*, 2003*b*). In addition, LRX1 was shown to be crucial for maintaining a correct cell wall structure at the root hair level. A detailed *in vivo* deletion study of the EXT domain of LRX1 showed that a minimal EXT domain containing six putative *O*-glycosylated Ser-Pro$_{(3–5)}$…xY̲x̲ repeats with one Y̲VY̲ motif (where x is a variable aminoacid) is required for proper LRX1 function (Ringli, 2010*a*). Changing Tyr residues to Phe in LRX1 showed its involvement in hydrophobic stacking, possibly as a mechanism of self-protein assembly, as was suggested for EXT3 (Hall and Cannon, 2002). However, LRX1 lacking Tyr in the EXT domain remain insoluble in the cell wall, indicating strong interactions of LRX1 within the cell wall that are not mediated only by Tyr-crosslinking (Ringli, 2010*a*). In agreement with that, other self-assembly mechanisms through hydrophobic associations between strictly periodic proteins could also help the EXT-network formation (Cannon *et al.*, 2008). However, it is still unclear how the LRR domain contributes to the overall function of LRX1.

Interestingly, two suppressors of *lrx1* were found to introduce secondary changes in the cell wall on top of the lack of LRX1 protein. The repressor of *lrx1_1*, *rol1* (repressor of *lrx1_1*) was characterized as a protein involved in the biosynthesis of UDP-L-Rhamnose, a precursor of a major monosaccharide component (rhamnose) of the pectin-type of polysaccharide rhamnogalacturonan I (RGI) and II (RGII). Furthermore, the mutant, *rol1-2* in the *lrx1* background caused changes in the expression of a number of cell wall-related genes including several EXTs and peroxidases (PERs). This suggests that the rescue of the *lrx1* mutant phenotype by *rol1-2* is mediated by compensatory changes in the cell wall (mostly pectic polysaccharides) and also in the EXT-associated network (Diet *et al.*, 2006). In addition, another suppressor of *lrx1*, *rol5* was linked to the target of rapamycin (TOR) pathway (Leiber *et al.*, 2010). ROL5, functionally similar to yeast Ncs6p ('needs Cla4 to survive 6 protein'), influences the TOR signalling pathway (Mahfouz *et al.*, 2006) and is required for the modification of tRNAs in Arabidopsis. Estradiol-induced *tor-es* mutants as well as a treatment with Rapamycin (a heterocyclid macrolide that binds FKBP12 and inhibits TOR kinase) repressed root hair growth mediated by glucose–TOR signalling (Xiong and Sheen, 2012). Both, inhibition of TOR signalling by Rapamycin and *rol5* mutation in *lrx1* background, led to suppression of the *lrx1* mutant phenotype and caused several cell wall changes including in RGI components (Leiber *et al.*, 2010). Overall, both *rol* suppressors of *lrx1*, *rol1* and *rol5* introduce secondary cell wall changes that are able to compensate the lack of

LRX1; although the molecular mechanism is still far from being elucidated.

Regarding hybrid AGP-EXT (HAE), each protein contains both AGP- and EXT-motifs in the same protein. AGPs are apoplastic proteins heavily *O*-glycosylated with up to 90–95% of glycans composed mostly of arabinose and galactose (Hijazi *et al.*, 2014). Since HAE hybrid proteins contain both domains (EXT and AGP), based on the *contiguity hypothesis*, they are expected to be processed throughout the secretory pathway carrying both *O*-arabinosides as well as larger arabinogalactans, although this needs to be experimentally confirmed. In addition, HAE proteins have an extra domain outside AGP and EXT-motifs in their C-terminal sequences (Fig. 1A). There are only four HAE proteins in *Arabidopsis* (Showalter *et al.*, 2010). HAE1 has a pectin methyl esterase inhibitor (PMEI) domain and HAE4 has a plant lipid transfer domain, while both HAE2–HAE3 contain domains with unknown functions (DUF). Despite the fact that the biological functions of HAEs are still not known, it can be speculated that the arabinogalactan glycan addition in the AGP sites would reduce their susceptibility to protease degradation as well as enhance their secretion into the apoplastic space, as was shown for fusion chimeric proteins containing AGP sites (Xu *et al.*, 2010; Xu and Kieliszewski, 2012). In addition, when chimeras of HAE plus an elastine domain were overexpressed in tobacco cells, they triggered substantial cell wall changes in polymer composition, hydration and biomass accumulation (Tan *et al.*, 2014). In addition, when genetically encoded-synthetic peptides with AGP- and EXT-motifs were overexpressed in Arabidopsis plants, a strong effect on root growth and root meristematic size was reported highlighting that both motifs, AGP and EXT, in HAE proteins have the ability to trigger disturbance effects during root growth and cell expansion (Estevez *et al.*, 2006).

PERK

The PERK gene family consists of RLK proteins with an extracellular domain rich in prolines followed by a typical transmembrane and intracellular kinase domains (Fig. 1A). In Arabidopsis, the PERK family contains 15 related-members (AtPERK1–15) (Silva and Goring, 2002). By using an InParanoind blast search analysis (Sonnhammer and Östlund, 2015) of AtPERKs orthologues, we found putative PERKs orthologues in at least 12 other plant species (see Supplementary Fig. S1, available at *JXB* online). These include four monocots—of which three loci are found in *Oryza sativa* and two in *Hordeum vulgare*—and eight eudicots including six loci in *Solanum tuberosum* and 19 in *Brassica rapa*, suggesting that the PERK family is well conserved in angiosperms (Florentino *et al.*, 2006).

The first member characterized was BnPERK1 (a putative orthologue of AtPERK1) in *Brassica napus*, which is ubiquitously expressed and induced by wounding (Silva and Goring, 2002). All AtPERKs show similar gene structure, with high variability in the first two exons that explains the differences in length between the extracellular domains (Fig. 2), with the exception of AtPERK2 that is encoded by one single exon. Considering the alignment of the cytoplasmatic domains and map locations, Silva and Goring (2002) proposed that PERKs came from duplication events. While the lengths of the AtPERK extracellular domains vary considerably, kinase phylogeny showed, as expected, a high degree of sequence similarity (Silva and Goring, 2002; Nakhamchik *et al.*, 2004). The extracellular domain of all AtPERKs contains proline-rich regions of Ser-Pro$_{(3–5)}$ type where Ser-Pro$_{(2–3)}$ motifs predominate (Silva and Goring, 2002; Nakhamchik *et al.*, 2004) (Fig. 2).

In Table 2 we summarize the expression patterns and functions of the already characterized members of the PERK
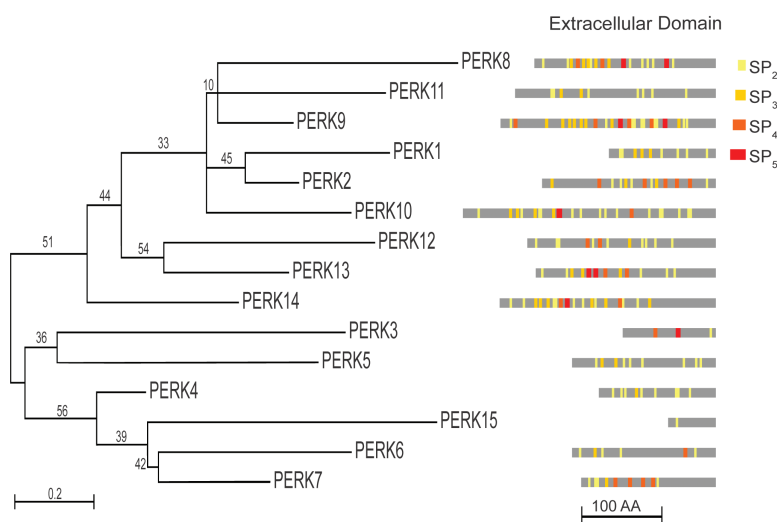

Fig. 2. Phylogeny and Ser-Pro$_n$ motifs map of the extracellular domain for the Arabidopsis PERK cluster. (Left) Neighbour-joining tree constructed based on the alignment of amino acid sequences of the extracellular domain of Arabidopsis PERK family members. The rooted tree represents a consensus tree generated by 500 bootstrap replicates, each inferred from parametric distances by the neighbour-joining method (Saitou and Nei, 1987). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura *et al.*, 2007). (Right) Extracellular domain organization. Ser-Pro$_n$ motifs are represented with different colours: SP$_2$=light yellow; SP$_3$=dark yellow; SP$_4$=orange; SP$_5$=red.

Table 2. *Overview of PERKs with emphasis on those present in polarized expanding cells (pollen tubes or root hairs)*

Protein/ Gene code	Tissue expression[a]/ (Subcellular localization)	Assumed function	References
BnPERK1	Stem, petal, and pistil/(PM)	Control of root and stem branching. Perception and response to a wound or pathogen stimulus. *In vitro* serine/threonine kinase activity	(Haffani *et al.*, 2006; Silva and Goring, 2002)
AtPERK1 (NsAK)/ At3g24550	Ubiquitously expressed/–	Related to virus infection	(Nakhamchik *et al.*, 2004; Florentino *et al.*, 2006; Haffani *et al.*, 2006)
AtPERK3/ At3g24540	Rosette leaf vein, stem, and pollen/–		(Nakhamchik *et al.*, 2004; Haffani *et al.*, 2006)
AtPERK4/ At2g18470	Root, bolt, germinated seed, and cotyledon /(PM)	Control of root growth in response to ABA. *In vitro* serine/threonine kinase activity	(Honys and Twell, 2004; Nakhamchik *et al.*, 2004; Bai *et al.*, 2009a, 2009b; Qin *et al.*, 2009)
AtPERK8/ At5g38560	Seedling, root, bolt, and floral buds/–	Redundantly control of primary root growth	(Nakhamchik *et al.*, 2004; Humphrey *et al.*, 2015)
AtPERK9/ At1g68690	Root, bolt, and floral buds/–	Redundantly control of primary root growth	(Nakhamchik *et al.*, 2004; Humphrey *et al.*, 2015)
AtPERK10/ At1g26150	Bolt and floral buds/–	Redundantly control of primary root growth	(Nakhamchik *et al.*, 2004; Humphrey *et al.*, 2015)
AtPERK12 (IGI1)/ At1g23540	Root hair, anther, pollen, stem, and immature silique/–	Control of apical dominance	(Nakhamchik *et al.*, 2004; Hwang *et al.*, 2010)
AtPERK13 (RHS10)/ At1g70460	Root hairs/–	Control of root hair elongation	(Nakhamchik *et al.*, 2004; Won *et al.*, 2009; Humphrey *et al.*, 2015)

[a] Based on reporter, RT-PCR, Northern blot analysis, or Genevestigator (Hruz *et al.*, 2008).
At = *Arabidopsis thaliana*; Bn= *Brassica napus*; PM=Plasma membrane.

gene family. AtPERK promoter:*GUS* analysis predicted that AtPERK1 is expressed mainly in the vascular tissues of cotyledons, developing leaves, and roots, while AtPERK3 is expressed in rosette leaf veins, stems and pollen (Haffani *et al.*, 2006). It was also shown that AtPERK12 is expressed in root hairs in young plants, while in adult plants is highly expressed in anthers, presumably in pollen (Hwang *et al.*, 2010). Using a promoter:*GFP* reporter, it was shown that AtPERK13 is also expressed in root hairs (Won *et al.*, 2009). All these results are in agreement with the microarray data obtained from different tissues of Arabidopsis. However, (Bai *et al.*, 2009a) found that AtPERK4 was present in roots, germinated seeds, cotyledons, stems and flowers, while pollen microarrays describe that AtPERK4 is highly expressed in mature pollen and pollen tubes (Honys and Twell, 2004; Qin *et al.*, 2009) but not in all of the sporophytic tissues that were analysed (Honys and Twell, 2004).

As typical plant receptor kinases, PERKs are expected to be located in the plasma membrane, as indeed has been reported for BnPERK1 and AtPERK4 (Silva and Goring, 2002; Bai *et al.*, 2009a). All AtPERK family members and BnPERK1 lack a signal peptide; however, a stretch of positively charged amino acids downstream of the transmembrane domain would be responsible for placing the kinase domain in the cytosol and the proline-rich EXT domain outside of the cell (Nakhamchik *et al.*, 2004). Thus, PERKs belong to the type Ib integral membrane protein family (Silva and Goring, 2002) and are hence proposed to be inserted into the membrane throughout the usual ER-translocator protein machinery (Singer, 1990). Serine/threonine kinase activity of BnPERK1

and AtPERK4 has been demonstrated *in vitro* (Silva and Goring, 2002; Bai *et al.*, 2009a). Moreover, Mayank *et al.* carried out a phosphoproteomic study on mature Arabidopsis pollen grains and found that AtPERK6 was phosphorylated in a serine located in the C-terminal domain (Mayank *et al.*, 2012).

Even though single mutants for most of the 15 AtPERKs do not show any detectable phenotype, apparently because of functional redundancy within the PERK gene family (Haffani *et al.*, 2006), there are some reports where forward and reverse genetic approaches have been used to shed light on the specific action of several AtPERKs during plant development. By an activation tagging approach, a new mutant called *igi1* (*inflorescence growth inhibitor 1*) that showed decreased height and increased branching, was characterized. These phenotypes are presumably caused by ectopic overexpression of the *IGI1* gene. In homozygous mutant plants (*igi1/igi1*), the expression of *IGI1* was approximately 3000 fold higher compared with that of the wild type, producing sterility because of the absence of inflorescences. By TAIL-PCR it was identified that the T-DNA was inserted in the promoter region of *IGI1* that turned out to be AtPERK12. These results suggest that, under physiological conditions, a member of the PERK family—not PERK12 because it is expressed in pollen but not in meristems—would negatively regulate apical dominance in Arabidopsis (Hwang *et al.*, 2010).

An *in silico* screening for gene promoters containing a root hair *cis* element (RHE) combined with the analysis of root hair transcriptomes, led to the identification of 19 root hair-specific genes. One of them (*RHS10*) is AtPERK13, which

was shown to be specifically expressed in root hairs. A T-DNA line for AtPERK13 gene showed longer root hairs than wild type plants, while AtPERK13 overexpressing plants under the root hair specific expansin EXPA7 promoter showed shorter roots, indicating the restraining effect of AtPERK13 on root hair elongation (Won *et al.*, 2009).

When *Arabidopsis* plants were transformed with the antisense version of BnPERK1 under the control of 35S promoter, growth defects such as loss of apical dominance, increased secondary branching, shorter stems and fewer seeds per silique were found. These antisense plants showed a complete inhibition of the expression of AtPERK1 and AtPERK3, the two AtPERK genes most closely related to BnPERK1, presumably responsible for the observed phenotype. On the other hand, ectopic expression of BnPERK1 in *Arabidopsis* under the control of 35S promoter produced enhanced growth phenotype with increased lateral shoot production, number of ovules per pistil and seed set (Haffani *et al.*, 2006). However, it was also described that the BnPERK1 antisense transgenic *Arabidopsis* plants showed longer hypocotyls when compared to wild type, while in the overexpressing lines hypocotyls were shorter (Haffani *et al.*, 2006), suggesting a still undeciphered complex regulation that will demand further experimentation.

It also has been shown that T-DNA mutants of AtPERK4 were hyposensitive to abscisic acid (ABA) during seed germination and primary root tip and seedling growth (Bai *et al.*, 2009*a*). The decreased sensitivity of *perk4* mutants to ABA during root tip growth brought out an enhanced cell elongation rather than increased cell division. The increase by ABA of cytoplasmic calcium and activation of calcium channels was lower in *perk4* mutants than in wild type plants. When expressed in yeast, recombinant PERK4 was autophosphorylated when stimulated by the addition of 1 μM ABA or calcium. All these results suggest that the inhibition of root growth caused by ABA is at least partially mediated by PERK4 (Bai *et al.*, 2009*a*).

Last, Humphrey *et al.* conducted a reverse genetics approach to study the function of AtPERK8, AtPERK9, and AtPERK10 (Humphrey *et al.*, 2015). They obtained single and multiple mutants for these three PERKs and analysed root growth under different sucrose conditions. While wild type and single mutant plants growing in 1/2 MS with 4.5% sucrose showed shorter primary roots compared with a medium without sucrose, the *perk8-1 perk9-1 perk10-1* triple mutant displayed increased root length compared with that of wild type plants. In contrast, overexpression of PERK10 led to a rapid arrest in primary root growth with ectopic depositions of lignin and callose (Humphrey *et al.*, 2015).

Collectively, these reports suggest that PERKs are regulators of plant growth and development. Root growth, sensitivity to ABA, and apical dominance are traits where PERKs show a negative effect. Mutant analysis of the remaining PERKs will be necessary to further validate and strengthen this hypothesis.

For the time being, all PERKs should be considered as orphan receptors because their ligands are still unknown. It seems likely that PERK extracellular domains are embedded in the cell wall similarly to the WAKs, suggesting that they could also function by sensing changes in the cell wall (Silva and Goring, 2002). Considering that the extracellular domains of WAKs bind pectin, which activates the WAK kinase domain (Kohorn and Kohorn, 2012), it is possible to speculate that PERKs also retain the ability of being activated by cell wall components and transduce signals to a downstream cascade. PERKs could also bind small peptides, as other receptor kinases do, so it would be also of great interest to test them as potential activators of PERK signalling pathways. Using a yeast two-hybrid approach, it has been found that the kinase domains of AtPERK8, AtPERK9, and AtPERK10 interact with two related kinesin-like calmodulin-binding protein (KCBP)-interacting protein kinase (KIPK), members of the *Arabidopsis* AGC VIII kinase family. Mutant analysis suggests that PERKs (8,9,10) and KIPKs (1,2)-KCBP are part of the same pathway that negatively regulates root growth (Humphrey *et al.*, 2015).

Arabidopsis wild type microarrays show that pollen and different root sections are the tissues with the maximum number of highly expressed PERKs. While PERK8 and PERK13 are specifically expressed in root hairs, PERK4, PERK5, PERK6, PERK7, PERK11 and PERK12 are found in pollen. These expression patterns seem to be logical considering that both root hairs and pollen tubes are tip growing cells that have to build new cell walls in a fast and regulated manner.

Most Group I Formins contain EXT-like motifs

Formins are actin-nucleating proteins that enhance rapid actin polymerization in animals, yeast, and plants (Ingouff *et al.*, 2005). Formins also bind actin filaments leading to their fragmentation and inducing actin filaments to form actin bundles, at least *in vitro*. Most of the formins studied are cytoplasmic proteins recruited to the cell membrane but, a subset of plant formins, classified as Group I, have evolved with an N-terminal extension of a transmembrane and an extracellular EXT-motif with up to three Ser-Pro$_{(3-4)}$ repeats (Fig. 1A). In this review, we will cover only the function of Group I formins. In *Arabidopsis* there are 11 formins in Group I (AtFH1–11) out of a total of 21 encoded formins (Table 3). These Group I formins are multidomain proteins with a predicted signal peptide (except AtFH7), a variable N-terminal domain, a proline-rich domain with Ser-Pro$_{(2-5)}$ motifs, a formin homology-1 domain (FH1), and an activity domain (FH2) (Fig. 1A,B). From the 11 predicted type I formins, AtFH2–AtFH4, AtFH7, and AtFH11 do not contain any Ser-Pro$_{(2-5)}$ but most of them still contain polyproline motifs of Pro$_{(2-4)}$. Based on the *contiguity hypothesis* it is highly possible that they could be *O*-arabinosylated on Hyp units within the Ser-Hyp$_{(2-5)}$ repeats. On the other hand, formin proteins do not contain Tyr-crosslinking motifs as present in classical EXT.

In most of the studied *Arabidopsis* formins, the FH2 domain is sufficient for nucleating actin while the FH1 domain interacts with profilin enhancing FH2's activity. However, in the case of AtFH3 and AtFH5, both FH1 and FH2 domains

Table 3. *Overview of formins with emphasis on those present in polarized expanding cells (pollen tubes or root hairs)*

Protein/ Gene code	Tissue expression[a]/ (Subcellular localization)	Assumed function/ mutant phenotype	References
AtFH1/ At3g25500	Ubiquitous/ (PM)	Actin nucleation and binding / *fh1* hypersensitive to the actin-polymerization inhibitor latrunculin B (LatB). Thicker and shorter roots, aberrant root hairs in LatB. Overexpression in tobacco short pollen tubes and supernumerary short actin cables	(Cheung and Wu, 2004; Michelot *et al.*, 2005; Martiniere *et al.*, 2011; Rosero *et al.*, 2013)
AtFH3/ At4g15200	Pollen tube	Actin nucleation and binding/ Overexpression in tobacco pollen tubes increase the number of longitudinal actin cables and causes swollen pollen tubes. RNAi against AtFH3 leads to short pollen tubes and a reduction in the levels of actin filaments	(Grunt *et al.*, 2008; Ye *et al.*, 2009)
AtFH4/ At1g24150	Cotyledons, leaves/ (PM and ER. Cell to cell junctions).	Actin nucleation and binding. Interaction with microtubules via its GOE domain/–	(Deeks *et al.*, 2005, 2010)
AtFH5/ At5g54650	Endosperm, pollen, root/ (PM, cell plate in dividing cells, pollen tube tip).	Actin nucleation and binding/ Delayed cellularization during endosperm development	(Ingouff *et al.*, 2005; Cheung *et al.*, 2010)
AtFH6/ At5g67470	Roots. Vascular tissue. Vascular bundles of leaves, stipules and apical hook/ (PM)	Actin nucleation and binding/–	(Favery *et al.*, 2004)
AtFH8/ At1g70140	Root/ (PM, preferentially in cell to cell junctions)	Actin nucleation and binding/ Root hair development. AtFH8-overexpressor lines with shorter root hairs. *fh8* mutants with shorter roots and fewer lateral roots (in presence of LatB)	(Deeks *et al.*, 2005; Yi *et al.*, 2005)
OsFH1/	Root/ (PM).	–/Short root hairs in liquid media	(Huang *et al.*, 2013)
NtFH5/	Ubiquitous/–	–/RNAi or antisense against NtFH5 leads meandering pollen tubes	(Cheung *et al.*, 2010)

[a] Based on reporter, RT-PCR, Northern blot analysis, or Genevestigator (Hruz *et al.*, 2008).
ER= endoplasmic reticulum; PM= plasma membrane; At=*Arabidopsis thaliana*; Nt=*Nicotiana tabaccum*; Os=*Oryza sativa*.

are necessary for proper activity. Accordingly, several Group I formins have been characterized to interact with actin microfilaments (e.g. AtFH1, AtFH3–AtFH6, and AtFH8) (Cheung and Wu, 2004; Ingouff *et al.*, 2005; Deeks *et al.*, 2010), while only AtFH4 was reported to also bind to microtubules via its Group Ie (GOE) domain—located between the transmembrane and FH1 domains (Deeks *et al.*, 2010). Group I plant formins are involved in several cell processes, from cell division to polarized cell growth (Cheung and Wu, 2004; Ingouff *et al.*, 2005; Yi *et al.*, 2005; Vidali *et al.*, 2009; Deeks *et al.*, 2010) (Table 3).

The best studied plant formins are those involved in polarized cell expansion. It has been described that any disturbance in formin protein levels causes visible actin defects. AtFH3 and AtFH5 are predominantly involved in pollen development and pollen tube growth (Grunt *et al.*, 2008; Ye *et al.*, 2009), whereas AtFH1, OsFH1 (*Oryza sativa*), and AtFH8 are important in root hair growth (Deeks *et al.*, 2005; Yi *et al.*, 2005; Huang *et al.*, 2013) (Table 3). Overexpression of AtFH1 and AtFH8 induces morphological defects in tip growing cells such as pollen tubes or root hairs, respectively, while *fh1* mutant showed aberrant and shorter root hairs (Cheung and Wu, 2004; Yi *et al.*, 2005). Expressing the N-terminus of AtFH8 is sufficient to induce wavy, swollen, or branched root hairs suggesting that this domain could disrupt cell wall organization during cell expansion (Yi *et al.*, 2005). By using several experimental approaches, it was shown that the N-terminal part of AtFH1 links formins to the extracellular matrix (plant cell wall) changing its lateral mobility and providing an anchoring point to the plant cell (Martinière *et al.*, 2012). By a series of deletions it was found that a small portion of the extracellular domain of AtFH1, containing a single Ser-Pro$_{(4)}$ motif, was fundamental for providing a physical link between AtFH1 and the cell wall, although the biochemical nature of this interaction still remains to be elucidated (Martinière *et al.*, 2012). It is unclear whether it is based on a protein–protein interaction or if the presence of predicted carbohydrates in the Ser-Pro$_{(4)}$ EXT-motif (if processed as a classical EXT protein) could play a role in the proposed anchoring function.

Concluding remarks

In recent years it is becoming clear that there are several molecular links that connect the plant cell surface continuum, which is highly important in many biological processes ranging from pathogen and symbiotic perception, chemotactic responses, to abiotic stress, nutrient and water sensing. Any

disturbance in the plant cell wall has a direct impact on the plasma membrane and cytoskeleton (e.g. cellulose–CESA–microtubules alignment) (Paredez *et al.*, 2006; Worden *et al.*, 2015). On the other hand, the disruption of any component in the cytoskeleton also influences the plant cell wall (e.g. MIDD1 alter cell wall composition) (Oda *et al.*, 2010; Oda and Fukuda, 2012; McKenna *et al.*, 2014). It is surprising that the EXT-motif with the short sequence of Ser-Pro$_{(3-5)}$ is found in several different protein contexts within the same extracellular space highlighting a putative conserved structural and functional role. It is still unknown whether the Ser-Pro$_{(3-5)}$ motif is usually present in the *O*-glycosylated form or if these posttranslational modifications only appear in EXTs and related structural proteins. Finally, a deeper understanding of the dynamic regulation and action of plant cell surface continuum and its relationship with the downstream signalling cascade is a crucial forthcoming challenge. The plant cell surface continuum concept is an exciting area of research that is rapidly evolving and great advances are expected to come in the following years.

Supplementary data

Supplementary Fig. S1. Examples of PERK protein sequences found for other monocots and dicot species using an InParanoind blast search analysis.

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