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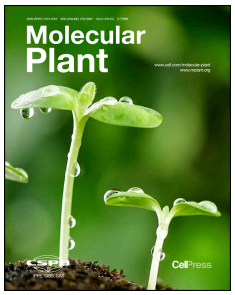

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Filling the Gaps to Solve the Extensin Puzzle

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Running Head: Extensin modifications and polar growth

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E.M. and C.B. reviewed text, references, and figures; M.B. and L.C. performed molecular modeling of extensins, designed Figure 3, and reviewed text; A.S. reviewed text and figures; D.R.R.G. reviewed text and references; J.M.E. conceived the project, designed figures, and wrote the article with contributions from all the authors.

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Key words

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Short summary

This review describes recent progress in our understanding of extensin post-translational modifications throughout the secretory pathway, extensin secretion and assembly in the cell walls, and possible sensing mechanisms at the interface between the apoplast and the cytoplasmic side of the cell surface.

ABSTRACT

Extensins (EXTs) are highly repetitive plant *O*-glycoproteins that require several post-translational modifications (PTMs) to become functional in plant cell walls. First, they are hydroxylated on contiguous proline residues; then, they are *O*-glycosylated on hydroxyproline (Hyp) and serine. After secretion into the apoplast, *O*-glycosylated EXTs form a tridimensional network organized by inter- and intra-Tyr linkages. Recent studies have made significant progress in the identification of the enzymatic machinery required to process EXTs, which includes prolyl 4-hydroxylases (P4Hs), glycosyltransferases (GTs), papain-type cysteine-endopeptidases (CEPs), and peroxidases (PERs). EXTs are abundant components of plant tissues, and are particularly important in rapidly expanding root hairs and pollen tubes, which grow in a polar manner. Small changes in EXT PTMs affect fast-growing cells, although the molecular mechanisms underlying this regulation are unknown. In this review, we highlight recent advances in our understanding of EXT modifications throughout the secretory pathway, EXT assembly in the cell walls, and possible sensing mechanisms triggered by the *Catharanthus roseus* cell surface sensor receptor-like kinases (CrRLK1Ls) located at the interface between the apoplast and the cytoplasmic side of the plasma membrane.

INTRODUCTION

In the model plant *Arabidopsis thaliana*, 10–15% of the genome is devoted to construction, dynamic architecture, sensing functions, and metabolism of the plant cell walls (Carpita, 2001; Cosgrove, 2015). In contrast to *N*-glycan glycosylation, the core mechanism for *O*-linked glycosylation in plants does not appear to be conserved among all eukaryotes. In mammals, the most common *O*-glycan results from the incorporation of an N-acetylgalactosamine (GalNAc) at serine or threonine residues (mucin-type *O*-glycosylation) (Bennet et al., 2012). In plant cells, *O*-linked glycans are usually attached to the hydroxyl group of hydroxyproline (Hyp); however, they are occasionally attached to the hydroxyl group of serine (e.g., in extensins, EXTs) (Kieliszewski, 2001). This *O*-linked glycosylation defines the molecular properties and biological function of the Hyp-rich glycoprotein (HRGP) superfamily and some secreted small peptides (e.g., CLE for CLAVATA3/Endosperm surrounding region). The HRGP superfamily is traditionally divided into three major subgroups: arabinogalactan-proteins (AGPs), EXTs, and the repetitive Proline-rich proteins (PRPs). However, the HRGP superfamily is better understood as a spectrum of molecules ranging from the highly glycosylated AGPs to the minimally *O*-glycosylated PRPs (for details, see Ellis et al., 2010; Lamport et al., 2011; Johnson et al., 2017). Bioinformatic analysis of the HRGP superfamily in *Arabidopsis thaliana* identified 59 genes encoding EXT-related glycoproteins, 18 encoding PRPs and 4 encoding AGP/EXT hybrid HRGPs (Showalter et al., 2016; Johnson et al., 2017). Here, we refer to EXTs in a wide sense to include related glycoproteins containing multiple Ser-$(Pro)_{3-5}$ repeats that may be *O*-glycosylated, Tyr (Y)-based motifs that could be crosslinked, and a putative *O*-glycosylated arabinogalactan (AG) motif since it contains a Ser-Pro-Ser-Pro sequence (**Figure 1A**) such as proline-rich proteins (PRPs) and leucine-rich repeat extensins (LRXs). We exclude the proline-rich extensin-like receptor kinases (PERKs) and formins (FHs) from this group because they contain additional protein domains (e.g., cytoplasmic kinase domains (PERKs) or actin-microtubule binding domains (formins)) that are highly important for their functions independently of the EXT-like domain.

EXTs require several modifications before they become functional at the plant cell surface. First, they are hydroxylated by prolyl 4-hydroxylase (P4H) enzymes (**Figure 1B-C**), and then *O*-glycosylated by several co-expressed glycosyltransferases (GTs) in the secretory pathway (e.g., ER and Golgi compartments) (**Figure 1D-E**). Finally, they are crosslinked in the apoplast (Velasquez et al., 2011) by unidentified secreted type-III peroxidases (PERs). For *O*-glycosylation, EXTs and small peptides require the conversion of specific peptidyl-proline residues to *trans*-4-hydroxyproline (Hyp) by P4H enzymes (Velasquez et al., 2011; Velasquez et al., 2015). Two mayor types of *O*-glycans are attached to Hyp in plant HRGPs. Linear chains of up to five arabinose units are added to clusters of Hyp residues in EXTs and small peptides (**Figure 1D**), whereas complex arabinogalactans are attached to AGPs or AGP-like proteins. The Hyp contiguity hypothesis

proposes that the addition of these two main types of *O*-glycan is controlled by a primary HRGP protein sequence (Kieliszewski, 2001). This hypothesis predicts that short arabino-oligosaccharides are added to contiguous Hyp_{3-5} residues in EXTs (**Figure 1D**), whereas complex arabinogalactans are transferred to clustered but non-contiguous Hyp residues in AGPs (Shpak et al., 1999; Tan et al., 2010). One exception to this rule are CLE-like peptides (e.g., Tob/Tom-HypSys, PSY1, CLV3, and CLE2), in which non-contiguous Hyp residues are arabinosylated. *O*-arabinosylation of small peptides is important for their stability and activity (Ohyama et al., 2009; Matsubayashi et al., 2010; Shinohara and Matsubayashi, 2013). Monomeric secreted EXTs form rod-like structures with a polyproline-II helical conformation, which are further stabilized by their Hyp-*O*-glycans (Stafstrom et al., 1986; Owens et al., 2010; Velasquez et al., 2011; Velasquez et al., 2015). In addition to EXT *O*-glycosylation, some EXTs are cross-linked and insolubilized into the plant cell wall by Tyr-based motifs (Lamport et al., 2011). Secreted type-III peroxidases (PERs) are thought to facilitate both intra-molecular and inter-molecular covalent Tyr-Tyr cross-links by generating *iso*dityrosine units and pulcherosine or di-*iso*dityrosine, respectively (Brady et al., 1996; Brady et al., 1998) (**Figure 1F**); however, the underlying molecular mechanisms are not completely determined. Finally, *O*-glycosylation defects on EXTs might be sensed and controlled in the secretory pathway by specific papain-type Cysteine EndoPeptidases (CEPs) (Helm et al., 2008). This review discusses recent discoveries of major PTMs of EXTs, the enzymes involved (i.e., proline hydroxylation by P4Hs, *O*-linked glycosylation by GTs, and Tyr-based crosslinking by PERs), and their functional implications. Several authoritative reviews provide comprehensive coverage of various aspects of EXT glycoproteins, small glycopeptides, and the associated enzymatic machinery (P4Hs, PERs, and CEPs) (Dunand et al., 2007; Gorres and Raines, 2010; Matsubayashi et al., 2010; Kieliszewski et al., 2011; Lamport et al., 2011; Hierl et al., 2013; Borassi et al., 2016).

PROLINE HYDROXYLATION OF EXTENSINS BY PROLYL 4-HYDROXYLASES

P4H enzymes are 2-oxoglutarate (2OG) dioxygenases (EC 1.14.11.2) that catalyze the formation of *trans*-4-hydroxyproline (Hyp/O) from peptidyl-proline; this reaction requires Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate cofactors. While plant P4Hs contain only the catalytic α-subunit (Koski et al., 2007; Koski et al., 2009), in animal cells P4Hs form tetramers of $\alpha_2\beta_2$ subunits where β-subunits have protein disulfide isomerase activity responsible for retention and solubility of α-subunits in the endoplasmic reticulum (Myllyharju 2003). Studies on synthetic repetitive peptides (Shpak et al., 1999; Held et al., 2004) and native purified P4H substrate proteins (Cannon et al., 2008) indicate that classical P4H-mediated proline hydroxylation of EXT with 2–4 contiguous proline units next to a serine residue [$Ser(Pro)_{2-4}$] mostly runs to complete proline-hydroxylation [Ser-$(Hyp)_2$ to Ser-$(Hyp)_4$], whereas the extent of this modification is much more difficult to predict in non-classical sequence contexts (Duruflé et al., 2017). The *Arabidopsis thaliana* genome encodes 13 putative *P4Hs*; the *in vitro* activity of P4H1, P4H2, and P4H5 has been characterized (Hieta and

Myllyharju, 2002; Tiainen et al., 2005; Velasquez et al., 2011; Velasquez et al., 2015b). On the other hand, the *Chlamydomonas reinhardtii* genome encodes 10 *CrP4H*-like polypeptides (Keskiaho et al. 2007) and only the activity of CrP4H1 has been assessed *in vitro* (Koski et al., 2007; Koski et al., 2009). Moreover, there are 6 homologous sequences of *AtP4Hs* in the moss *Physcomitrella patens*, but only PpP4H1 was shown to be enzymatically active by using a heterologous human erythropoietin (hEPO) substrate (Parsons et al., 2013).

Recent work has described the actions of *Arabidopsis thaliana* P4H2, P4H5, and P4H13 on EXTs, specifically in root hair cells (Velasquez et al., 2015). Treatment of root hairs with P4H inhibitors DP (α,α-dipyridyl) and EDHB (ethyl-3,4-dihydroxybenzoate) blocked peptidyl-proline HRGP hydroxylation and drastically inhibited cell elongation, suggesting the existence of a direct link between proline hydroxylation and root hair growth (Velasquez et al., 2011). Expression of P4H:GFP fusions under control of their endogenous promoters revealed that *P4H2*, *P4H5*, and *P4H13* were expressed primarily in root epidermal trichoblast cells and growing root hairs, and were localized in the ER and Golgi compartments (Velasquez et al., 2011, Velasquez et al., 2015b). These results suggest that proline hydroxylation of HRGPs might be initiated in the ER and completed in the Golgi.

P4H5 and P4H13 contain an [RK]X[RK] motif (ERG domain) in their N-terminus, which is important for Golgi targeting (Velasquez et al. 2015), as shown previously for P4Hs in tobacco (*Nicotiana tabacum*) cells (Yuasa et al., 2005). According to the biochemical inhibition phenotype, the analysis of T-DNA insertional mutants for *P4H2*, *P4H5*, and *P4H13* also showed the arrest of cell elongation and the presence of truncated root hair phenotypes. The *p4h5* mutant displayed the most drastic phenotype, with an altered cell structure resembling of the triple mutant *p4h2 p4h5 p4h13*. By contrast, *P4H5* overexpression induced an over-elongated root hair phenotype (Velasquez et al. 2011; Velasquez et al. 2015b). Genetic complementation or by a P4H promoter-swapping approach as well as over-expression studies (Velasquez et al., 2015b) showed that P4H2 and P4H13 have similar function during root hair growth while P4H5 displayed a unique role (**Figure 1B-C**). Meanwhile, root Hyp levels were reduced in all these mutants. P4H5 preferentially hydroxylates three of the first four proline units (SOOOP) in EXT following a specific order, rather than acting on other proline-rich peptides (Velasquez et al., 2015b). This result allows us to propose that, in root hair cells, P4H5 has a main role in the initiation and continuous proline hydroxylation of EXTs, whereas P4H2 and P4H13 terminate the hydroxylation on these contiguous prolines (Velasquez et al., 2015b; **Figure 1B**). In agreement with this notion, P4H5 clearly preferred an EXT substrate even in the presence of four-time higher AGP peptide concentration (Velasquez et al., 2015b). In addition, P4H5 interacts with P4H2 and P4H13, and most P4Hs can form dimers, suggesting that one or several multiprotein P4H complexes could function *in vivo* to hydroxylate EXTs (**Figure 1C**). The combined evidence suggests that EXT proline hydroxylation is mediated by

P4H2,5,13 protein complexes (with unknown stoichiometry). The proline hydroxylation modification on EXTs is strictly required for the subsequent *O*-glycosylation steps and control of root hair cell expansion. Further studies are needed to characterize other AtP4Hs *in vitro* to examine how primary and/or secondary HRGP sequences might regulate the degree and pattern of proline hydroxylation.

GLYCOSYLTRANSFERASES (GTs) INVOLVED IN *O*-GLYCAN ASSEMBLY IN EXTENSINS

EXTs are characterized by repetitive Ser-Hyp_{3-5} repeats, where the contiguous Hyp residues are substituted with up to 4–5 units of L-arabinofuranose (L-Ara*f*). These modifications generate the structure Hyp-(1→4)-â-L-Ara*f*-(1→2)-â-L-Ara*f*-(1→2)-â-L-Ara*f*-(1→3)-á-L-*t*Ara*f*; the linkage of the fifth arabinose is not yet resolved, and the serine is substituted with D-galactose as Ser-(1→3)-α-Gal*p* (**Figure 1D**). Several known arabinofuranosic transferases catalyze the sequential addition of arabinose residues on Hyp (**Figure 1D**). The first arabinose is added by three Hydroxyproline *O*-β-arabinosyltransferases 1-3 (HPAT1-HPAT3), which belongs to glycosyltranferase GT95 family (Ogawa-Ohnishi et al., 2013). The Reduced Residual Arabinose 1–3 (RRA1-RRA3) of the GT77 family (Egelund et al., 2007; Velasquez et al., 2011) is thought to transfer the second arabinose, while the third residue addition is catalyzed by the Xyloglucanase113 (XEG113), which also belongs to the GT77 family (Gille et al., 2009) although the *in vitro* transferase activities of RRA1-RRA3 and XEG113 remain to be determined. These putative assignments of their enzymatic activity were based on the analysis of underglycosylated EXTs isolated from *rra1-rra3* and *xeg113* mutants (Velasquez et al., 2011). *O*-arabinosylation with β-linked-L-arabinofuranosides at Hyp has a key role in regulating short peptides hormones in the CLE family (*i.e.,* Tob/Tom-HypSys, PSY1, CLV3, and CLE2) using identical linkages/stereochemistry as used for the innermost three arabinoses found in the EXTs (Ito et al. 2006; Ohyama et al., 2009; Matsuzaki et al., 2010), suggesting that similar P4Hs and GTs might participate in these PTMs.

Two additional enzymes were identified recently that provide a more comprehensive model of the PTM machinery acting on EXTs in *Arabidopsis thaliana* (**Figure 1E**). A unique Serine-galactosyltransferase (SGT1/SerGT1) adds Galactose to Serine in the repeated SOOOO motif in EXTs (Saito et al., 2014). SerGT1 is the first example of a glycosyltransferase with type-I membrane protein topology with no homology to known glycosyltransferases, indicating that it is a novel plant specific glycosyltransferase in the new GT96 family (Saito et al., 2014). The second enzyme is Extensin Arabinose Deficient (ExAD), which transfers the fourth arabinofuranose residue with α-(1→3) linkage. ExAD belongs to clade-E of inverting GT47 (Rune Møller et al., 2017). The corresponding *exad1* T-DNA mutant displayed a similar truncated root hair phenotype (as observed for *hpats*, *rra1-3*, *xeg113*) and EXTs in the *exad1* mutant only contained side chains of three arabinoses without any trace of Ara_4 or Ara_5 chains (Rune Møller et al., 2017). The

arabinosyltransferase that adds the fifth and final arabinose unit remains to be identified. An early study identified a Hyp-$(Ara)_5$ species (Campargue et al., 1998), which was validated by mass spectrometry analysis (Velasquez et al., 2011). Although, the linkage has not been determined and the corresponding GT candidates are unknown, they may include the EXT AraTs described above. *P4H2*, *P4H5*, and several GTs described before (*SerGT1*, *HPAT3*, *RRA3*, and *XEG113*) are expressed together in the same transcriptional co-expression network (**Figure 1E**), suggesting that they may be physically associated in the Golgi compartment. Further experiments are needed to determine whether these GTs work as multiprotein complexes in EXTs *O*-glycosylation as shown for several GTs modifying plant cell wall polysaccharides such as GAUT1 and GAUT7 in pectin biosynthesis (Atmodjo et al., 2011) or exostosins in heparan sulphate synthesis (Busse-Wicher et al., 2014).

A recent evolutionary analysis indicated that the PTM enzymatic machinery for EXTs could have arisen before EXTs; this should be considered a vascular plant innovation since no Tyr-crosslinking motif in EXT-like proteins is found in unicellular photosynthetic organisms (Liu *et al.,* 2016). The green algae *Chlamydomonas reinhardtii* genome comprises putative *RRAs* and *XEG113* orthologues and several GTs belonging to the GT47 family (all belong to Clade-E). These might catalyze the synthesis of *O*-glycosylated EXT-like surface proteins (Rune Møller et al., 2017). In agreement with this possibility, RNAi-mediated inhibition of a single P4H, CrP4H1, in *C. reinhardtii* drastically disrupted the cell wall (Keskiaho et al., 2007). These combined results suggest a conserved function for proline hydroxylation/*O*-glycosylation of structural cell wall glycoproteins in ancient green algae and vascular plants (Keskiaho et al., 2007; Velasquez et al., 2011; Velasquez et al., 2015).

IS QUALITY CONTROL OF MISGLYCOSYLATED EXTENSINS REGULATED BY CYSTEINE ENDO-PEPTIDASES?

Plant and animal N-glycoproteins comprise more than 33% of the total cell proteomes; they have a highly regulated and conserved quality control mechanism that rectifies protein folding errors throughout the secretory pathway (Caramelo and Parodi, 2015). When N-glycoproteins cannot fold correctly, they are degraded by ER-associated protein degradation (ERAD), which is linked to ubiquitination and proteasomal degradation (Vembar and Brodsky, 2008). A second degradation mechanism (Xu et al., 2013) involves an unfolded response based on protein *O*-Mannosylation (UPOM). Both of these mechanisms are crucial to maintain glycoprotein homeostasis, also known as glycoproteostasis (Kim et al., 2013). By contrast, little is known about how plant *O*-glycoprotein folding (including EXTs) is sensed and controlled along the secretory pathway. The first evidence of a possible EXT regulator comes from a unique group of papain-type cysteine-endopeptidases (CEPs), which contains a C-terminal KDEL endoplasmic reticulum (ER) retention signal, and has no homologous proteins in mammals and yeast (Hierl et al., 2013). CEPs are usually synthesized as

pre-pro-enzymes; the N-terminus pro-peptide and C-terminal KDEL are both removed in the ER when the enzyme becomes active (Hierl et al., 2012; Hierl et al., 2013). CEPs can also be stored in ER-related compartments such as "ricinosomes" and ER-bodies in Brassicaceae (Hierl et al., 2013). CEPs are involved in programmed cell death (PCD), such as PCD in *Ricinus* endosperm (Schmid et al., 1999), megagametophyte cell death after seed germination (He and Kermode, 2003), and tapetal cell death during functional pollen formation (Zhang et al., 2014). The *in vitro* activity of a CEP from *Ricinus communis* (RcCysEP) can digest broad sequences, including the *O*-glycosylated peptide [VY↓K↓SOOOO] (↓= cleavage site) commonly present in EXT-repeats (Helm et al., 2008). The three *CEPs* present in *Arabidopsis* (*AtCEP1–AtCEP3*) are widely expressed throughput the plant (Helm et al., 2008; Zhou et al., 2016). Only AtCEP1 is experimentally linked to tapetum PCD during pollen development (Zhang et al., 2014). It is also tempting to hypothesize that CEPs may control EXT proteolysis in the ER (and possibly also the Golgi) when they are over- or under-*O*-glycosylated, but further evidence is required to determine whether there is an *in vivo* link between CEP and EXT PTM processing in the secretory pathway. It is unknown if CEPs are secreted and reach the apoplast where they could theoretically regulate EXT network disassembly under specific conditions.

TRANSCRIPTIONAL CONTROL OF EXTENSINS AND PEROXIDASES

Several transcription factors (TFs) have been shown to control the expression of numerous EXTs in diverse organs of the model plant *Arabidopsis thaliana*. Although the *Arabidopsis* genome encodes 59 EXTs, only 1 EXT mutant [*root shoot hypocotyl-defective* (*rsh,* also *ext3)*] has a near-lethal phenotype (Cannon et al., 2008) suggesting a high degree of genetic redundancy in the EXT protein family. Root hair cells are the exception to this rule, because single *lrx1* and *lrx2* mutants have aberrant root hair morphologies (Baumberger et al., 2001; Baumberger et al., 2003; Ringli et al., 2010). In addition, several classical EXT mutants display short root hairs (Velasquez et al., 2011). These EXTs (*ext6-7, 12-14, 18*) were first identified by analyzing a co-expression network with other well-known cell wall genes that are important for root hair growth, including the bHLH-type transcription factor *RSL4* for Root Hair Defective 6 Like-4 (Datta et al., 2015; Mangano et al., 2017), *LRX1* (Baumberger et al., 2001; Baumberger et al., 2003), and *PRP3* (Bernhardt and Tierney, 2000) (**Figure 2A**). These six EXTs have been identified in most of the available root hair transcriptomes (Birnbaum et al., 2003; Brady et al., 2007; Deal and Henikoff, 2010; Bruex et al., 2012). These classical EXTs contain a highly conserved repeated motif of 25 amino acids that contains *O*-glycosylation and crosslinking motifs, but they are clearly differentiated by their N-terminus sequences where they share very low similarity, and variability in the total protein length (**Figure 2B**). These two variables within the overall similarity between EXTs could explain why a single plant cell needs to express at least six classical EXTs with the same repetitive motifs, and why most plants display a

short root hair phenotype when the encoding genes are mutated by T-DNA (Velasquez et al., 2011).

In support of the notion that RSL4 regulates the transcription of several EXTs, these EXTs (i.e., *EXT1*2, *EXT14*, and *EXT18*) are down-regulated in the loss-of-function mutant *rsl4-1* and strongly upregulated in RSL4 overexpression lines (e.g., $35S_{pro}$-RSL4; Yi et al., 2010); therefore, it is plausible that RSL4 directly controls their expression. In agreement with this possibility, some of these EXTs contain several root hair-specific *cis*-Elements (RHEs) that function as RSL4-binding domains at their regulatory regions (Hwang et al., 2017; **Figure 2B**). Furthermore, recently it was shown that RSL4 binds to the promoters of genes encoding root hair-related EXTs such as *LRX*1 and *PRP3*, and positively controls their expression (Hwang et al., 2017). These combined results indicate that EXTs have essential roles in root hair cell elongation under the control of RSL4 (Marzol et al., 2017). Several other TFs also regulate EXT expression in a RSL4-dependent or RSL4-independent manner. Very recently, a negative regulator of RSL4 the trihelix transcription factor GT-2-LIKE 1 (GTL1) was characterized (Shibata et al. 2018). GTL1 binds to GT3 boxes in the RSL4 promoter region (GGTAAA at -556 and TTTACC at -785 from the starting transcription site) and negatively regulates *RSL4* expression to repress root hair growth. GTL1 also repress the expression of several EXTs (e.g. *EXT12* and *LRX2*) by down-regulating *RSL4* (Shibata et al. 2018). In addition, recent work showed that ETHYLENE INSENSITIVE 3 (EIN3) physically interacts with RHD6, and together these proteins control *RSL4* expression. On the other hand, several EXTs (e.g., EXT11, EXT13, and LRX1) are believed to be involved in the root hair initiation controlled by EIN3/EIL1 and RHD6/RSL1 independently of RSL4 (Feng et al., 2017). In response to ethylene stimulation, EIN3 directly triggers EXT expression (e.g., *EXT13* and *EXT14*) (Song et al., 2016).

The HDG11 transcription factor, belonging to the HD-ZIP IV subfamily, and related transcriptional regulator proteins such as MEDIATOR25 (MED25) [also called PHYTOCHROME AND FLOWERING TIME1 (PFT1)] specifically regulate the expression of several EXTs in root hairs. Several root hair EXTs exhibit an HD-binding *cis*-element containing the (T)TTAATT(T) or the complementary (A)AATTAA(A) sequence (e.g., *EXT6*, *EXT7*, *EXT10*, *EXT12*), which can be bound by HDG11 *in vitro* and *in vivo* (Xu et al. 2014; **Figure 2B**). The *med25*/*pft1* mutants display truncated root hairs and downregulation of several EXTs, suggesting that MED25 and PFT1 are positive regulators of EXT-mediated root growth (Sundaravelpandian et al. 2013). Recent work showed that auxin treatment promoted the interaction between MED25/PFT1 and ARF7/ARF19, thereby releasing the repressor Aux/IAA14 for degradation together with other components (e.g., CKM for the CDK8 kinase module of the mediator complex and TPL for Topless) and triggering the transcriptional activation of target genes mediating lateral root development (Ito et al., 2006). A similar mechanism could exist in root hair cells where ARF7/ARF19 could be activated by high auxin levels, thereby promoting root hair growth (Mangano et al., 2017). It is not clear if RSL4, GTL1, HDG11, and

MED25/PFT1 act in a coordinated manner to control EXT expression, or if they are activated by different signals, such as low Pi-auxin and ethylene in the case of RSL4 (Yi et al., 2010; Datta et al., 2015; Feng et al., 2017), high auxin for MED25/PFT1, and still unknown signals for HDG11 and GTL1.

Recent work identified four PERs that are highly expressed in root hairs (*PER1*, *PER44*, *PER60*, *PER73*), co-expressed, and associated with the transcriptional regulator RSL4 (Mangano et al., 2017; **Figure 2A**). These PERs were partially characterized, and they were linked to lower levels of reactive oxygen species (ROS) in the tip of root hair cells. A mild root hair phenotype was observed with multiple PER T-DNA mutants (*per44 per73*), whereas single mutants displayed almost normal growth, suggesting a high degree of genetic redundancy (Mangano et al., 2017). RSL4 directly promotes the expression of these PERs through binding to the regulatory regions of these genes. These four PERs are placed together with six EXTs in the same transcriptional co-expression network under the direct control of RSL4; therefore, they are excellent candidates for performing EXT-crosslinking in the root hair cell walls. At least two more transcription factors negatively regulate *PER44* expression: KUODA1 (KUO1), a MYB-like transcription factor, was shown to bind to two motifs (ATCACA) in the *PER44* promoter and repress its expression in expanding leaves (Lu et al., 2014), and to the double B-Box BBX24 in growing hypocotyls (Crocco et al., 2015). Further experiments are required to establish how PER expression is coordinated at the transcriptional and post-transcriptional levels to control EXT crosslinking and ROS homeostasis (Mangano et al., 2016; Mangano et al., 2017). In addition, several PERs isolated from numerous plant systems designated as extensin peroxidases (EP) have been previously shown to crosslink EXT substrates *in vitro* (Schnabelrauch et al. 1996; Wojtaszek et al. 1997; Jackson et al. 2001; Price et al. 2003; Dong et al. 2015), although their *in vivo* roles remain elusive.

EFFECTS OF POST-TRANSLATIONAL MODIFICATIONS ON EXTENSIN CONFORMATION AND FUNCTION: THE CHALLENGE OF MODELING AN EXTENSIN GLYCOPROTEIN

Plant cell wall integrity depends on the correct assembly of its individual components. Many defects in the cell wall or extracellular matrix of green plants (Showalter et al. 2010) and related algae (Keskiaho et al., 2007) involve Hyp-rich EXT proteins (Cannon et al. 2008). A recent study analyzed root hair growth in mutants deficient in Hyp-*O*-arabinosylation (*hpat1/hpat2*, *rra3*, *xeg113*, and *exad*), mutants low in serine-*O*-galactosylation (*sergt1*), and multiple mutants deficient in both *O*-glycosylation types (*p4h5 sergt1* and *rra3 sergt1*) or coupled with P4H inhibitors in the *sergt1* mutant background (EDHB + *sergt1* or DP + *sergt1*) (Velasquez et al 2015a). Mutants in each individual *O*-glycosylation type displayed attenuated polar growth. Mutants in both *O*-glycosylation types (Hyp-*O*-arabinosylation + Ser-*O*-galactosylation) displayed more severe inhibition of polar growth, suggesting that both *O*-glycans are required for correct EXT function in

root hair growth (Velasquez et al 2015a). The degree of Hyp-*O*-arabinosylation in an EXT monomer affects the degree of Tyr-crosslinking performed by a tomato PER in an *in vitro* system (Chen et al., 2015). These combined results indicate that even small changes in EXT *O*-glycosylation may interfere with its self-assembly into the EXT network (Cannon et al., 2008), as observed for the *sergt1* mutant, which lacks a single monosaccharide unit (Velasquez et al., 2015a).

To elucidate the effects of these changes on EXT, a short peptide [Ser(Pro)$_4$] was used as a very simple EXT model to analyze the molecular dynamics of non-hydroxylated, hydroxylated, and *O*-glycosylated states. The modeling revealed that *O*-glycosylation stabilizes the helical conformation of the model peptide, whereas incomplete *O*-glycosylation enhances its flexible conformation (Velasquez et al., 2011). It was also theorized that EXTs with incomplete hydroxylation/*O*-glycosylation could significantly affect the interaction of EXTs with the surrounding environment, including lateral alignment interactions with other EXTs (Cannon et al., 2008), possibly forming triple EXT helix-like conformations comparable to collagen (with similar interaction energies). Next, a larger EXT repeat peptide (SPPPPYVYSSPPPPYYSPSPKVYYK) was analyzed with Hyp-*O*-arabinosylation, Ser-*O*-galactosylation, and complete *O*-glycosylation in all Hyp units (Velasquez et al., 2015a). High levels of *O*-glycosylation in certain EXT segments were found to physically restrict the EXT lateral alignments (**Figure 1F**), possibly by acting as a branching point (Velasquez et al., 2015). Although these attempts to generate a simple EXT model were useful, one of the major limitations in our understanding of how EXT function in the plant cell walls is the lack of a full-length EXT protein.

To have a more detailed understanding of how EXT molecules might behave in the cell wall, we have built a larger model of an EXT sequence that includes 10 conserved repeats of SPPPPYVYSSPPPPYYSPSPKVYYK with a total length of 250 amino acids by using a course-grained molecular dynamics approach (**Figure 3A**). Due to the absence of coarse grain force fields that include parameters for carbohydrates, the system was modeled in the non-glycosylated state. Parameters for the O-glycosylated form are being developed and we expect to be able to simulate the glycosylated system in the near future. The EXT molecules were modeled in two different configurations: as a single chain and as a trimeric helical conformation similar to collagen as performed before (Velasquez et al., 2015a). The results obtained in these simulations indicate the importance of the triple helix conformation in the overall protein stability and specially the conservation of the fibril-like structure (**Figure 3B-C**). This is easily observed by comparing the number of H-bonds along the MD trajectories in both systems (**Supplemental Figure S1 and Supp**lemental Text 1). We observed that the number of H-bonds is significantly greater in the triple helix due to H-bonds between the different chains. This would provide structural stabilization to the fibril. Future simulations in the *O*-glycosylated forms are required to address the structural and dynamic effect of this PTM. Current experimental and modeling lines of

evidence are in agreement with the proposed role of the proline hydroxylation and carbohydrate moieties in keeping the EXT molecule in an extended helical polyproline-II conformation state (Stafstrom and Staehelin, 1986; Owen et al., 2010; Ishiwata et al., 2014). This extended conformation might allow EXTs to properly interact with themselves and with the surrounding apoplast environment, including PERs and pectins, to form a proper cell wall network (Nuñez et al., 2009; Valentin et al., 2010).

ARE LRX PROTEINS LINKERS BETWEEN THE EXTENSIN NETWORK AND CELL SURFACE INTEGRITY SENSORS?

It is unknown how dynamic changes in EXT network assembly/disassembly are sensed by the root hair cell and pollen tubes to coordinate oscillations in growth, cell wall relaxation, and cell wall rigidification. The cell surface sensors *Catharanthus roseus* receptor-like kinase (CrRLK1L) ANXURs (ANX1/ANX2) and BUDHA'S PAPER SEAL1 and 2 (BUPS1/BUPS2) located at the interface between the apoplast and the cytoplasmic side of the plasma membrane control cell expansion in pollen tubes (Boisson-Dernier et al., 2013; Ge et al., 2017). On the other hand, the membrane receptor FERONIA (FER) acts in root hairs and growing root cells as well as in female fertility during plant reproduction (Duan et al., 2010; Nissen et al., 2016; Barbez et al., 2017; Li et al., 2016). In addition, PERK4 and PERK13 have been shown to control root and root hair growth, respectively (Bai et al., 2009; Humphrey et al., 2015; Hwang et al., 2016), while several *PERKs* are highly expressed in pollen tubes (Borassi et al., 2016). Therefore, PERKs and CrRLK1Ls are good candidates for sensing EXT network changes and triggering downstream responses. Moreover, it was recently suggested that only LRR4, a truncated version of LRX4 with the Leucine-Rich Repeat domain but lacking the C-terminal EXT domain, physically interacts with the FER´s malectin domain (Dünser et al., 2017). This might imply a direct connection between LRXs through the EXT domain to the EXT network on one side, and throughout the LRR domain to cell surface CrRLK1L sensors close to the plasma membrane (**Figure 4**). In agreement with this notion, root hair LRXs (LRX1 and LRX2) as well as pollen LRXs (LRX8-LRX11) are key components for proper polar growth and both groups of LRXs were proposed as sentinels of cell wall integrity in these rapidly expanding cells (Baumberger et al., 2001; Baumberger et al., 2003; Ringli, 2010; Fabrice et al., 2017; Sede et al., 2017; Wang et al., 2017). LRX3, LRX4, and LRX5 control cell expansion in root cells (Draeger et al. 2015). On the other hand, *LRX8* and *LRX9* (possibly also *LRX10* and *LRX11*) are highly expressed in pollen tubes, and were able to bind to Rapid Alkalinization Factor 4 and 19 (RALF4 and RALF19), which are secreted to the media by pollen tubes. Additionally, RALF4 and RALF19 are able to bind to both ANX1/ANX2 and BUDS1/BUDS2 (Ge et al. 2017). LRXs, CrRLK1Ls (ANX1/ANX2 and BUDS1/BUDS2), and RALF4/RALF19 all act together as an autocrine (i.e., signals from the same cell type) mechanism to monitor the cell wall integrity status during pollen tube growth (Ge et al., 2017; Mecchia et al., 2017). In addition, RALF23 was shown to bind to FER during plant immunity responses (Stegmann

et al., 2017) and RALF1-FER associates to acidify the apoplast, thereby suppressing cell expansion (Haruta et al., 2014). FER interacts not only with RALF and LRX proteins, but also with many other proteins, including RLKs, glycosylphosphatidyl-inositol anchored proteins, phosphatases, and small guanosine triphosphatases (Daun et al., 2010; Li et al., 2016; Liao et al., 2017). In addition, it was shown that FER extracellular domain as well other malectin domains from several CrRLKL1s are able to interact with pectins and sense the integrity status of the plant cell walls (Miyazaki et al. 2009; Ge et al. 2017; Feng et al. 2018). All together indicate that CrRLK1Ls may represent a central hub orchestrating signals and mediating growth and immune responses at the cell wall.

Known signaling cascade components, including the receptor-like cytoplasmic kinase (RLCK) MARIS (MRI) downstream of FER-ANX cell surface sensors (Boisson-Dernier et al., 2015), and two related kinesin-like calmodulin binding protein (KCBP)-interacting protein kinases (KIPKs) (members of the *Arabidopsis* AGC-VIII kinase family) downstream of PERKs (for PERK8-PERK10) (Humphrey et al., 2015), have been characterized. Multiple interactions were recently proposed between LRXs, possibly involving the EXT-network, the cell surface CrRLK1L sensors, and RALF secreted peptides in the control of polar growth (**Figure 4**). It is unclear how these components are coherently synchronized to coordinate polar growth. The exact role of RALFs and how they are connected at the molecular level to changes in the EXT network merits further investigations.

PERSPECTIVES

Plant-specific PTMs on EXTs are key chemical modifications required for EXT protein conformation and function in plant cell walls. These PTMs are evolutionarily conserved from unicellular green algae to complex vascular plants, indicating their essential roles in cell growth and development. Despite significant progress in our understanding of PTMs and EXT functions, it is unknown how EXTs are coordinately assembled, disassembled, and recycled. Future challenges include identifying EXT transcriptional regulators in different cell types; developing a realistic model of an entire EXT molecule in contact with the surrounding apoplastic environment; and determining PERs mechanism of action on the crosslinking of EXTs. One of the major constraints on plant glycobiology research is the limited ability to track PTMs in glycoproteins *in vivo*. This could be overcome in the near future by combining recent developments in super-resolution microcopy, which have already been used in plant cells for stimulated emission depletion (STED), (Kleine-Vehn et al., 2011), stochastic optical reconstruction microscopy (STORM) (Liesche et al., 2013), or total internal reflection microscopy (TIRF) (Gronnier et al., 2017), with new genetic sensors (Geilfus et al., 2014; Ast et al., 2017; Waadt et al., 2017), and *in vivo* sugar tagging approaches such as click-chemistry (Anderson et al., 2012, McClosky et al., 2016). The field of EXT glycobiology is in its nascent stages, but is poised to grow rapidly.

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Legend

Figure 1. EXT Post-Translational Modifications (PTMs): Proline Hydroxylation, *O*-Glycosylation, and Tyr-Crosslinking.

(**A**) Protein structure and motifs for a classical EXT include a variable N-terminus domain and several highly conserved repetitive motifs. Each of these includes usually two EXT *O*-glycosylation motifs, a putative arabinogalactan (AG) motif since it contains a Ser-Pro-Ser-Pro sequence, and several Tyr residues involved in EXT-crosslinks. Several repetitive motifs can occur in tandem (e.g., 13–23 times in root hair EXTs). There are unusual and variable repeats between the classical repeats. One repeat sequence type is shown, although wide variations exist for EXTs. (**B**) Proline hydroxylation is catalyzed by prolyl 4-hydroxylases (P4Hs) to yield a *trans*-4-hydroxyprolyl residue (Hyp). The *in vitro* activity of the *Arabidopsis thaliana* P4H5 had a preference for the three prolines within SPPPP. It is postulated that P4H2 or P4H13 would hydroxylate the fourth proline as a termination process. (**C**) Physical interaction between P4H5, P4H2, and P4H13 (see details in Velasquez et al. 2015). Several lines of evidence indicate that P4H2 and P4H13 are functionally (and possibly catalytically) interchangeable. P4H5 can partly replace P4H2 and P4H13, but not vice versa. Black arrows indicate functional replacement. Doubled-headed arrow indicates interchangeable proteins. Line thickness indicates the strength of the protein–protein interaction. (**D**) *O*-glycosylation patterns on EXT repeat. Sugar moieties and chemical linkages are shown (on the left). Site of action of currently known glycosyltransferases that transfer specific sugars on the EXT-backbone are indicated together with the GT CAZy family (on the right). (**E**) Transcriptional co-expression analysis on root hair genes revealed that *P4H2/P4H5* are associated with several GTs involved in the *O*-glycosylation of EXTs such as *SERGT1, HPAT3, RRA3,* and *XEG113*. Co-expression values are based on *Pearson* correlation coefficients (r-value range from –1 for absolute negative correlation to 1 for absolute positive correlation). Co-expression networks for P4H2, P4H5, RRA3, XEG113 and SERGT1 (cluster 172) were identified from PlaNet/AraNet (http://aranet.mpimp-golm.mpg.de/aranet) (Mutwil et al. 2011) and trimmed to facilitate readability. Each co-expression of interest was confirmed independently using the expression angler tool from Botany Array Resource BAR (http://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi) (Toufighi et al. 2005) and ATTED-II (http://atted.jp) (Obayashi et al. 2018). BiFC=Bimolecular Fluorescent Complementation. FRET= Fluorescence Resonance Energy Transfer. (**F**) Hypothetical EXT structure of Tyr-crosslinked peptides by a lateral alignment of three short EXT chains and Tyr-intra (*iso*dityrosine) -and interchain-crosslink types (pulcherosine and di-*iso*dityrosine). Only Tyr residues are depicted with their chemical structure.

Figure 2. Transcriptional control of RSL4, EXTs, and PERs in root hair cells.

(**A**) Root hair transcriptional co-expression network; classical EXTs, PERs, and the transcriptional regulator RSL4 are highlighted. RSL4 as well as PRP3 and LRX1 were used as gene baits to narrow

down the co-expressed genes. Transcriptional connections are highlighted for EXT genes (in red) and PER genes (in blue) with bait genes (in black). The co-expression network was identified from AraNet/PLaNet (http://aranet.mpimp-golm.mpg.de/aranet) (Mutwil et al. 2011) and trimmed to facilitate readability. Each co-expression of interest was confirmed independently using the expression angler tool from Botany Array Resource BAR (http://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi) (Toufighi et al. 2005) and ATTED-II (http://atted.jp) (Obayashi et al. 2018). Only those genes that are connected with genes of interest are included. (**B**) Root hair EXT protein domain structure. Number of regular repeats (x, in black) plus unusual repeats (y, in light grey) (n=x+y). Length of the N-terminus as well as the total protein length in each EXT is shown. The overall conserved protein repeat sequence is indicated on the bottom, highlighting the *O*-glycosylation motif and Tyr involved in the EXT-crosslinking obtained with WebLogo (Crooks et al. 2004). On the left, Root Hair *cis*-Element (RHE-RSL4), HDG11 HD *cis*-element (HD-HDG11), and EIN3 binding sites in the regulatory regions (promoters) of EXTs are shown. Only 1.5–2.0 kb upstream of the transcription initiation site is shown.

Figure 3. EXT conformational coarse-grained (CG) protein model.

EXT CG model, including 10 conserved repeats of SPPPPYVYSSPPPPYYSPSPKVYYK (total length of 250 amino acids). EXTs were modeled as single and triple chains, following the structure taken from Velazquez et al. (2015a). (**A**) Construction of the EXT model, showing the simulation solvent box. (**B**) Specific interactions observed in the single chain simulation are shown. In single-stranded non-glycosylated EXTs, Pro-Ser-Pro and $(Pro)_4$ motifs favor coiled conformations over the initial extended conformation. (**C**) Selected snapshots of the triple chain MD trajectory, showing the stability of the triple strand along the 2 μs simulation. The results obtained in these simulations highlight the importance of the triple helix EXT in the overall protein stability, and especially, the maintenance of the fibril-like structure. O-glycans were not included in the model. See also **Supplemental Text S1** and **Figure S1**.

Figure 4. Molecular components that connect the EXT glyco-network with the cell surface sensors to trigger downstream growth responses.

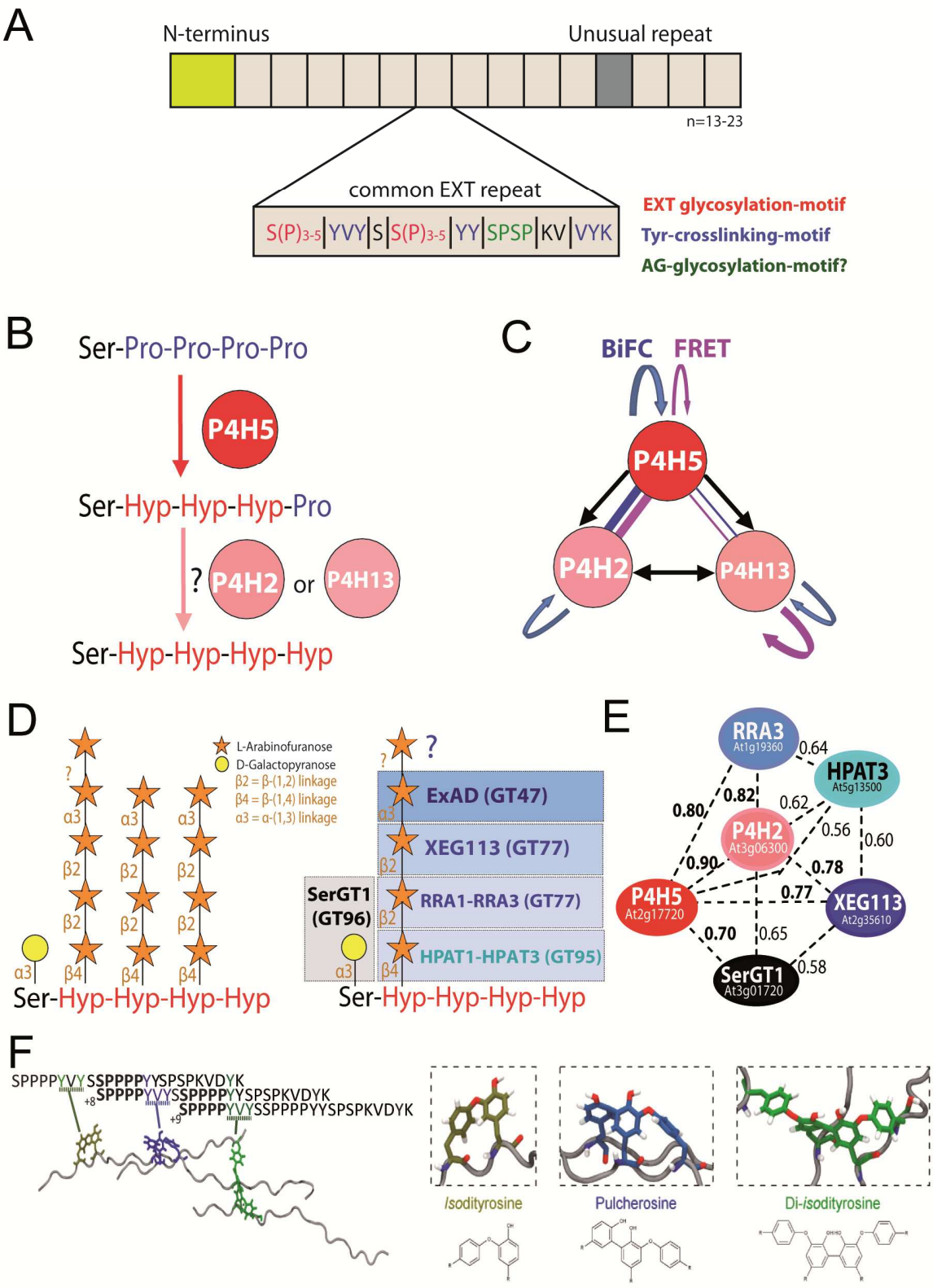
Cell surface sensors including *Catharanthus roseus* receptor-like kinases (CrRLK1Ls) and Proline-rich Extensin-like Receptor Kinases (PERKs) are candidates to sense the EXT network changes and trigger downstream responses. Several interactions have been recently identified between LRXs-FERONIA (FER, a CrRLK1L), between LRXs-RALFs for Rapid ALkalinization Factors, and between RALFs-FER. LRX1/LRX2 and LRX8-LRX11 are key components for proper root hair (Baumberger et al. 2001; Baumberger et al 2003; Ringli 2010) and pollen tube polar growth (Fabrice et al 2017; Sede et al. 2017), respectively, and both LRXs might act as sentinels of cell wall integrity in these

rapidly expanding cells. In addition, LRX3-LRX5 are important for root cell elongation. At least two RALFs (4 and 19) from pollen tubes were determined to interact with several LRXs (8, 9, 10, 11) as well as with both ANX1/ANX2 and BUDS1/BUDS2, thus indicating an autocrine cell mechanism of cell wall integrity check-out during growth (Mecchia et al. 2017; Ge et a. 2017). In addition, the LRR4 domain from LRX4 (present in growing roots) interacts with FER. Based on this, it is proposed that LRX proteins would be able to connect throughout their C-terminal EXT domain and through the LRR domain to cell surface CrRLK1L sensors close to the plasma membrane (Dünser et al. 2017). In this manner, LRXs might physically link the status of EXT assembly-disassembly with the surface CrRLK1Ls during growth. Finally, RALF1/RALF23 was shown to interact with FER (Haruta et al. 2015; Stegmann et al. 2017). PERKs would also be involved in sensing the EXT glyco-network since they contain several EXT-like domains, although little is known about this mechanism (Borassi et al. 2016). PERK13 is expressed in root hairs, while several PERKs are present in pollen tubes. Known downstream components include the receptor-like cytoplasmic kinase (RLCK) MARIS (MRI) for the surface sensors FER-ANXs (Boisson-Dernier et al. 2015) and two related kinesin-like calmodulin-binding protein (KCBP)-interacting protein kinases (KIPKs) in the case of PERKs (Humphrey et al. 2015). It is unclear how these multiple components (e.g., CrRLK1Ls, EXTs, LRXs, PERKs, and RALFs) are orchestrated in a coherent manner to coordinate polar growth. Two-side arrowhead indicates physical interactions between two proteins. ?, not experimentally confirmed; K, ser-thr kinase; l, leaves; PM, plasma membrane; pt, pollen tubes; r, roots; rh, root hairs; TM, transmembrane domain.

Acknowledgements

We apologize for the inadvertent omission of any pertinent original references owing to space limitations. No conflict of interest is declared.

Figure 1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Tyr-crosslinking-motif
AG-glycosylation-motif?
B
Ser-Pro-Pro-Pro-Pro
P4H5
Ser-Hyp-Hyp-Hyp-Pro
? P4H2 or P4H13
Ser-Hyp-Hyp-Hyp-Hyp
C
BiFC FRET
P4H5
P4H2
P4H13
D
L-Arabinofuranose
D-Galactopyranose
β2 = β-(1,2) linkage
β4 = β-(1,4) linkage
α3 = α-(1,3) linkage
Ser-Hyp-Hyp-Hyp-Hyp
ExAD (GT47)
XEG113 (GT77)
RRA1-RRA3 (GT77)
HPAT1-HPAT3 (GT95)
SerGT1 (GT96)
Ser-Hyp-Hyp-Hyp-Hyp
E
RRA3 At1g19360
HPAT3 At5g13500
P4H2 At3g06300
P4H5 At2g17720
XEG113 At2g35610
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0.80
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0.90
0.78
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0.65
0.58
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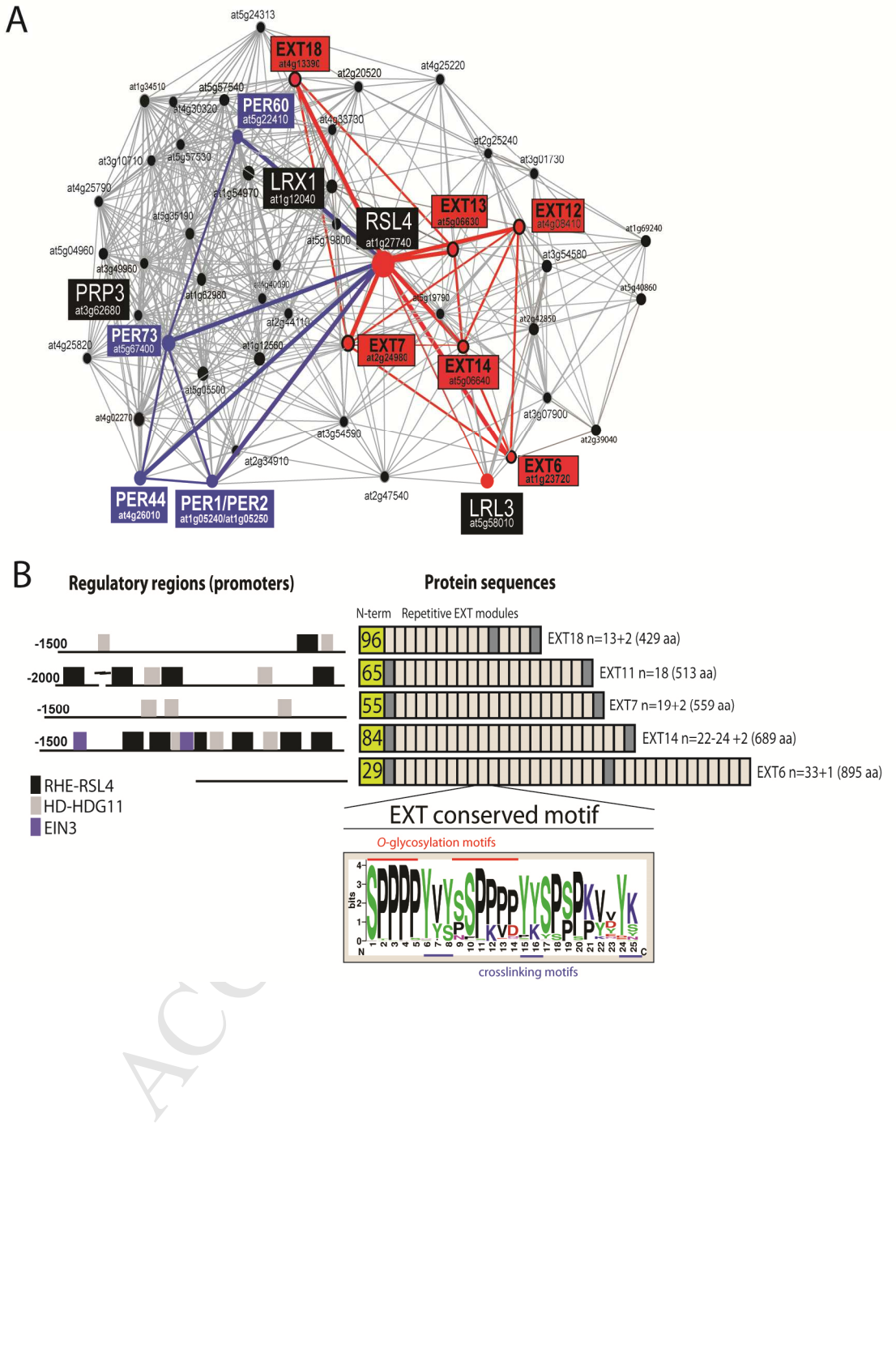
+9
Isodityrosine
Pulcherosine
Di-isodityrosine

Figure 2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EXT12
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at2g34910
EXT6
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PER1/PER2
at1g05240/at1g05250
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LRL3
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Regulatory regions (promoters)
Protein sequences
N-term
Repetitive EXT modules
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EXT7 n=19+2 (559 aa)
84
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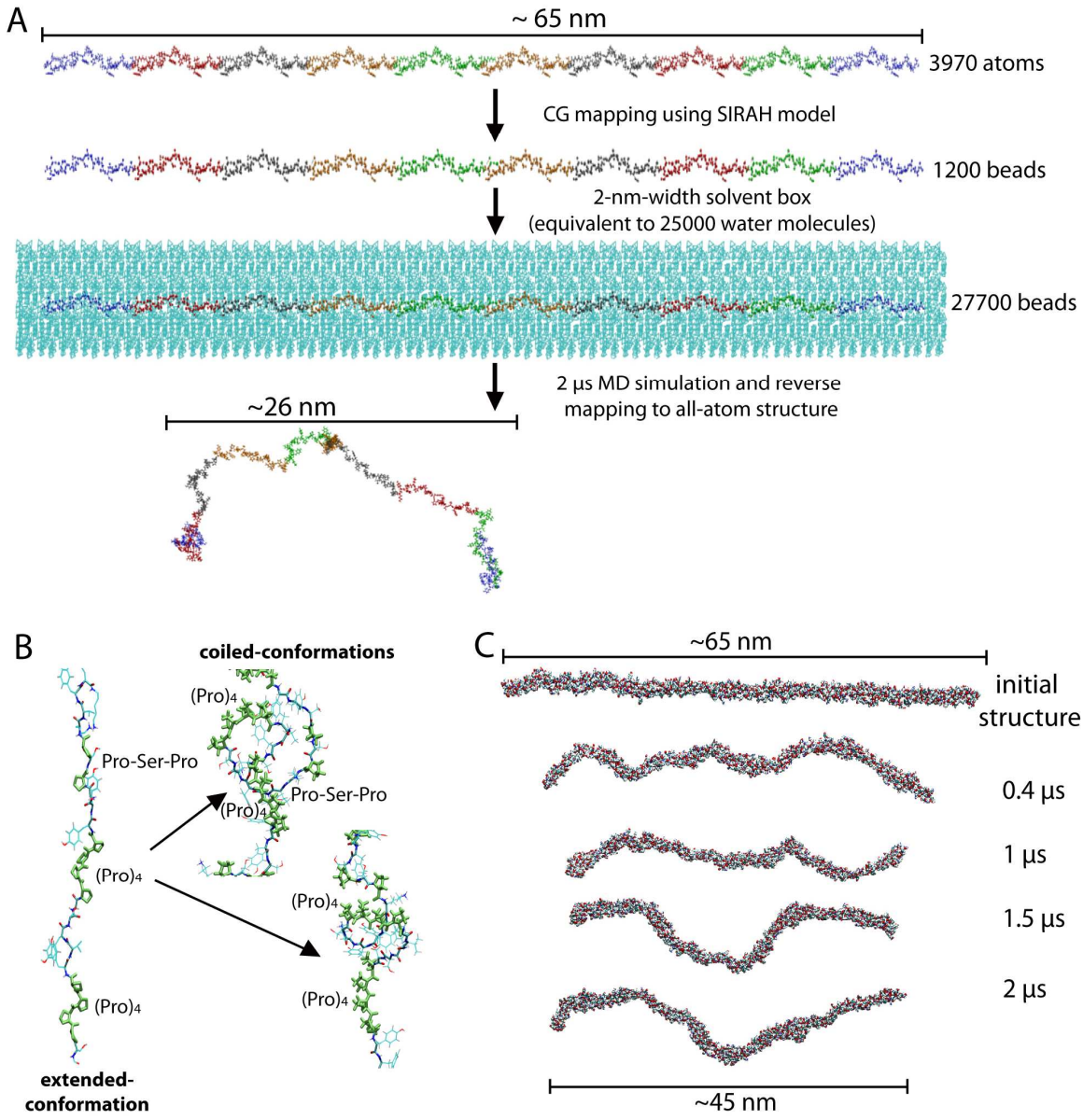
EIN3
EXT conserved motif
O-glycosylation motifs
bits
crosslinking motifs

Figure 3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1200 beads
2-nm-width solvent box
(equivalent to 25000 water molecules)
27700 beads
2 μs MD simulation and reverse
mapping to all-atom structure
~26 nm
B
coiled-conformations
(Pro)4
Pro-Ser-Pro
(Pro)4
Pro-Ser-Pro
(Pro)4
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extended-
conformation
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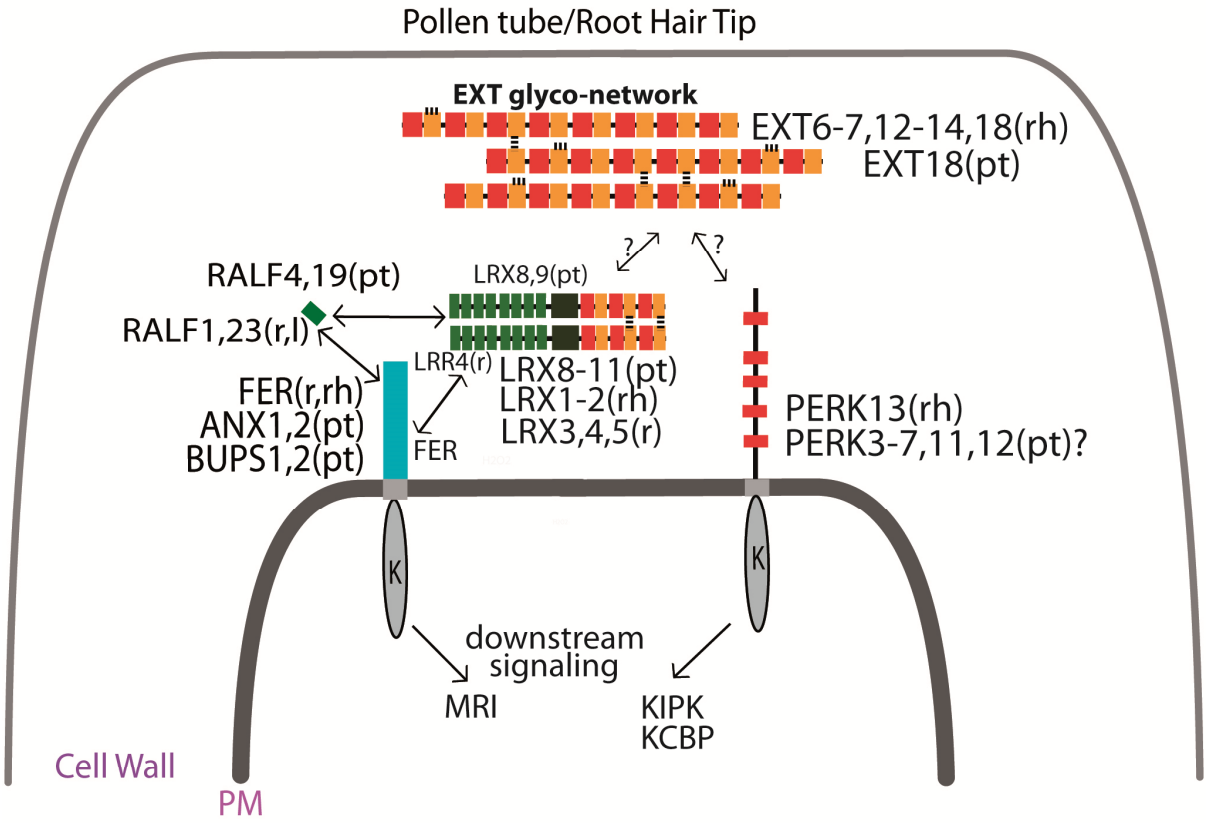
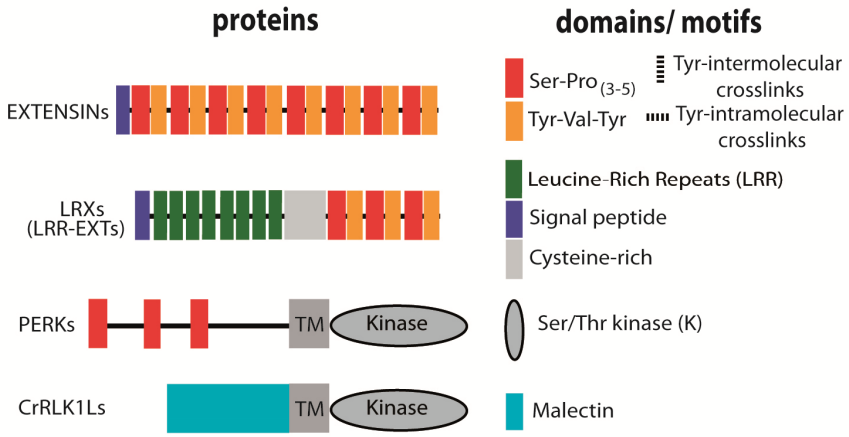

Pollen tube/Root Hair Tip
EXT glyco-network
EXT6-7,12-14,18(rh)
EXT18(pt)
RALF4,19(pt)
LRX8,9(pt)
RALF1,23(r,l)
LRR4(r)
LRX8-11(pt)
LRX1-2(rh)
LRX3,4,5(r)
FER(r,rh)
ANX1,2(pt)
BUPS1,2(pt)
FER
PERK13(rh)
PERK3-7,11,12(pt)?
K
K
downstream
signaling
MRI
KIPK
KCBP
Cell Wall
PM


proteins
domains/ motifs
EXTENSINs
LRXs
(LRR-EXTs)
PERKs
TM
Kinase
CrRLK1Ls
TM
Kinase
Ser-Pro (3-5)
Tyr-Val-Tyr
Tyr-intermolecular crosslinks
Tyr-intramolecular crosslinks
Leucine-Rich Repeats (LRR)
Signal peptide
Cysteine-rich
Ser/Thr kinase (K)
Malectin