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Emerging models on the regulation of intercellular transport by plasmodesmata- associated callose

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Abstract

The intercellular transport of molecules, through so-called plasmodesmata, membranous channels that traverse the cell walls, is of fundamental importance for plant development. Regulation of plasmodesmata aperture (and transport capacity) is mediated by changes in the flanking cell walls, mainly via the synthesis/degradation (turnover) of the (1,3)-β-glucan polymer, callose. The role of callose in organ development and in plant environmental responses is well recognized but detailed understanding of the mechanisms regulating its accumulation and its effects on the structure and permeability of the channels is still missing. We compiled information on the molecular components and signalling pathways involved in callose turnover at plasmodesmata and, more generally, on the structural and mechanical properties of (1,3)-β-glucan polymers in cell walls. Based on this revision, we propose models integrating callose, cell walls and the regulation of plasmodesmata structure and intercellular communication. We also highlight new tools and interdisciplinary approaches that can be applied to gain further insight on the effects of modifying callose in cell walls and its consequences for intercellular signalling.

Introduction

Systemic coordination, achieved through cell-cell signalling, is essential for multicellular organisms to develop appropriately and to respond to changes in their environment. Systemic coordination in plants is made more complex by the cell wall, a defining feature of this kingdom, which presents a physical barrier to intercellular transport and signalling. One method that plants have evolved in overcoming this barrier is the symplastic pathway (Stahl and Simon, 2013). The symplastic pathway is enables direct cell-cell transport via plasmodesmata (PD), membrane lined pores which bridge the cell walls of neighbouring cells to form a cytoplasmic continuum, termed the symplast. The symplast forms a network of molecular highways that not only allow for the flux of small molecules, such as photosynthates and phytohormones but can also accommodate the transport of larger macromolecules, such as proteins and RNAs. The superficial structure of PD could give the illusion that they are merely pipes, allowing uncontrolled flux between cells. However, it is

clear that PD are major sites for the regulation of intercellular transport, which has profound effects on numerous developmental events and responses to biotic and abiotic stresses (Sager and Lee, 2014). Regulation of symplastic transport is linked to changes in PD structure and composition and their consequences vary depending on the developmental context (Benitez-Alfonso, 2014).

PD consist of a specialised membrane domain, which is a continuation of the plasma membrane (PM), and a central structure (known as the desmotubule) formed from appressed endoplasmic reticulum (ER) (Barton *et al.*, 2011). These domains are embedded within distinct cell wall regions enriched in the (1,3)-β-glucan polymer callose (Fig. 1). Primary PD are formed during cytokinesis but PD can also arise *de novo* and undergo structural modifications within established cell walls (secondary PD) (Burch-Smith *et al.*, 2011). PD can be configured with a single channel, known as simple PD, or can be more complex, either with twinned channels or multiple branched channels (Roberts *et al.*, 2001). Another form of PD, named 'funnel', occur between protophloem sieve element cells (PSE) and phloem pericycle pole cells (PPP), where the wide aperture, or funnel end, is positioned within the PSE (Ross-Elliott *et al.*, 2017). Mathematical modelling suggests that 'funnel' PD are more efficient at unloading solutes into PPP via mass flow and diffusion than a simple PD configuration (Ross-Elliott *et al.*, 2017). How branched or 'funnel' PD are generated and how they impact the permeability of the channels for transport remains a topic for debate. The PD of developing tobacco leaf cells predominantly display a simple configuration but, as these cells develop, the PD gain more elaborate configurations (Roberts *et al.*, 2001). In general, the transition from immature to mature tissues correlates with increased PD branching and a constriction in transport (Oparka *et al.*, 1999) but this is at odds with work showing that increase frequency of branched PD in embryonic cells, in the mutants *increased size exclusion limit 1 and 2,* lead to increase permeability to symplastic dyes (Kobayashi *et al.*, 2007; Stonebloom *et al.*, 2009, 2012). The data suggest that other factors (such as cell wall thickness) might influence the effect of the different PD configurations on symplastic transport.

Other factors, including modifications in PD-associated cell walls, influence symplastic communication. A key feature of this regulation is the modulation of the pore aperture by the accumulation of the cell wall polysaccharide callose (a (1,3)-β-glucan polymer),

especially at PD neck regions, which limits molecular flux (De Storme and Geelen, 2014). In this review we discuss recent advances in understanding the role of callose in PD regulation, its effects on the structural and physical properties of cell walls and the molecular and signalling components that influence its synthesis/degradation. Based on this research, models are proposed to explain the effect of PD-associated callose in intercellular communication. The regulation and timing of intercellular signalling via the flux of molecules through PD regulate: organ positioning and emergence (Benitez-Alfonso *et al.*, 2013), cell fate specification (Guseman *et al.*, 2010) and the response to various pathogens (Faulkner *et al.*, 2013). In this context, the implications of modifying callose at PD for plant development and environmental responses are discussed.

The regulation of callose turnover at plasmodesmata

The synthesis and subsequent degradation (turnover) of callose at PD sites is key to the regulation of intercellular signalling. The characterization of a number of proteins involved in callose turnover have provided insights into the mechanisms underlying this process (Zavaliev *et al.*, 2011). Callose synthesis is carried out by callose synthases (CalS), otherwise known as glucan synthase-like (GSL) (Schneider *et al.*, 2016), which have high substrate specificity for uridine diphosphate glucose (UDP-Glc) which is assembled into chains with β-1,3-links. CalS proteins are large, containing 14-16 transmembrane domains (Schneider *et al.*, 2016), an extracellular domain and a large cytoplasmic domain (Thiele *et al.*, 2009). The synthesis of callose at the PM and its subsequent deposition into the cell wall is mediated by multi-subunit callose synthase complexes (CalSC). Besides CalS, CalSC is likely to comprise a sucrose synthase enzyme (SuSy), which degrades sucrose to UDP-glucose, and a UDP-glucose transferase (UGT1) which transfers the substrate to the catalytic site of CalS (Schneider *et al.*, 2016). A monomeric GTPase is thought to form part of the CalSC and regulate its activity. The GTPase ROP1, an Arabidopsis homolog of yeast Rho1, interacts with CalS at the cell plate. GTPase RabA4C has specifically been shown to interact with CalS12 and overexpression leads to enhanced callose deposition (Ellinger *et al.*, 2014). Another CalSC component found in cotton fibres is Annexin, which appear to play a role in balancing callose and cellulose synthesis (Andrawis *et al.*, 1993). It is not clear if CalS associations

differ between cell / tissue types. For example, phragmoplastin was found to interact with the CalSC in the cell plate (Hong *et al.*, 2001; Hong, 2001) but is not yet linked to the regulation of callose at PD (De Storme and Geelen, 2014).

There are 12 callose synthase genes in *A. thaliana,* the majority of which have been, at least partially, characterised (Cui and Lee, 2016). There is a degree of spatial and developmental regulation of CalS expression between plant tissues and organs. For example, CalS7 is specifically found in the phloem while CalS10 has broad expression in multiple plant tissues (Guseman *et al.*, 2010; De Storme *et al.*, 2013). Despite some differences in their expression profile, multiple CalS genes appear to be expressed at a given time in a given tissue/organ. CalS expression is affected by developmental and stress conditions such as infection with downy mildew (Dong *et al.*, 2008; Coker *et al.*, 2015), mechanical wounding (Zavaliev *et al.*, 2011), in microspore development (Shi *et al.*, 2015) and during the exine layer formation in pollen (Enns *et al.*, 2005). Ectopic expression of CalS5 also appears to regulate cell wall permeability to H_2O and the response to osmotic stress (Xie *et al.*, 2012).

The importance of CalS activity at PD has been demonstrated. An inducible mutation in CalS3 (icals3m), leading to increased expression, shows increased accumulation of callose and a decrease in symplastic transport (Vatén *et al.*, 2011). Ectopic expression of these hyperactive mutant versions restrict the movement of the transcription factor SHORT-ROOT (SHR), and associated downstream miRNAs, regulate cell polarity and cell elongation leading to abnormal cell expansion and altered cellular patterning in the developing root (Vatén *et al.*, 2011; Wu *et al.*, 2016). With this tool, it has been demonstrated that regulation of callose defines cell identity and the proper localisation of PIN efflux carriers that determine auxin distribution in the root (Wu *et al.*, 2016). On the other hand, CalS7 loss-of-function mutant shows a reduced number of PD in the phloem sieve elements (Xie *et al.*, 2011) and a reduction in the formation of callose linings and in the movement of assimilates (Barratt *et al.*, 2011). Similarly, increase expression of the tomato homolog, CALLOSE-SYNTHASE-7 LIKE (CAS7), in response to infection with *Candidatus Phytoplasma solani,* also correlates with an increase in the deposition of callose in the phloem (Marco *et al.*, 2016). A mutation in CalS10, otherwise known as *chorus*, is important for the regulation of callose deposition at PD in epidermal cells (Chen *et al.*, 2009; Cui and Lee, 2016) as loss of function mutants display abnormal stomatal clustering phenotypes associated with increase

mobilisation/transport of the transcription factor SPEECHLESS, which promotes cellular entry into the stomatal lineage (Chen *et al.*, 2009; Simmons and Bergmann, 2016). CalS10/GSL8 is also involved in the phototropic response in hypocotyls, a phenotype that correlates with changes in auxin distribution (Han *et al.*, 2014). CalS10, and homologs in other species, also play important roles in male gametophyte development, root growth, vascular patterning and stabilisation of ploidy, although the involvement of PD in some of these processes is not fully understood (De Storme and Geelen, 2014; Song *et al.*, 2016). More recent work indicates that CalS1 and CalS8 also regulate PD permeability in response to stress signals (Cui and Lee, 2016). Callose deposition at PD is regulated in response to salicylic acid (SA) and to reactive oxygen species (ROS) but the pathways mediating these responses appear independent requiring CalS1 for the SA response but CalS8 in the ROS response. The mechanism mediating these differences is unknown and might involve non-PD genes, such as thioredoxin-m3/ GAT1, that regulate PD-callose and the plant response to these signals (Benitez-Alfonso *et al.*, 2009).

The accumulation of callose at PD is also determined by the activity of PD-located callose-degrading enzymes, named (1,3)-β-glucanases (BG; Glycosyl Hydrolase family 17; GH17). There are at least 50 BG genes in *Arabidopsis* (Doxey *et al.*, 2007) which can be classified into 5 groups based upon the protein domain, structure/sequence. BG expression regulate plant defence, seed germination, cell division, flowering, pollen-tube growth, abiotic stress response and fruit ripening (Balasubramanian *et al.*, 2012). Proteomic analysis of PD-enriched cell wall fractions identified a number of BG genes in *Arabidopsis* (Levy *et al.*, 2007; Fernandez-Calvino *et al.*, 2011). PD-located (1,3)-β-glucanases (PDBG) belong to one clade of GH17 proteins whose evolutionary root appears to correlate with the development of complex PD structures and regulatory mechanisms during land plant colonization and the development of increasingly complex plant forms (Gaudioso-Pedraza and Benitez-Alfonso, 2014).

As with CalS, miss-expression of PDBG affects cell-to-cell connectivity, development and stress responses. Antisense expression of a tobacco BG, for example, leads to increased callose accumulation, decreased intercellular transport of the tobacco mosaic virus movement protein (TMV-MP) and reduced spread of the pathogen (Iglesias and Meins, 2000). A knockout mutant in *AtBG_pap*, an Arabidopsis PD-associated BG, leads to a

reduction in the trafficking of 'free' (cytoplasmic) GFP, an increase in callose accumulation (Levy *et al.*, 2007) and affected virus movement (Zavaliev *et al.*, 2013). Three other PDBGs have been identified in Arabidopsis; PDBG1 (at3g13560), PDBG2 (at2g01630) and PDBG3 (at1g66250). *pdbg1,2* double mutant shows increased callose deposition, reduced symplastic connectivity and altered lateral root patterning (Benitez-Alfonso *et al.*, 2013). The expression of orthologues of these proteins in *Populus* is induced in response to gibberellins (GA) and correlates with bud dormancy release and shoot branching (Rinne *et al.*, 2011, 2016).

Another family of proteins (termed Plasmodesmata Callose Binding Proteins or PDCB) are also involved in callose regulation although is not clear how they interact with the processes of synthesis/degradation. PDCBs only encode a carbohydrate binding module (CBM43), otherwise known as X8 domain, and a glycophosphatidylinositol (GPI) anchor to target the PD membrane (Simpson *et al.*, 2009). PDCB YFP-fusions and immunogold labelling suggest localization predominately at PD neck regions (Simpson *et al.*, 2009) co-localising with callose and PDBG1 (Benitez-Alfonso *et al.*, 2013). PDCB overexpressing lines showed increased callose deposition and reduced symplastic transport, a phenotype that correlates with increased lateral root density as described for *pdbg1,2* (Simpson *et al.*, 2009; Maule *et al.*, 2013). The mechanism underlying this effect is unknown but it is possible that increasing PDCB availability to bind callose restricts the activity (or substrate accessibility) of PDBG affecting callose turnover and symplastic communication (Fig. 1).

In summary, enzymes involved in callose metabolism target PD to regulate symplastic transport in response to developmental and environmental cues. PD-located CalS, PDBG and PDCB family members have been identified in *Arabidopsis*, and their role in PD function has been partially characterized. Other proteins may be directly, or indirectly involved in callose regulation but the precise mechanisms are as yet unknown.

Developmental and environmental signals influence callose levels at PD

The dynamic nature of callose turnover allows plants to differentially modulate symplastic signalling in response to varying environmental and developmental cues. Recent research highlights the importance of PD regulation during pathogen infection and identified

receptor proteins that localize at PD and participate in this process via regulation of callose deposition (see Stahl and Faulkner, 2016 for a recent review). This is the case of the protein family PLASMODESMATA LOCATED PROTEINS (PDLPs), which are receptor-like proteins isolated in the PD proteome of *A. thaliana* (Thomas *et al.*, 2008). PDLP5 functions in SA signalling and mediates callose deposition during plant immune responses (Lee *et al.*, 2011; Lim *et al.*, 2016). The exact mode of action of PDLPs have not been fully determined but it is thought that involves induction of callose synthesis at PD (Fig. 1). Consistent with this hypothesis, SA-dependent induction of CalS1, and consequent callose deposition at PD, is dependent on PDLP5 (Cui and Lee, 2016). PDLP5 may induce callose to isolate infected cells from healthy tissue triggering, ultimately, programmed cell death (Lee *et al.*, 2011).

PDLP5, and PDLP1, have also been recently found to have a role in systemic acquired resistance (SAR), a longer-term immune response that is essential for priming distal tissues against an impending threat, by regulating the transport of the defence-related signals azelaic acid (AzA) and glycerol-3-phosphate (G3P) (Lim *et al.*, 2016). Interestingly, the induction of SAR against pathogenic *Botrytis cinerea* and aphids, in plants primed with benign *Bacillus cereus* AR156 and *Bacillus velezensis,* also correlate with induction in callose accumulation (Nie *et al.*, 2017; Rashid *et al.*, 2017). Whether PD regulation is required for these responses remains to be seen. PDLP1 is also associated with callose accumulation during the encasement of the haustorium, specialised feeding structures that allow pathogens, such as *Hyaloperonospora arabidopsidis*, to get nutrients from host cells (Caillaud *et al.*, 2014).

Other receptors have been identified as part of the sensory machinery required for pathogen-induced PD-callose accumulation. LYM2 (LYSIN MOTIF DOMAIN-CONTAINING GPI-ANCHORED PROTEIN 2) is involved in regulating PD in response to *Botrytis cinerea* and chitin perception whereas FLS2, a LRR receptor–like kinase, mediates PD closure in response to bacterial flagellin (Gómez-Gómez and Boller, 2000; Faulkner *et al.*, 2013). It has recently been shown that PD-localised CALMODULIN-LIKE- 41 acts downstream of FLS2 and directly promotes callose accumulation at PD in response to flagellin-22 (Xu *et al.*, 2017)

Callose is also deposited in response to toxic metal ions such as aluminium, lead, arsenic and cadmium and, in some instances, this has been correlated with reduced symplastic transport (Sivaguru *et al.*, 2000; Ueki and Citovsky, 2005; Piršelová *et al.*, 2012;

Samardakiewicz *et al.*, 2012). Very little research explores the link between callose, regulated symplastic transport and plant response to soil nutrients and water stress. Callose was involved in restricting root meristem growth in Arabidopsis in response to iron-dependent Pi-deficiency (Müller *et al.*, 2015) via a mechanism mediated by the ferroxidase LOW PHOSPHATE ROOT 1 (LPR1) and the P5-type ATPase PHOSPHATE DEFICIENCY RESPONSE 2 (PDR2).

Developmental signals also participate in callose regulation at PD. Auxin appear to regulate the expression of PDBG and PDCB family members that participate in root branching and patterning (Maule *et al.*, 2013; Benitez-Alfonso *et al.*, 2013). PD-located enzymes and callose are also regulated in the shoot in response to GA. During dormancy, axillary buds in hybrid aspen appear symplastically isolated but GA accumulation, during long periods of chilling or after decapitation of the main shoot apical meristem, induces BG expression to establish symplastic transport of the FLOWERING LOCUS T homologue, required to reinstate organ development (Rinne *et al.*, 2011, 2016).

Conditions/signals that alter the composition and/or fluidity of membranes (such as temperature) might also control callose by affecting the targeting of PDBG and PDCB proteins. A GPI anchoring domain is an important feature in PDBG and PDCB proteins. Its removal from AtBG_pap, PDBG1 and PDCB1 is sufficient to prevent these proteins from localising to PD (Zavaliev *et al.*, 2016). Correct GPI integration depends in membrane composition. PD membranes are enriched in sterols and sphingolipids and altering this composition, by inhibition of sterol production using the drugs fenpropimorph and lovastatin, led to an increase in callose deposition, miss-localization of PDCB1 and PDBG2 and a concurrent reduction in the intercellular movement of GFP (Grison *et al.*, 2015). Readers are referred to a recent review in this topic (Iswanto and Kim, 2017).

Clearly, regulation of callose at PD is an essential component of many plant responses to biotic and abiotic stresses, and also developmental cues. The identification of components of signalling cascades involved in regulating PD-callose have provided a valuable insight into the dynamic nature of symplastic regulation in plant development. Despite these advances, there remains many questions about how PD-callose is regulated and how it affects signalling. It would be of great interest, for example, to know whether PD- callose is regulated in response to the availability of soil nutrients and to what extent it play a role in

the responses to soil conditions for example by modulating root branching. The phenotypic characterization of mutants in callose turnover at PD can provide insights into this process but other areas of research, such as establishing how these signals affect the molecular and mechanical properties of callose in cell walls, needs to be explored.

Callose structural and mechanical properties and potential links to PD regulation

Callose is deposited into the paramural space where microscopy suggests that it forms a collar surrounding the pore which acts as a sphincter to control PD aperture (Fitzgibbon *et al.*, 2010). In contrast to the (1,4)-β-glucan cellulose, which forms highly crystalline structures, callose is more disordered forming amorphous helical structures (Kim, 2016; Przekora *et al.*, 2016). It has been proposed that callose gelling properties act as a leak sealant in response to wounding (Parre and Geitmann, 2005) and at sieve plates, where it causes the occlusion of sieve pores. Callose can also function as a load bearing component as described in pollen tubes (Parre and Geitmann, 2005). In *Solanum chacoense* pollen, digestion of callose correlates with a decrease in esterified pectins and in cell wall stiffness, leading to an increase in pollen tube diameter, reduced pollen tube growth and germination (Parre and Geitmann, 2005; Chebli *et al.*, 2012). Digestion of callose also affects cellulose distribution in pollen tubes (Chebli *et al.*, 2012). Interaction between callose and cellulose are also proposed at sites of fungal attack, presumably acting as a protective barrier (cement-like) to cell wall digestion by fungal degrading enzymes (Eggert *et al.*, 2014; Voigt, 2016).

Very little is known about how callose deposition impacts the mechanical properties of PD and the consequences of changes in its regulation for cell growth and shape *in planta*. It has been proposed that closing up PD (as for sieve pores) affects the cell osmotic potential/pressure and growth (Anisimov and Egorov, 2002) as alters the diffusion of small molecules such as water and sucrose. On the other hand, as described for pollen, callose might interact with other cell wall components (such as pectins and cellulose) more generally affecting cell wall architecture and mechanical properties.

Besides callose, other cell wall components are known to be differentially regulated at PD although their function remain unclear (for a review consult Knox and Benitez-Alfonso, 2014). Imaging of tomato pericarp and tobacco leaves revealed that pit fields (regions where PD occur in high density) have a low cellulose content while certain pectins epitopes are differentially regulated (Fig. 2) (Casero and Knox, 1995; Faulkner *et al.*, 2008). In particular unesterified pectins, labelled with the JIM5 antibody, appear associated with PD cell walls, whereas immunolabelling with the antibody LM5 reveals that a linear-(1→4)-β-galactan epitope is absent (Fig. 2) (Roy *et al.*, 1997; Orfila and Knox, 2000). Treatment with the calcium chelator 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) suggests that pit field pectin is not cross-linked by calcium ions, thus it is not rigid but instead flexible unlike the classical egg-box models (Orfila and Knox, 2000). The side chains of pectin (1-5)-α-L-arabinan is also enriched at PD (Orfila and Knox, 2000). The effect in cell wall mechanics of pectin esterification and the pectic side-chains is highly context dependant (Braybrook and Peaucelle, 2013; Amsbury *et al.*, 2016). Low levels of esterification can lead to both a stiffening or a loosening of the cell wall network depending on environmental conditions (Braybrook and Peaucelle, 2013; Atmodjo *et al.*, 2013). Both arabinan and galactan side chains are thought to interact with cellulose (Zykwinska *et al.*, 2007; Lin *et al.*, 2015). An increase in galactan content has been shown to correlate with an increase in rigidity (Jones *et al.*, 1997; McCartney *et al.*, 2000) and a reduction correlates with cell wall softening during fruit ripening (Gross and Wallner, 1979). On the other hand, pectic arabinan is involved in cell-cell adhesion (Peña and Carpita, 2004; Neumetzler *et al.*, 2012; Cankar *et al.*, 2014) and in maintaining wall flexibility by preventing close association of pectic chains (Jones *et al.*, 2003).

The presence of unesterified pectin with enriched arabinan side chains and the absence of linear galactans suggests that the cell wall at pit fields is both flexible and adhesive. It is possible that this flexibility is required to allow the active transit of large molecules which are greater than the PD aperture observed by electron microscopy and that the adhesive properties of pectic arabinans allows anchoring at the site where PD breach the cell-wall. The importance of cellulose and pectins in stabilizing these connecting points is supported by the discovery of spoke-like structures while imaging PD in the algae *Chara corallina* and their partial destruction by digestion with cellulases and pectinases (Brecknock *et al.*, 2011).

These spokes are also present in *Azolla* pinnata roots and in tobacco plants *(Nicotiana tabacum)* suggesting that they are a conserved feature (Ding *et al.*, 1992; Brecknock *et al.*, 2011). In yeast β-1,3-glucans are proposed to connect the cell wall to the PM (Muñoz *et al.*, 2013), thus it is possible that callose play a role in stabilising PD-cell wall contact points or membrane contact sites (MCS) between the PM and the DT (Tilsner et al., 2016). Future analysis of PD spokes after changes in callose abundance will provide insights into its role as a cell wall adhesive.

Pectin abundance can be linked to cellulose (Wang *et al.*, 2012; Lin *et al.*, 2015), other pectic components and structural proteins (Tan *et al.*, 2013). It is not yet known to what extent callose interacts with these components of the cell wall or what significance these putative interactions may have. Demonstrating in-vivo interactions of cell wall components at the scale of PD is challenging and the development of *in vitro* techniques and models will be crucial to gain knowledge in this area of research. The development of detection tools for novel pectic components might change our current picture on PD cell wall composition and reveal new insights on their mechanical properties. Using polymer blends, (1,3)-β-glucans were shown to modify the elasticity, reduce the compressive strength and increase the adhesive properties of chitosan/hydroxyapatite gels and of polyvinyl alcohol (Basha *et al.*, 2016; Przekora *et al.*, 2016). A similar approach using cell wall relevant polysaccharides (such as cellulose or xyloglucans) could provide information on the role of callose in the regulation of PD mechanical properties and general cell wall properties. This approach was successful in demonstrating interactions between arabinoxylan and mixed linkage (1,3)-(1,4)-β-glucans influencing the mechanics of cell walls (Lopez-Sanchez *et al.*, 2016).

To summarize, information on how structurally callose integrates with the cellulosic and pectic components of cell walls is lacking. Interactions between these components might influence the properties of callose and thus reveal new mechanisms for PD regulation. New models/ approaches are required to further advance on understanding how cell walls and callose regulation are concerted to mediate specific PD and cell responses to developmental and environmental cues.

Conclusions, emerging models and perspectives

Plasmodesmata dynamically adjust their aperture in order to regulate the intercellular flux of a wide range of macro- and micro-molecules, providing a mechanism for integration of both short and long range signals in the plant. The plasticity of this signalling network is maintained by the reversible accumulation and degradation of callose at the neck regions of the pore. Little is known about other cell wall components involve in PD function but the presence of cellulose-depleted and pectin-rich domains might be of significance to provide cell walls with the flexibility required to accommodate the transport of macromolecules bigger than PD aperture. It is likely that the cell wall structure establishes the mechanical limit for macromolecular transport while callose allows dynamic regulation within this range (Fig. 3). The presence of pectin-modifying enzymes, such as pectinases and pectin methylesterases, in the PD proteome suggests that the microstructure of the pectin network is closely regulated at PD. The mechanical properties of pectin are strongly influenced by both pH and Ca^{2+} availability (Geitmann, 2010), thus it is possible that these signals participate in PD regulation by modulating the rigidity of the cell wall surrounding PD. It has been suggested that callose and cellulose interact but it is not yet clear to what extent this interaction occurs at PD sites and/or if pectins (or other cell wall components) are involved. Since cell walls are modified to accommodate for changes in PD structure, outstanding questions on the role of callose (and other polymers) in this process remain (Fig.3).

The availability of PD proteomic data, new imaging platforms (such as AFM, FESEM, cryo-electron tomography, etc.), genetic tools to modify callose accumulation (such as icals3m), and systems to mimic PD-cell wall environment will provide information on the structural and mechanical properties of callose in the cell wall and insights on its function in the establishment and maintenance of symplastic connectivity during development. It can answer if callose functions through merely reducing PD aperture or via modifications in the elasticity (dilation capacity) of the channel or by inducing changes in PD structural conformations (Fig.3). β-1,3-glucans are also of commercial interest as a thickener in food production (Kim, 2016) and are applied in the medical field as both a flexible scaffold for the re-growth of damaged skin (Basha *et al.*, 2016) and as an additive to improve the flexibility and porosity of scaffolds used for bone tissue engineering (Przekora *et al.*, 2016). Thus

research on callose properties in cell walls might be also of interest in light of these applications.

In the context of plant development, another unexplored topic is the importance of the temporal and spatial dynamics in callose regulation. For example short term stimuli (such as exposure to abiotic factors) might lead to reversible callose accumulation but long term effects might be irreversible and necessary to determine symplastic domains during tissue/organ differentiation. More research is necessary to confirm or reject this hypothesis which might be key to dissect the differences between callose role in regulating organ development, the response to (fungal, bacterial and viral) pathogens and also to understand how abiotic factor, such as nutrient and water availability, impact on PD transport and plant development.

It is not yet clear if the modulation of callose deposition is the sole mechanism for regulating PD cell walls in response to developmental and environmental factors. Evidence of callose interactions in cell walls and with other regulatory mechanisms are emerging in other systems highlighting the need for more research on the regulation of these fascinating structures.

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Figure legends:

Figure 1. Callose regulation at Plasmodesmata (PD). Callose turnover at the neck region of PD is regulated by Callose synthases (CalSs) and PD localised β-(1,3)-glucanases (PDBGs). Callose is stabilised by PD-callose binding proteins (PDCBs); which might inhibit PDBG activity. PDBGs and PDCBs have specialised GPI-anchors which tethers them to micro-domains of the PM at PD which are rich in sphingolipids. Callose deposition is enhanced by salicylic acid (SA) and by Reactive Oxygen Species (ROS), a mechanism mediated by PDLP proteins. PDBGs are regulated by auxins (AUX). Callose restricts PD aperture and the size of macromolecules (such as non-cell autonomous proteins, NCAPs) that can pass through the cytoplasmic sleeve formed between the desmotubule (DT) and the PM. Placement of proteins reflects putative localisation within the PD.

Figure 2. Plasmodesmata are embedded in distinct cell wall regions. Immunofluorescence on sections of wax-embedded tomato pericarp with pit fields indicated by arrowheads. (A, C) Confocal microscopy of the outer face of tomato cells shows a reduction in cellulose at pit fields revealed by staining with calcofluor white. (B,D). Immunolabelling, using Alexa-488 conjugate as secondary (green signal), and as primary either anti-callose (B) or the antibody LM5 (D) reveals abundant callose and absence of a linear-(1→4)-β-galactan pectin epitope at pit fields. Scale bars = 5µm

Figure 3. Hypothetical models on the regulation of symplastic transport by changes in callose. Representation of simple PD showing that cell walls are flexible to accommodate the transport of both small and large molecules. Three potential modifications in PD transport capacity mediated by changes in callose turnover are proposed: (a) Callose deposition reduces the size of PD cytoplasmic aperture thus molecular flux, (b) Callose accumulation affects the mechanical properties (elasticity) of cell walls thus their capacity to transport large macromolecules and (c) Changes in the structural properties of cell walls (through callose interactions with other cell wall polymers) are likely linked to the formation of funnel, branched or twinned PD architectures which differ in transport capacity.

Figure 1

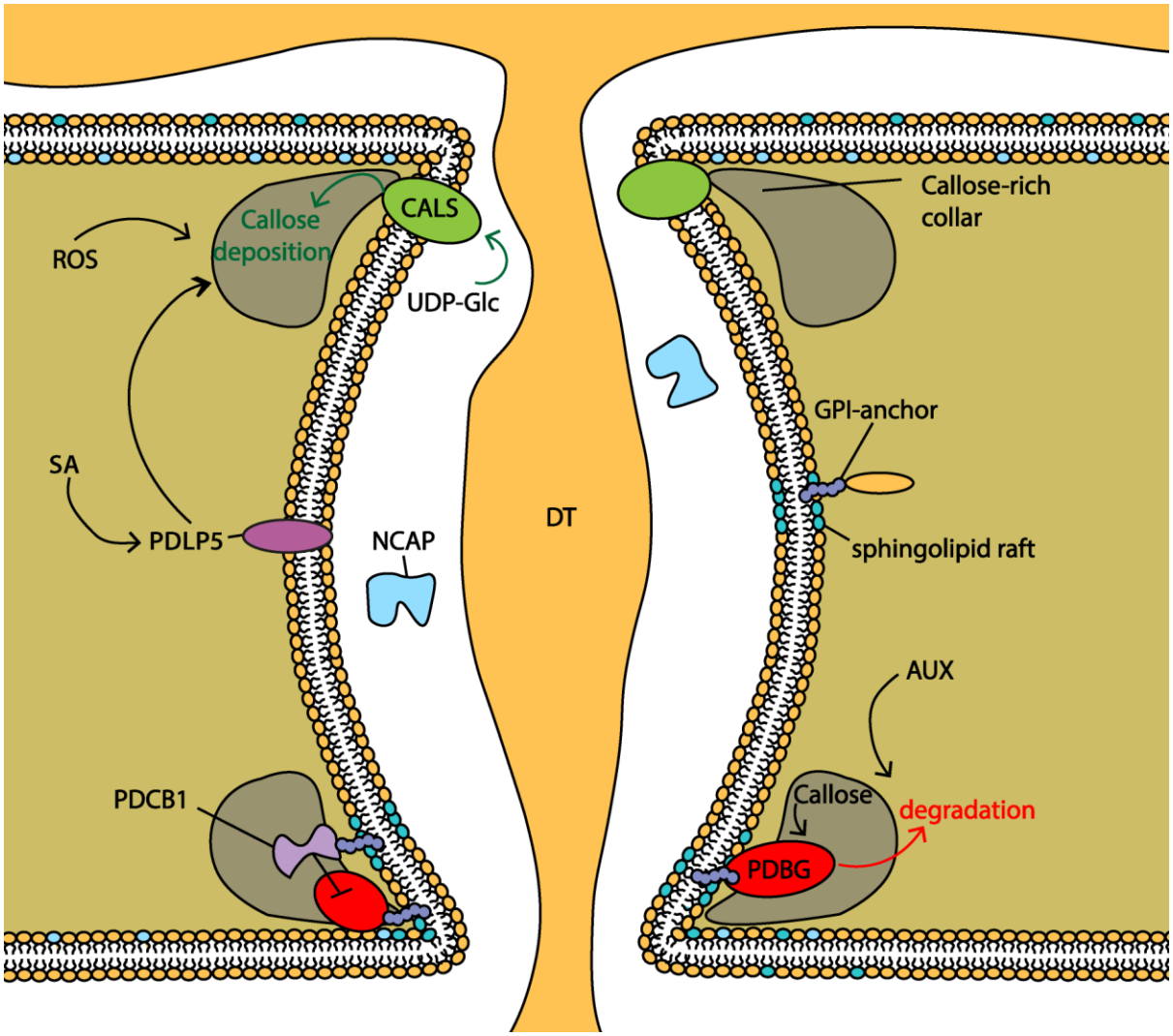
ROS
Callose deposition
CALS
UDP-Glc
SA
PDLP5
NCAP
DT
PDCB1
Callose-rich collar
GPI-anchor
sphingolipid raft
AUX
Callose
degradation
PDBG

Figure 2

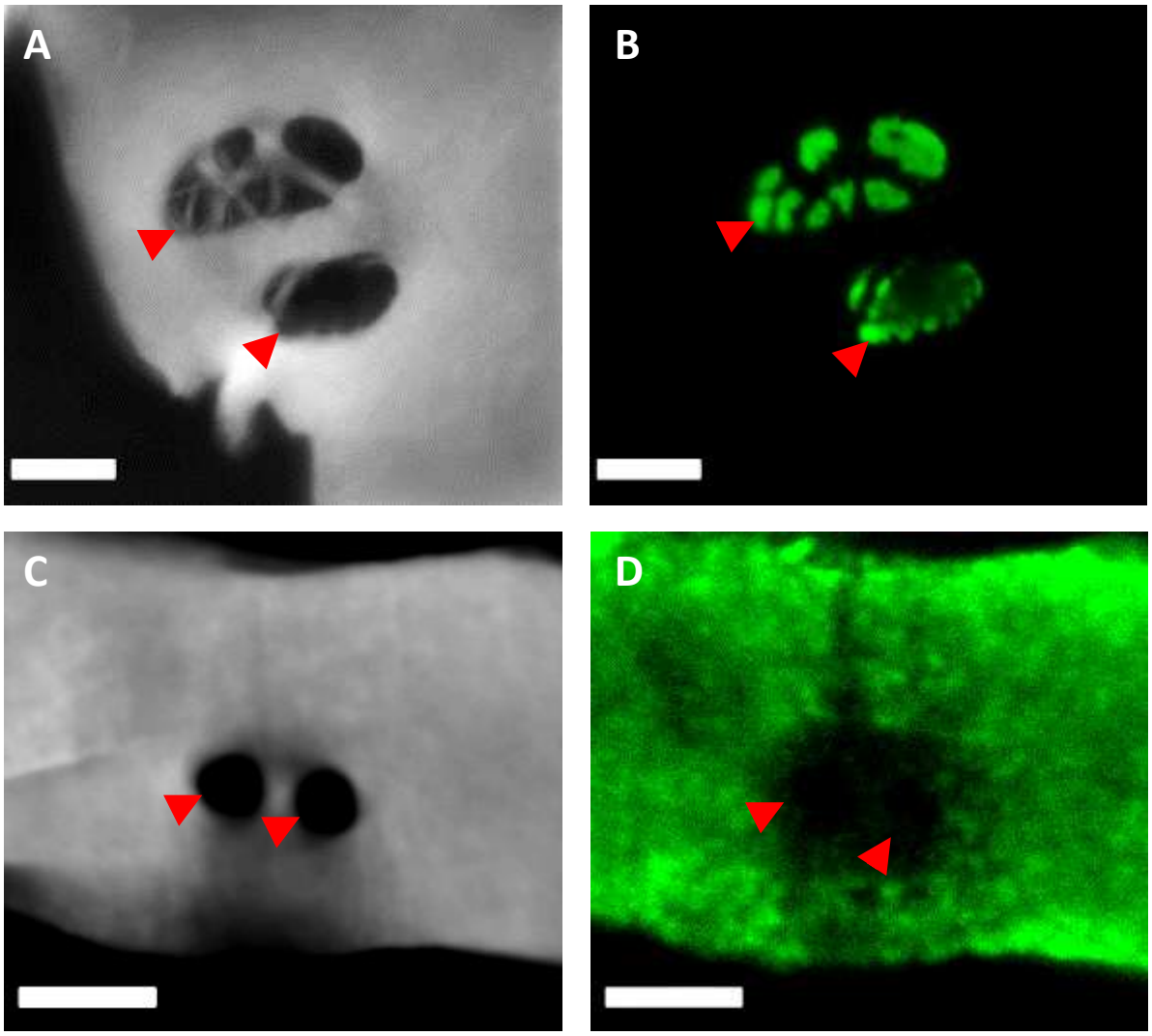

Figure 3