Arabidopsis AGC3 kinases and PIN plasma membrane abundance

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Chapter 1

The evolution of AGC3 and D6 kinases and PIN phosphorylation and its role in plant development

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Abstract

The plant hormone auxin plays a central role in the growth and development of plants. Auxin acts in a concentration dependent manner and polar cell-to-cell transport of this hormone determines its distribution in the tissues of plants. This polar auxin transport is mediated by several families of auxin transporters, including the PIN FORMED (PIN) auxin efflux carriers that determine the direction of transport by their polar localization at the plasma membrane. The plasma membrane abundance and polarity of PINs (and thereby of polar auxin transport) is regulated by their post-translational modification, of which phosphorylation is best studied. PIN proteins in *Arabidopsis* consist of two transmembrane domains separated by a 'long' (PIN1,2,3,4,6,7) or by a 'short' (PIN5,8) hydrophilic loop. Phosphorylation of 'long' PINs in their central hydrophilic loop by the AGC3 kinases PINOID, WAG1 and WAG2 triggers shootward (apical) or outer-lateral polarity. The AGC1-type D6 kinases also phosphorylate the PIN hydrophilic loop, however this was reported to result in auxin transport activation rather than subcellular polarity establishment. Here we investigate the conservation and phylogeny of AGC3 and D6 kinases and their phosphorylation sites in PINs from the earliest land plants to flowering plants. In early land plants, many of the same proteins and conserved motifs can be found, however it is in monocots and dicots that conservation of PIN phosphorylation by AGC3 and D6 kinases is strongest. The expansion and increased conservation of AGC3 and D6 kinases and PINs in later lineages such as monocot and dicot flowering plants, is in line with their important role in the formation of reproductive organs and in the tropic growth responses that allow plants to adapt to changes in their environment.

Auxin as central regulator of plant development

Plant development progresses through strictly programmed and more flexible phases. Embryogenesis for example is strictly regulated, producing embryos with a uniform body plan, while the organ formation in the juvenile or adult plant is more flexible, as the position and number of organs formed may be affected by environmental factors. The plant hormone auxin is a central regulator during the programmed phases, but is also involved as an integrator of many environmental signals. Auxin acts in a concentration dependent manner; auxin minima and maxima determine the positions where new organs are formed or where new tissues differentiate, for example during lateral root formation (Benková et al., 2003; De Smet et al., 2007; Swarup et al., 2008) or the phyllotaxis of leaves and flowers (Deb et al., 2015; Kierzkowski et al., 2013; Reinhardt et al., 2003).

Auxin perception takes place in the nucleus of the plant cell, where it acts as a molecular glue to enhance the interaction between the AUX/IAA transcriptional repressors and the TIR1/AUXIN-RESPONSIVE F-BOX (AFB) co-receptor proteins, which are part of an E3 ubiquitin ligases complex. When auxin levels in the cell are low, AUX/IAA proteins bind to AUXIN RESPONSE FACTOR (ARF) transcription factors and together with co-repressor TOPLESS they inhibit the transcription of auxin responsive genes. When auxin levels in the cell rise, the TIR/AFB E3 ligases are able to recruit the AUX-IAAs to label them for degradation by the 26S-proteasome through ubiquitination. This de-represses the ARFs, which can subsequently initiate auxin related transcriptional responses (reviewed by: Wang and Estelle, 2014). ARF3/ETTIN is an ARF involved in gynoecium development that senses auxin in a non-canonical way. It is not repressed through an AUX-IAA dependent mechanism, but it controls transcription by binding to other transcription factors in an IAA dependent manner, having interactions with different factors, such as INDEHISCENT or SPATULA, depending on the amount of IAA present (Moubayidin and Ostergaard, 2017; Simonini et al., 2016). Next to the TIR-AFBs the F-box protein S-Phase Kinase-Associated Protein2B (SKP2B) can bind auxin (Jurado et al., 2010) and *skp2b* loss-of-function alleles show some resistance to auxin treatment. Furthermore SKP2B can regulate cell cycle processes during lateral root formation in an auxin dependent manner (Manzano et al., 2012). The other candidate auxin receptor AUXIN BINDING PROTEIN 1 (ABP1) is the first protein for which auxin binding was unequivocally demonstrated (Lobler and Klambt, 1985; Woo et al., 2002). The role of ABP1 in plant development has been extensively studied (review: Grones and Friml, 2015), but the recent finding that the embryo lethal phenotype of the *abp1-1* allele is not caused by loss-of-function in the *ABP1* gene, but rather by a mutation in the *BELAYA SMERT (BSM)* gene located adjacent to *ABP1* (Dai et al., 2015) has brought the importance of ABP1 as auxin receptor into question. As *ABP1* is a single copy gene in *Arabidopsis,* and other *abp1* mutant alleles are wild type in all tested aspects (Gao et al., 2015), the re-installment of ABP1 as genuine auxin receptor requires the identification of mutant alleles that do show clear phenotypes.

The auxin minima and maxima are generated by a combination of local auxin biosynthesis and metabolism and polar cell-to-cell transport of auxin (Benková et al., 2003; Deb et al., 2015; Robert et al., 2013; Sorefan et al., 2009; Stepanova et al., 2008). For auxin biosynthesis and metabolism we refer to excellent recent reviews (Ljung, 2013; Tivendale et al., 2014; Zhao, 2010). In this chapter we focus on polar auxin transport, which is mediated by several families of auxin transporters: The PIN-FORMED (PIN) auxin efflux carriers (reviewed by Křeček et al., 2009), PIN-LIKES (PILS) auxin carriers (reviewed by Feraru et al., 2012), ABC B-type/ P-glycoproteins (ABCB/PGP) transporters (both influx and efflux, reviewed by Cho and Cho, 2013), the AUXIN1/LIKE AUXIN1 (AUX/LAX) auxin influx carriers (Peret et al., 2012) and the vacuolar auxin transporter WAT1 (Ranocha et al., 2013). AUX/LAX proteins are very important for loading of auxin in the cell (Boot et al., 2016) and contribute to development in a significant manner (Robert et al., 2015; Swarup et al., 2008). Together with the ABCB/ PGPs (Blakeslee et al., 2007) they determine the amount of auxin that is available in the cell for polar efflux by the PIN proteins. Depending on the tissue PINs are localized at the plasma membrane of plant cells in an apical (shootward), basal (rootward), lateral, or apolar manner, and this localization correlates well with the anticipated direction of transport (Benková et al., 2003; Friml et al., 2003). Later it was demonstrated that PINs are the rate-limiting components of polar auxin transport system that determine the direction of transport through their asymmetric distribution at the plasma membrane (Petrásek et al., 2006; Wisniewska et al., 2006). Auxin itself promotes PIN polarity in a self-organizing process, making it possible for the plant to transport auxin towards higher auxin concentrations (Berkel et al., 2013), thereby generating the strong maxima needed for proper development.

PINs are involved in almost all developmental processes, but they have specialized functions. PIN1, PIN4 and PIN7 are mostly involved in programmed development, such as embryo patterning and the formation of organs at the shoot apical meristem (Friml et al., 2003; Furutani et al., 2004; Kierzkowski et al., 2013; Reinhardt et al., 2003), root apical meristem maintenance (Blilou et al., 2005; Friml et al., 2002a; Friml et al., 2003) and leaf development (Hay et al., 2006). PIN1, PIN6 and PIN8 together are important for vascular development (Sawchuk et al., 2013). PIN2, 3, and 7 are involved in tropic responses to light and gravity (Friml et al., 2002b; Friml et al., 2003; Keuskamp et al., 2010; Luschnig et al., 1998), while PIN3, PIN4 and PIN7 also regulate tissue patterning in the shoot (Le et al., 2014) and root (Marhavý et al., 2013; Rosquete et al., 2013). Since PIN polarity is so important for development and tropisms of plants we will discuss the regulation of it in the sections below.

PIN polarity and plasma membrane abundance regulated by post translational modification

Like all transmembrane proteins, PINs are delivered from their site of biosynthesis in the rough endoplasmatic reticulum (ER) to their site of action at the plasma membrane by vesicle transport via de Trans Golgi Network and Early Endosomes (TGN/EE). PIN proteins have two sets of five transmembrane domains, which in PIN1, 2, 3, 4, 6 and 7 (long PINs) are separated by a long hydrophilic loop (HL) of around 300 to 400 amino acids that extends into the cytosol, while PIN5 and 8 (short PINs) have very short hydrophilic loops of 50 to 60 amino acids (Křeček et al., 2009; Nodzynski et al., 2016) (Table 2). The long PINs are generally found at the plasma membrane and the short PINs at the ER. However in some (artificial) cases PIN5 and PIN8 can appear at the plasma membrane (Ganguly et al., 2014) and PIN6 can be found both at the plasma membrane and ER (Simon et al., 2016a). The dynamic membrane polarity of long PINs is achieved through a continuous process of endocytosis and recycling back to the plasma membrane (Kleine-Vehn and Friml, 2008). The endocytosis of PINs from the plasma membrane is clathrin-mediated and involves recognition of targeted cargo by the AP-2 complex (review: Baisa et al., 2013), while vesicle trafficking to endosomal compartments depends on the ARF GTPase SCARFACE (Dhonukshe et al., 2007; Sieburth et al., 2006). Exocytosis back to the plasma membrane is dependent on the GNOM and GNOM-LIKE1 (GNL1) ARF GTP Exchange Factor (GEF) in the case of rootward PIN polarity, and occurs GNOM- or GNL1-independent in the case of shootward polarity (Dhonukshe et al., 2008; Doyle et al., 2015; Geldner et al., 2001; Geldner et al., 2003; Kleine-Vehn et al., 2008a; Kleine-Vehn et al., 2009).

PIN polarity and plasma membrane abundance is regulated by post-translational modification, of which phosphorylation is currently best studied. Phosphorylation of the long PINs by the AGCVIII-type protein serine/threonine kinase PINOID (PID) and its close homologs WAG1 and WAG2 at conserved TPRXS(S/N) motifs in their HL causes PIN1, PIN2 and PIN4 to accumulate at the shootward plasma membrane via GNOM- or GNL1-independent trafficking (Dhonukshe et al., 2010b; Friml et al., 2004; Huang et al., 2010; Kleine-Vehn et al., 2009; Michniewicz et al., 2007). The AGCVIII-type protein serine/threonine kinase D6 Protein Kinase (D6PK) phosphorylates the PIN HL as well, however with a different outcome: No plasma membrane polarity switch is observed, but a change in PIN auxin transport activity (Zourelidou et al., 2014). Phosphorylation of PIN1 is important for embryo development, lateral root formation, inflorescence meristem development, gynoecium patterning and leaf epidermis lobbing (Dhonukshe et al., 2010b; Friml et al., 2004; Huang et al., 2010; Li et al., 2011; Marhavý et al., 2014; Michniewicz et al., 2007; Moubayidin and Østergaard, 2014), whereas phosphorylation of PIN2 and PIN3 is important for gravitropism, phototropism and planar polarity of root epidermis cells and fruit dehiscence (Dhonukshe et al., 2010b; Ding et al., 2011; Ganguly et al., 2012; Girin et al., 2011; Rakusová et al., 2011; Sorefan et al., 2009; Stanislas et al., 2015; Willige et al., 2013).

Phosphorylation of PINs by PID creates a feed forward loop, since *PID* itself is induced by auxin (Benjamins et al., 2001; Friml et al., 2004). This process is important in for example phyllotaxis, where the auxin promoted induction of *PID* and thereby the promotion of apical PIN1 leads to an up-the-gradient transport of auxin (Furutani et al., 2014; Reinhardt et al., 2003). Besides auxin *PID*, *WAG1* and *WAG2* are all regulated by light and in this way can relocate PIN3 in phototropic responses of the hypocotyl (Ding et al., 2011). In this way hormones and abiotical signals can affect PIN localization and thereby auxin transport, which leads to tropic and developmental outputs.

Another posttranslational modification that has been found to be important for the cycling and/ or removal of PIN proteins from the plasma membrane is ubiquitination: the covalent binding of ubiquitin proteins to certain conserved lysine residues in long PIN HLs (Leitner et al., 2012) (Habets and Offringa, 2014). For PIN2 it has been shown to occur in two different manners: K48 mono- and K63 poly-ubiquitination (K48 and K63 refer to the lysine residue on ubiquitin where it is attached to the target). Mono-Ubiquitiniation is possibly a signal to either remove or transport PIN2 between the Trans-Golgi-Network/ Early-Endosome (TGN/EE) and the plasma membrane (Leitner et al., 2012). Poly-ubiquitination can lead to TGN/EE targeting and/or subsequent targeting towards a multi-vesicular body (MVB), which is followed by degradation in the lytic vacuole (Korbei and Luschnig, 2013; Offringa and Huang, 2013). PINs can be sorted towards a multi-vesicular body (MVB) through the action of the ESCRT homolog CHARGED MULTIVESICULAR BODY PROTEIN/CHROMATIN MODIFYING PROTEIN1 (CHMP1) (Spitzer et al., 2009) and the de-ubiquinating protein ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM3 (ASMH3) (Isono et al., 2010). There is also the possibility of PINs moving back from the TGN/EE to the plasma membrane through the action of the retromer, consisting of among others SORTING NEXIN 1 (SNX1), VACUOLAR PROTEIN SORTING 29 (VPS29) and VPS35 (Jaillais et al., 2006; Jaillais et al., 2007; Kleine-Vehn et al., 2008b). Degradation of PIN proteins in the vacuole (vacuolar targeting) is an important way for the plant to regulate directed growth such as gravitropism and root negative phototropism by reducing PIN plasma membrane abundance at one side of the cell (Abas et al., 2006; Baster et al., 2012; Kleine-Vehn et al., 2008b; Leitner et al., 2012; Wan et al., 2012). Vacuolar targeting of PIN1 and PIN2 in the root is dependent upon COP1 light signaling from the shoot (Sassi et al., 2012) and phototropin signaling in the root (Wan et al., 2012). The RING domain ligase 1 and 2 (RGLG1&2) have been linked to PIN1-GFP abundance and may be involved in the transfer of ubiquitin to the PIN HL (Yin et al., 2007). Lysine 63 ubiquitination is subsequently recognized by a family of TOL proteins that determine the targeting of the vesicle to the MVB. The precise molecular mechanisms that facilitate the trafficking of PIN containing vesicles to the MVB and vacuole has been extensively reviewed (Korbei and Luschnig, 2013).

The effects of AGC3 and D6 kinase phosphorylation of the PIN hydrophilic loop

As described above, phosphorylation of the PIN HL by PID and two closely related kinases WAG1 and WAG2 results in apical (shootward) polar localization of PINs (Dhonukshe et al., 2010b; Habets and Offringa, 2014) and stimulation of polar auxin transport (Benjamins et al., 2001; Friml et al., 2004). In contrast, the antagonistic action of PP2A/PP6-type phosphatases leads to PIN dephosphorylation, resulting in the opposite rootward PIN localization (Dai et al., 2012; Michniewicz et al., 2007). Together with a fourth kinase AGC3-4, PID, WAG1 and WAG2 form the AGC3 subclade of the AGCVIII kinases (Galván-Ampudia and Offringa, 2007; Rademacher and Offringa, 2012) At least for PID, WAG1 and WAG2 it has been shown that they phosphorylate the hydrophilic loop of long PINs at the first serine in the conserved TPRXS(S/N) motif (S1, S2 and S3) (Huang et al., 2010). Mutation S1, S2 and S3 to an alanine causes a rootward localization of PIN2 in the root tip epidermis and of PIN1 in the apical meristem, leading to loss of gravitropism, lack of cotyledons and pin-shaped inflorescences. Mutating these residues to a glutamic acid (S to E) to mimic phosphorylation causes a shootward localization of PIN1 in the root meristem, resulting in a reduced auxin maximum in the root meristem. In the shoot meristem constitutive apical localization results in pin structures, due to the loss of dynamic PIN1 localization that is required to generate strong auxin maxima that are initiation points for organogenesis (Huang et al., 2010). This mimics some of the phenotypes observed in PID, WAG1 and WAG2 overexpression lines (Dhonukshe et al., 2010b; Friml et al., 2004). Phosphorylation by AGC3 kinases also seems to control the plasma membrane abundance of PIN1 and PIN2, since loss of phosphorylation versions of these PINs show a decrease of the plasma membrane signal (Dhonukshe et al., 2010b; Huang et al., 2010). Transmembrane proteins are degraded via targeting to vacuole and it remains to be investigated if AGC3-mediated PIN phosphorylation is coupled to a higher degree of vacuolar targeting and degradation of PINs.

A group of closely related protein serine/threonine kinases kinases belonging to the AGC1 subfamily of AGCVIII kinase, D6PK and three D6PK LIKES (D6PKLs), can also phosphorylate the PIN1 and PIN2 hydrophilic loop (Zourelidou et al., 2009). The quadruple *d6pk* loss-of-function mutant mimics the phenotypes of the *pin-formed* mutant, while triple mutants are defective in hypocotyl bending (Willige et al., 2013; Zourelidou et al., 2009). D6PK and the D6PLs, below referred to as D6PKs, preferentially phosphorylate the PIN1 HL and PIN3 HL at two serine residues named S4 and S5, however other residues in the PIN3 HL still account for 28% of phosphorylation performed by D6PK. D6PK overexpression does not result in the same basal-to-apical shift in PIN polarity that was observed for PID overexpression (Zourelidou et al., 2009; Willige et al., 2013). (Zourelidou et al., 2014). A recent publication using phosphorylation specific anti-PIN1 antibodies suggests that there is little difference

between D6PK and AGC3 specificity and that other factors besides phosphorylation of S1,2,3 and 4 phosphorylation sites account for the difference observed between D6PK and PID function (Weller et al., 2017). However, when these sites are mutated to gain or loss of function in *PIN1*, they mirror *PID* overexpression and *pid* mutant effects and not those of *D6PK* (Dhonukshe et al., 2010a; Huang et al., 2010; Zourelidou et al., 2009). DPK is rootward localized and this seems to be important for the promotion of rootward polarization of trichoblasts (Barbosa et al., 2014; Stanislas et al., 2015). Internalization of D6PK after BFA treatment suggests that its trafficking to the rootward plasma membrane is GNOM-dependent (Barbosa et al., 2014), which is different from PID. Co-expression of PINs and D6PK in *Xenopus* oocytes suggests that it activates the auxin transport capacity of PIN1 (Zourelidou et al., 2014). D6PK also promotes the rootward polarization of trichoblasts, however there appeared to be no effect on PIN polarity due to D6PK phosphorylation (Barbosa et al., 2014; Stanislas et al., 2015). Although further in planta evidence is required to prove the proposed role of D6PK phosphorylation as an activation signal for PIN-based auxin transport, the current data indicate that the D6PKs act in concert with the AGC3 kinases to regulate polar auxin transport

Next to the AGC kinases, CDPK-RELATED-KINASE5 (CRK5) was reported to phosphorylate the PIN2 HL *in vitro* at unknown residues. Inactivation of this kinase leads to a decrease in PIN2 exocytosis. CRK5 is specifically involved in the modulation of root and shoot gravitropism (Rigó et al., 2013), however to date no *in vivo* phosphorylation was reported on this kinase regarding PIN2 phosphorylation.

Other phosphorylation sites on PIN HLs

Besides the phosphorylation sites associated with the AGC3 and D6PK's, other important sites of phosphorylation in PIN HLs were found that have to date not yet been directly associated with the action of a kinase. A group of phosphorylation sites collectively called M3, partially overlapping with the AGC3 kinase motifs are important for the plasma membrane localization of PIN3. This region also includes the first TPRXS(S/N) motif, however a mutant of three Serines to Alanines in M3 where the TPRXS(S/N) was left intact (3m1) only partially disrupted PIN3 plasma membrane localization (Ganguly et al., 2012). This 3m1 phosphorylation site, when mutated in PIN1 and PIN7 slightly decreases plasma membrane abundance. Mutating this site in PIN2 causes increased loss of PIN2-GFP plasma membrane signal in the dark, which was rescued with wortmannin application (an inhibitor of vacuole directed vesicle trafficking), showing that this mutant is enhanced in vacuolar targeting and subsequent degradation (Sasayama et al., 2013). However, no specific kinase has been identified that phosphorylates the 3m1 sites at the other serines apart from the TPRXS(S/N) motif. Serine 337 and Threonine 340 were identified as potential

phosphorylation sites through mass spectrometry (Michniewicz et al., 2007) and are found in PIN1, 3 and 4. Mutation of these two residues caused floral and cotyledon development defects, as well as a disrupted phyllotaxis (Zhang et al., 2010). Furthermore phosphomimic mutants in these sites have similar action on root meristem collapse as *35S::PID*, however *in vitro* phosphorylation experiments showed no direct phosphorylation of these sites by PID (Zhang et al., 2010) and to date no other kinase has been shown to phosphorylate Ser337/Thr340.

Translation of AGC3-phosphorylation into PIN plasma membrane targeting

Phosphorylation of the PIN HL is important for PIN function. What exactly 'senses' the phosphorylation status of PINs and subsequently recruits factors that initiate trafficking or activation is not yet known (Huang et al., 2010). One possibility is that phosphorylation causes conformational changes in the hydrophilic loop. Pin1at is a PIN1-type peptidyl-prolyl cis/trans isomerase (PPIase) which specifically recognizes phosphorylated Serine/Threonine residues preceding Proline and catalyzes a cis/trans conformational change between these residues (Landrieu et al., 2002). It has been proposed that this might be a mechanism through which AGC3 kinase-mediated phosphorylation is sensed (Xi et al., 2016). However, the serines phosphorylated by PID in the TPRXS(S/N) motif do not precede a proline (Dhonukshe et al., 2010b; Huang et al., 2010) and the strongest conformational change induced by Pin1at was found in a peptide that does not contain a TPRXS(S/N) motif at all. Pinat1 was found to induce a change of polarity in PIN1 (not in other PINs), and overexpressing Pin1at disrupts gravitropism (Xi et al., 2016). However, the subcellular localization of Pin1at is nuclear and cytoplasmic and not on the plasma membrane, making it highly unlikely that this protein directly induces a conformational change in PIN1 as a result of AGC3 kinase-mediated phosphorylation.

Alternatively, the PID-interacting protein TWISTED DWARF1 (TWD1) might induce the conformational change in PIN proteins (Henrichs et al., 2012), since it is a PPIase, similar to Pin1at, and it is found at the plasma membrane. Although TWD1 was found to interact with PID, ABCB1 and ABCB19 and to affect auxin transport (Henrichs et al., 2012; Wang et al., 2013) and recently it was shown that TWD1 binds NPA and interacts with ACT7 (part of actin cytoskeleton) (Zhu et al., 2016). Besides this TWD1 associates with BRASSINOSTEROID INSENSITIVE1 (BRI1) (Zhao et al., 2016), showing that it is a more general adapter of protein interactions at the plasma membrane.

Recently, certain residues in the PIN1 HL have been identified that bind the μ-subunit of the AP-2 complex involved in clathrin-mediated endocytosis in plants (Baisa et al., 2013; Sancho-Andrés et al., 2016). A conformational change induced by AGC3 kinase-mediated

phosphorylation in the PIN1 HL could expose or hide these residues to the AP-2 complex, thus affecting the clathrin-mediated endocytosis of PIN proteins into vesicles, a process that is important for PIN polarity establishment (Kleine-Vehn et al., 2011). This does not explain the targeted exocytosis to the plasma membrane and it remains to be seen which other residues in and interactors with the PIN HL affect its endocytosis, exocytosis and localization.

Plant AGC kinases have a unique structure

Based on previous phylogenetic analyses, the plant AGCVIII kinases in *A. thaliana* have been grouped into four subclades called the AGC1 to AGC4 kinases (Bögre, 2003; Galván-Ampudia and Offringa, 2007). The typical hallmarks discerning the AGCVIII kinases from other AGC kinases are a DFD instead of the generally conserved DFG in the magnesium binding loop resulting in reduced kinase activity, and an insertion domain between kinase subdomains VII and VIII (Bögre, 2003; Galván-Ampudia and Offringa, 2007; Rademacher and Offringa, 2012). This insertion domain is a variable stretch of amino acids between the conserved kinase domains and can be identified by the DFD motif in subdomain VII, an AEP motif in the middle and the conserved APE motif at the end of the activation loop in subdomain VIII. This activation loop is important for the regulation of the kinase activity though the auto- or transphosphorylation of a serine or threonine residue in a conserved motif ([S/T]PxxGTx[D/E]Y), which causes the insertion loop to change conformation, making the magnesium binding site available (Rademacher and Offringa, 2012; Zegzouti et al., 2006a). The insertion domain was shown to mediate plasma membrane localization to PID via interactions between charged phosphoinositide membrane lipids and Arginine residues in the insertion domain (Simon et al., 2016b; Zegzouti et al., 2006b). Although the insertion domain is highly variable across species and kinases, there are certain conserved residues that can be used to distinguish subtypes of AGCVIII kinases.(Rademacher and Offringa, 2012), Firstly, the different sizes of the insertion domains of AGC3, D6PK and phototropin kinases from different species are depicted in Table 1. D6PKs have a longer N-terminal domain and insertion domain compared to AGC3 kinases (Rademacher and Offringa, 2012). AGC3-4 differs in this respect, as it has an insertion domain that is comparable in size to what is found in AGC1 kinases.

For most plant AGC kinases, phosphorylation of the activation loop by the 3-phosphoinositide-dependent protein kinase 1 (PDK1) enhances their auto- and transphosphorylation activity (Zegzouti et al., 2006a; Zegzouti et al., 2006b). A hydrophobic motif (FXXF) present at the C-terminus of these kinases, referred to as PDK1 Interacting Fragment or PIF, was reported to enhance the activation of the AGC kinase by PDK1. However, a translational PID-GUS or PID-VENUS version were the PIF was deleted could still complement the *pid* loss-of-function mutant (Benjamins et al., 2001; Michniewicz et al., 2007), questioning the importance of the PIF for PID function.

Table 1: AGC kinases analyzed in this study.

Table showing the name of the kinase, insertion loop size (from DFD to APE motif), AGC clade and gene code.

gene name	insertion domain size	clade	gene code
VcPHOT2	68	PHOT	Vocar20005779
PpAGC3-1	110	preAGC3/1	Phpat.009G094900
PpAGC3-2	110	preAGC3/1	Phpat.015G093300
PpD6PKL1	38	D6PK	Phpat.009G080500
PpD6PKL2	134	D6PK	Phpat.009G080600
PpD6PKL3	134	D6PK	Phpat.015G074900
PpPHOT2-1	68	PHOT	Phpat.0079051000
PpPHOT2-2	56	PHOT	Phpat.014G037700
PpPHOT2-3	52	PHOT	Phpat.001G093900
PpPHOT2-4	69	PHOT	Phpat.019G036700
PpPHOT2-5	57	PHOT	Phpat.021G074300
SmAGC3-1	107	preAGC3/1	Sm62746
SmAGC3-2	106	preAGC3/1	Sm89681
SmD6PKL1	103	D6PK	Sm11420
SmD6PKL2	104	D6PK	Sm234346
SmPHOT2-1	58	PHOT	Sm230655
SmPHOT2-2	59	PHOT	Sm172224
BdPID1	80	AGC3	Bradi1g13860
BdPID2	63	AGC3	Bradi4g01700
BdWAG	82	AGC3	Bradi1g13860
BdD6PKL1	106	D6PK	Bradi1g43350
BdD6PKL2	108	D6PK	Bradi3g39070
BdD6PKL3	105	D6PK	Bradi4g33110
BdD6PKL4	103	D6PK	Bradi4g08700
BdPHOT1	55	PHOT	Bradi4g45310
BdPHOT2	55	PHOT	Bradi5g907360
OsAGC3-4	111	AGC3	Os01g07940
OsPID1	75	AGC3	Os03g44020
OsPID2	73	AGC3	Os12g42020
OsWAG	76	AGC3	Os03g14840
OsD6PKL1	105	D6PK	Os02g49310
OsD6PKL2	105	D6PK	Os06g18830
OsD6PKL3	109	D6PK	Os08g38320
OsD6PKL4	105	D6PK	Os09g08420
OsD6PKL5	108	D6PK	Os09g30150
OsPHOT1-1	54	PHOT	Os11g01140
OsPHOT1-2	54	PHOT	Os12g01140
OsPHOT2	54	PHOT	Os04g23890
AcPID	92	AGC3	Aquca 001_00789
AcWAG	67	AGC3	Aquca 010_00269
AcD6PKL1	107	D6PK	Aquca 011_00517
AcD6PKL2	106	D6PK	Aquca 093_00028
AcD6PKL3	106	D6PK	Aquca 094_00011
AcPHOT1	53	PHOT	Aquca 010_00630
AcPHOT2	55	PHOT	Aquca 038_00012
AcPHOT2-LOV	55	PHOT	Aquca 095_00053
MgPID1	78	AGC3	Migut.H01340
MgPID2	86	AGC3	Migut.M01872
MgWAG1	60	AGC3	Migut.C00169
MgWAG2	62	AGC3	Migut.M00668
MgD6PKL1	95	D6PK	Migut.H00073
MgD6PKL2	114	D6PK	Migut.H01747
MgD6PKL3	96	D6PK	Migut.K01397
MgD6PKL4	107	D6PK	Migut.M01451
MgD6PKL5	104	D6PK	Migut.M01638
MgPHOT1	57	PHOT	Migut.N02640
PID	79	AGC3	at2g34650
AGC3-4	137	AGC3	at2g26700
WAG1	73	AGC3	at1g53700
WAG2	75	AGC3	at3g14370
D6PK	105	D6PK	at5g55910
D6PKL1	101	D6PK	at4g26610
D6PKL2	104	D6PK	at5g47750
D6PKL3	102	D6PK	at3g27580
PHOT1	57	PHOT	at3g45780
PHOT2	55	PHOT	at5g58140

Co-evolution of AGC kinases and PIN hydrophilic loop phosphorylation sites

Earlier analyses showed that AGC3s and D6PKs evolved at the same time as 'long' PIN proteins (Galván-Ampudia and Offringa, 2007). Since then more genome sequences of ancestral clades in plant life have been resolved, such as the whole genome sequences of the moss *Physcomitrella patens* (Rensing et al., 2008) and of the early vascular plant *Selaginella moellendorffii* (Banks et al., 2011). This allowed us to determine more accurately at what stage of plant life co-evolution between AGC3 and D6 kinases and PIN proteins was accompanied by conservation of phosphorylation sites in the PIN HLs. In addition, a new phylogenetic analysis of the AGC3s and D6PKs was performed to highlight the evolution of subtypes in more detail. Below we place this information into a biological context of developmental processes that co-evolved and for which the involvement of AGC kinase-mediated PIN phosphorylation has been proven.

Molecular phylogeny of the (land) plant AGC3 and D6 protein kinases

To create a phylogenetic tree depicting the evolution of AGCVIII PIN HL-phosphorylating kinases we used a broad range of model plant species **(Fig. 1A)**. Of the non-flowering land plants *Selaginella moellendorffii* was selected as an early vascular plant (lycophytes),with complex true roots (Banks, 2009), while the moss *Physcomitrella patens* was selected as non-vascular plant. In view of the importance of auxin transport for vascular differentiation, we expected non-vascular plants to show significant differences in polar auxin transport requirements. Furthermore, we included two species of monocots (*Oryza sativa* and *Brachypodium distachyon*), a basal eudicot (*Aquilegia coerulea*), and *Mimulus guttatus* (asterid) and *Arabidopsis thaliana* (rosid) as examples of advanced dicots, thereby covering all main branches of land plant life, except for gymnosperms. Although *O. sativa* is quite related to *B. distachyon*, both species were included because *O. sativa* has an *AGC3-4-like* gene, while this gene is lacking in *B. distachyon*.

For the *A. thaliana* AGCVIII kinases it has been established that the AGC3 kinases PID, WAG1 and WAG2 phosphorylate the PIN HL and induce switches in PIN polarity, whereas phosphorylation of the PIN HL by D6PKs (part of the AGC1 subclade) does not induce PIN polarity switches (Dhonukshe et al., 2010) Experiments in Xenopus cells suggest that phosphorylation (both by D6PKs and AGC3s) activates the PIN auxin transporters (Zourelidou et al., 2014). For *A. thaliana* PHOT1 it has been shown that its kinase domain cannot phosphorylate the PIN HL (Ding et al., 2011) and for kinases in the AGC2 clade it has been shown that their overexpression does not induce a switch in PIN polarity (Dhonukshe et al., 2010b). Whether AGC2 kinases phosphorylate the PIN HL is still

unknown, but since AGC2 function has not been correlated with auxin-related processes, we focused our phylogenetic analysis on the AGC3 and D6 protein kinases, and on the phototropins because of their role as founding members of the AGCVIII kinase family.

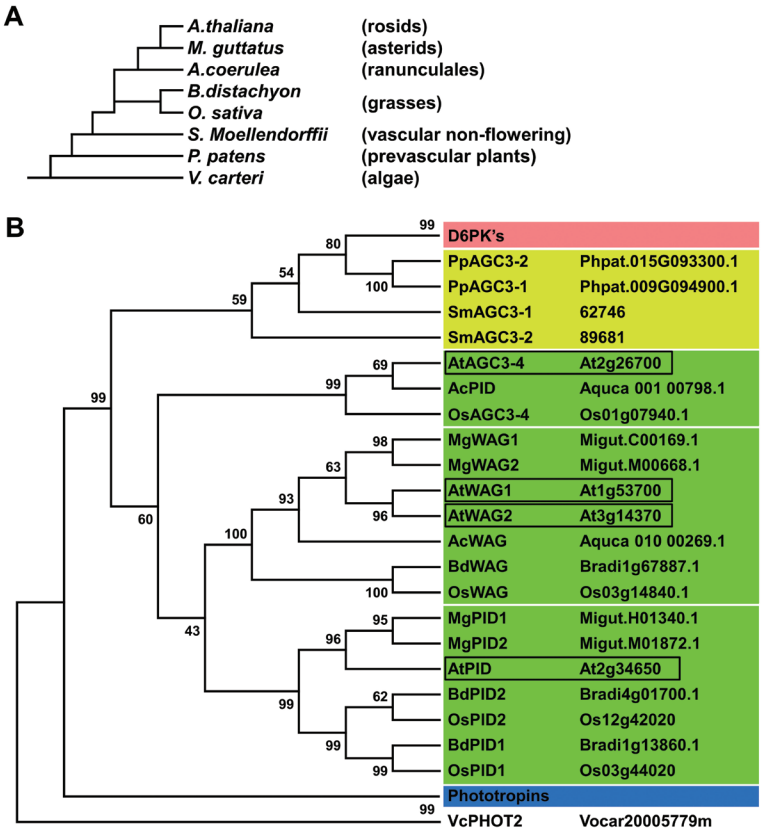

Figure 1: Molecular phylogeny of the AGC3 kinases, in relation to the D6 kinases and the phototropins. **(A)** Species used for the phylogenetic analysis and their evolutionary relations (adapted from Ruhfel et al., 2014). **(B)** Maximum likelihood phylogeny of the phototropins, AGC3 and D6PK kinase domains, focusing on the AGC3 kinases. Bootstrap values are depicted at each node (1000 repeats). The length of the horizontal branches does not represent the evolutionary distance. A phylogenetic tree focusing on D6PKs and phototropins is shown in **Fig. S1** and **Fig. S2**, respectively.

In line with this role, only phototropins can be found in Chlorophyta (green algae), and these are more closely related to *A. thaliana* PHOT2, while homologs of *A. thaliana* PHOT1 are only found in seed plants (Galván-Ampudia and Offringa, 2007). This makes the *Volvox carteri* PHOT2-like kinase a suitable outgroup for phylogenetic analysis. Chlorophyta do not possess *PIN* genes, however they do contain the ER localized *PIN-LIKES* in their genomes (Feraru et al., 2012), but until now no link between AGC kinase phosphorylation and *PIN-LIKES* has been reported. In the charophyte algae *PIN* sequences have been

identified (Bennett, 2015) and polar auxin transport measurements on cells of *Chara corellina* suggest that the origin of polar auxin transport lies already with the water living Characeae (Boot et al., 2012) rather than with the Bryophyta (Flores-Sandoval et al., 2015; Viaene et al., 2014).

From the species described above and summarized in Fig. 1A, the phototropins, AGC3 kinases and D6PKs were identified using the *A. thaliana* PHOT2, PID and D6PK protein sequences in Protein BLAST. The aligned protein sequences were used to construct a maximum likelihood phylogeny tree (Fig. 1B, S1 (D6PKs and S2 (PHOTs)). This phylogenetic tree confirms the observation from an earlier analysis of *A. thaliana* AGCVIII kinases (Galván-Ampudia and Offringa, 2007) that the phototropins (Fig. S2) form a separate clade from the D6PKs (Fig. S1) and the AGC3s (Fig. 1B). Within the AGC3 group there is a separation between the AGC3-4, WAG- and PID-like kinases. Both *P. patens* and *S. moellendorfii* genomes contain AGC3 kinase homologs that form a paraphyletic group within the larger sister group of AGC kinases comprising the D6PKs (Fig. 1B), which were also found in *S. moellendorffii* and *P.* patens (Fig. S1). PpAGC3-1, PpAGC3-2, SmAGC3-1 and SmAGC3-2 share characteristics of both D6PK/ and AGC3 kinases: they lack the D6PK N-terminal domain, but the C-terminal domain is somewhat similar to D6PKs and their insertion loop is neither typically D6PK nor AGC3 (alignment: supplemental file 01). This tree (Fig. 1B) does not perfectly reflect the evolutionary history of the plant species from which these kinases were taken. However, based on this analysis the last common ancestor of the Pp/SmAGC3 and AGC3 kinases is probably a protein sharing the characteristics of both the AGC3s and D6PK's. Pp/SmAGC3 and AGC3 kinases are between the D6PKs are thus further removed from the phototropins then the AGC3 kinases. This means that the last common ancestor may have resembled more the AGC3 kinases. From that the two types (D6PK and AGC3) evolved, while in mosses and early vascular plants the AGC3 kinases evolved into proteins able to combine the functions of AGC3s and D6PKs. D6PKs and AGC3s have two distinct functions: on the one hand activation of PIN-based auxin transport has been suggested for both AGC3s and D6PKs (Zourelidou et al., 2014) and on the other hand AGC3 kinases control polarity of PINs (Dhonukshe et al., 2010b; Huang et al., 2010). It is likely that in mosses both processes are already present, since they show complex development and tropisms (Cove et al., 1997). However, in more primitive ancestral plants only activation might have been present.

Phototropins as founding members of blue light-induced growth responses

Our current and previous analysis showed that of all plant AGC kinases only phototropins can be found in chlorophyte algae, suggesting that they are the founding members of the plant-specific AGCVIII kinase group. In higher plants, the phototropins are involved in orienting plant growth based on low (PHOT1) or high (PHOT2) intensity blue light perception (reviewed by: Liscum et al., 2014), by inducing changes in PIN polarity through signal transduction via NPH3-domain containing proteins (Ding et al., 2011). In addition, PHOT2 plays a role in protecting plant cells to photodamage by high intensity blue light by inducing chloroplast avoidance movement (Jarillo et al., 2001; Kagawa et al., 2001). A recent analysis of phototropin evolution concluded that the single *PHOT* gene of algae is neither a true PHOT1- or PHOT2-type (Li et al., 2015). Furthermore, this study concluded and that not all land plants have *PHOT1*- and *PHOT2*-type genes, something we observed as well in our analysis (Fig. S2). Some organisms have more than one of each type, but interestingly *P. patens* has five *PHOT2*-type genes and no *PHOT1*-type gene, which seems in line with the observation that it does not show strong tropic responses to blue light (Bao et al., 2015). Another peculiarity is the occurrence of a PHOT2-like kinase from *A. coerulea* that has lost its Light Oxygen Voltage (LOV) domain. Li et al. concluded that in early land plants there have been many gene duplications leading to sub-functionalization and convergent evolution, and it is from one of these events that probably the AGC3 kinase clade arose.

AGC3 and D6 protein kinases subfunctionalised in early land plants

Based on the phylogenetic analysis, ancestral forms of AGC3 and D6 protein kinases were found in *P. patens* and *S. moellendorffii* (Fig. S1), suggesting that sub-functionalisation already occurred in these early land plants. The recent finding of polarly localized PINs in *P. patens* is in line with such a sub-functionalisation (Vianne et al., 2014). Whether the ancestral AGC3 kinases were already involved in PIN polarity regulation requires further investigation. The phylogeny of the D6PKs itself was difficult to resolve with several low bootstrap values (Fig. S1). Interestingly, *A. thaliana* D6PKL2 (PK5) was placed separate from the other D6PKs with a well-supported bootstrap value. This implies that it is more related to the moss/lycophyte D6PKs. Still, its genetic redundancy with the other D6PKs indicates that is it functionally equivalent to D6PK (Zourelidou et al., 2009).

Even though early land plants, such as *P. patens* and *S. moellendorfii*, did develop gravitropism of caulonemata (Jenkins et al., 1986) and roots (Banks, 2009; Haberlandt, 1914), respectively, they do not have the AGC3 kinases that are typical for flowering plants.

Within this group, the PID-, WAG- or AGC3-like kinases all branch off in monophyletic clades. *M. guttatus*, *O. sativa* and *B. distachyon* have two PID-like kinases, while *A. thaliana* only has one. Remarkably, PID1 of *O. sativa* and *B. distachyon* are more similar than their respective PID2s, whereas for *M. guttatus* PID1 and PID2 group together, suggesting that the two PID-types probably originated from a separate gene duplication event in grass or asterid species. Since loss of *PID* function results in sterility (Benjamins et al., 2001; McSteen et al., 2007), it might be advantageous for a plant to possess two PID-like genes, thereby providing selective pressure for extra genetic redundancy. It is likely though that in the meantime the two *PID* genes in *O. sativa*, *B. distachyon* and *M. guttatus* have undergone subfunctionalization by amino acid substitutions and altered expression patterns. Another likely functional divergence between the two PID copies could be the subcellular localization at the plasma membrane or in the nucleus. At least for the PID ortholog in maize BARREN INFLORESCENCE2 (BIF2) is was shown that besides PINs it also interacts with and phosphorylates the nuclear transcription factor BARREN STALK1 (BA1) (Skirpan et al., 2008).

Similar to *PID*, flowering plants have either one or two copies of the *WAG* gene (Fig. 1B). In *A. thaliana* the *WAGs* act redundantly with *PID* in root gravitropism (Dhonukshe et al., 2010b; Santner and Watson, 2006), hypocotyl phototropism (Ding et al., 2011; Preuten et al., 2013) and in cotyledon development during embryogenesis (Cheng et al., 2008; Dhonukshe et al., 2010b). In addition, *WAG2* has been reported to play a role in fruit opening (Girin et al., 2011; Sorefan et al., 2009), whereas *WAG1 i*s also expressed in the shoot (Cheng et al., 2008)(eFP browser expression data). It may be that the original function of the *WAG* genes lies in shoot developmental processes (similar to PID) and that they were later adopted to function in fine-tuning root growth in higher plants.

The third clade of flowering plant-specific AGC3 kinases is represented by AtAGC3-4 (Fig. 1B). AtAGC3-4 has previously been named PID2 (Cheng et al., 2008), however it is clear from this phylogenetic analysis that it is a distinct protein from AtPID. Of the eight plant species used for our analysis only *A. thaliana*, *A. coerulea* and *O. sativa* have an AGC3-4-like kinase. *A. coerulea* has the smallest set of kinase genes: one *PID-like*, one *WAG-like* and three *D6PK-like* genes, which is the same for *P. patens.* AcPID is a kinase that is phylogenetically closer to AGC3-4. AtAGC3-4 and its orthologs in other species have an insertion domain which is much larger than that of PID and the WAGs (Table 1), however the insertion domain of AGC kinases is generally not well conserved, and there are distinctive differences between sequences at the N-terminal and C-terminal ends of the kinase domains that distinguish AGC3-4 from PID and the WAGs. The precise function of AGC3-4-like kinases is not yet clear and it is possible that AcPID combines normal PID function and a putative AGC3-4 function in one kinase. Why some species have lost an AGC3-4 kinase is also not clear, however, there are other species beside *A. thaliana* and *O. sativa* that have an AGC3-4 like kinase (Fig. S3), so it will be interesting to know more about the function of this particular type of kinase.

AGC3 and D6 kinase phosphorylation motifs in the PIN HL

Previously, it was shown that the hydrophilic loop of long *A. thaliana* PINs is phosphorylated by the AGC3 kinases PID, WAG1 and WAG2 at a conserved serine in the TPRXS(S/N) motif (Dhonukshe et al., 2010b; Huang et al., 2010). The TPRXS(S/N) AGC3 kinase motif is essential in determining polarity of PIN proteins at the plasma membrane, and as such is a defining sign in the evolution of polar PINs. Similarly, the phosphorylation motifs S4 (RXSNF) and S5 (SRRS) for the D6PKs (Zourelidou et al., 2014) are also important landmarks for PIN evolution. Here we checked for the eight plant species selected for our study and also for the angiosperm species *Phaseolus vulgaris* which long PINs contain these motifs, and combined this information with the evolutionary analysis of the AGC3 kinases to analyze how these components in the polar auxin transport pathway co-evolved during the evolution of plant life **(Fig. 2)**.

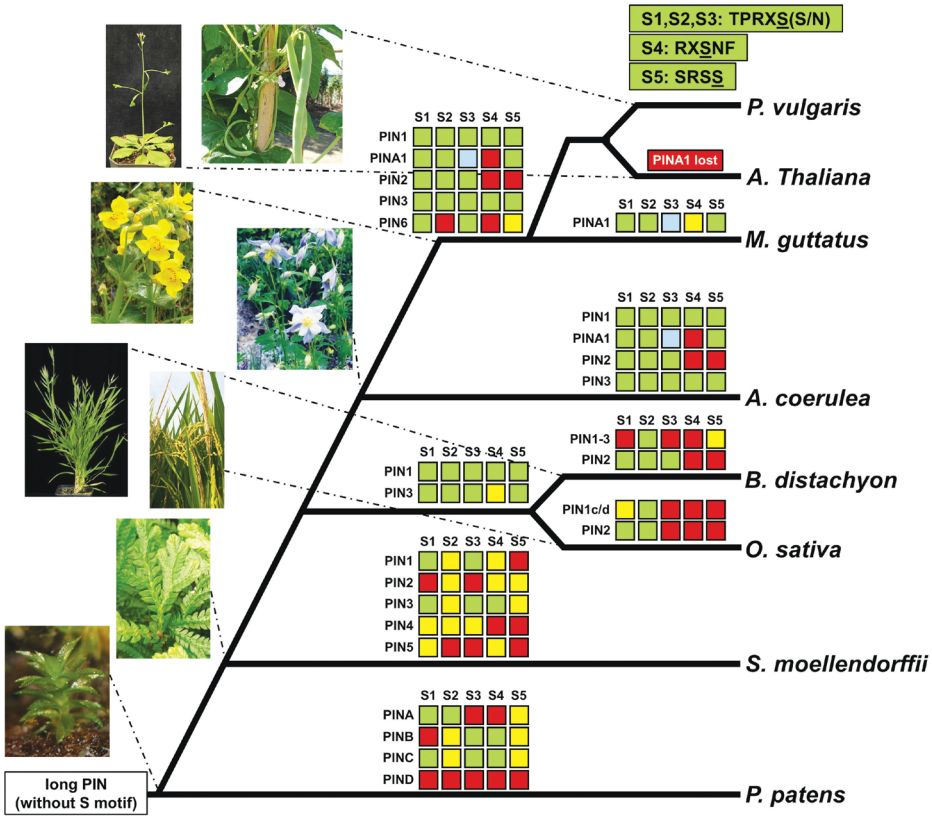

Figure 2: Conservation of AGC3 and D6 kinase phosphorylation motifs in PIN hydrophilic loops. Analysis of the AGC3 motifs S1, S2, S3 (TPRXS(S/N)), and the D6PK motifs S4 (RXSNF) and S5 (SRRS) in PINHLs from the land plant species indicated in the tree. Except for *P.patens* and *S. moellendorffii*, in other plants the PINs are organized into five different types: PIN1, PINA1, PIN2 PIN3 and PIN6. A green box indicates a canonical motif that is also found in *A. thaliana* PIN1 and PIN3, a yellow or blue box is a motif that is non-canonical and a red box indicates that the motif is missing. This analysis is based on the details provided in **Table 2**.

The reason to include *P. vulgaris* was that it contains a *PIN* gene called *PIN1A*, where the S3 and S4 motifs are absent or the S3 motif is modified (Table 2). *PIN1A*-type genes are also present in *A. coerulea, M. guttatus, B. distachyon* and *O. sativa,* but surprisingly this gene was lost in *A. thaliana*.

Previous phylogenetic analysis has shown that there is a clear subdivision of long PINs in the PIN1-type, PIN2-type and PIN3-type (which also includes PIN4 and PIN7), and of short PINs in PIN5- 6- and 8-types (Viaene et al., 2014). A renewed phylogenetic analysis with all the PIN protein sequences that were used to generate Table 2 an Fig. 2 confirmed that all mono- and dicot PINs classified as described before (Fig. S4). All long PIN types have the S1 to 3 motifs and the polarity of all of these PINs can be affected by PID or WAG phosphorylation in *A. thaliana* (Dhonukshe et al., 2010b; Ding et al., 2011; Huang et al., 2010). Interestingly, the PIN2-type PINs lack both D6PK S4 and S5 motifs, a feature that is conserved in both monocot and dicot PIN2 orthologs analyzed here (Fig. 2). Since the D6PK motifs S4 and S5 are thought to be necessary for activation of PIN transporters (Zourelidou et al., 2014), and PIN2 has been proven to transport auxin (Leitner et al., 2012), it is logical to think that PIN2 is constitutively active. A different possibility is that simply another, as of yet unknown residue in PIN2 is phosphorylated by D6PKs, or that PID phosphorylation leads to activation as well (Zourelidou et al., 2014).

In the early land plant species *P. patens* four PINs can be identified of which PpPIND has a relatively small hydrophilic loop (140 aa) that lacks any of the phosphorylation motifs and is more likely a forerunner of PINs with short (50-60 aa) loops. In the other long PpPINs a modified version of the S2 motif (TPR(D/E)SS) is present containing either an aspartic acid (D) or a glutamic acid (E), residues that are known to mimic a phosphorylated serine or threonine. The S5 D6PK motif is also slightly different (SSRS instead of SRRS). Interestingly, a recent study showed that PpPINA is localized on the plasma membrane and PpPIND in the ER (Viaene et al., 2014). This fact and the fact that it has a very short hydrophilic loop, devoid of canonical phosphorylation motifs, suggest that PpPIND might be a good example of the ancestor of the PIN5/8-type 'short' PIN. Both our and previous phylogenetic analyses show that PIN5/8 type PINs are evolutionary quite distant from the 'long' PINs, something that can also be said of PpPIND (Fig. S4) (Křeček et al., 2009; Viaene et al., 2013).

S. moellendorffii has 9 'long' PIN proteins that do not cluster together with the canonical long PINs in higher plants, and can be subdivided into 5 types based on their 'S' motifs (Table 2). SmPIN1 and SmPIN3 have the conserved S1, S3 and S4 motifs and they share the S2 motif with PpPINC. They are likely to locate at the PM and to act most similar to the flowering plant long PIN proteins. The remaining three SmPINs have an unorthodox makeup of motifs which are not found in the later plant lineages and might be specific for this clade of plant life. If PpPIND is an ancestor of the 'short' PIN type, then it has been lost in *S. moellendorffii*. Another possibility is that SmPIN5-1 or SmPIN5-2 are ancestors to the 'short' PIN type, but that for some reason they did not lose their complete HLs yet.

Table 2: PIN proteins analyzed in Figure 2.

Table showing the PIN proteins used in this study with their hydrophilic loop sizes (loop starts at EYR motif and ends at SYSS motif (Křeček et al., 2009)) and AGC3 and D6 kinase phosphorylation motifs. Green colour signifies a canonical motif as found in *A thaliana*, red means the motif is missing, while the other colours show a deviant motif with the colour corresponding to the organism where the motif is first encountered.

Gene name	hydrophilic loop size	AGC3 kinase motif# 1	AGC3 kinase motif# 2	AGC3 kinase motif# 3	D6 kinase motif# S4	D6 kinase motif# S5
PpPINA	**281**	TPRXS(S/N)	TPRXS(S/N)	-	-	SSRS
PpPINB	**396**	-	TPR(D/E)SS	TPRXS(S/N)	RXSNF	SSRS
PpPINC	**421**	TPRXS(S/N)	TPR(D/E)SS	TPRXS(S/N)	RXSNF	SSRS
PpPIND	**140**	-	-	-	-	-
SmPIN1-1	**333**	TPRXS(S/N)	TPR(D/E)SS	TPRXS(S/N)	RXSHF	-
SmPIN1-2	**325**	TPRXS(S/N)	TPR(D/E)SS	TPRXS(S/N)	RXSHF	-
SmPIN2-1	**310**	TPRXS(S/N)	TPR(D/E)SS	-	GNSSL	TARS
SmPIN2-2	**434**	TPRXS(S/N)	TPR(D/E)SS	-	GNSSL	SARS
SmPIN3-1	**376**	TPRXS(S/N)	TPR(D/E)SS	TPRXS(S/N)	RXSNF	SPRRS
SmPIN3-2	**379**	TPRXS(S/N)	TPR(D/E)SS	TPRXS(S/N)	RXSNF	SPRRS
SmPIN4-1	**383**	TPRXSD	TPTXST	TPREPPS	-	-
SmPIN5-1	**346**	TPGXSD	-	-	RGGSPF	-
SmPIN5-2	**343**	TPGXSD	-	-	RGGSPF	-
BdPIN1-1	**304**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
BdPIN1-2	**288**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
BdPIN1-3 / PIN1A	**254**	-	TPRXS(S/N)	-	-	SSRS
BdPIN2	**345**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	-	-
BdPIN3-1	**295**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RGSSF	SRQS
BdPIN3-2	**305**	TPRXS(S/N)	PRVSL	TPRXS(S/N)	RASSF	SRRS
OsPIN1a	**300**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
OsPIN1b	**302**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
OsPIN1c	**257**	QPRVSN	TPRXS(S/N)	-	-	-
OsPIN1d	**250**	QPRVSN	TPRXS(S/N)	-	-	-
OsPIN2	**329**	TPRXS(S/N)	TPRXS(S/N)	-	-	-
OsPIN10a	**322**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RGSSF	SRRS
OsPIN10b	**291**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RASSF	SRRS
AcPIN1	**328**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
AcPIN1A	**311**	TPRXS(S/N)	TPRXS(S/N)	SPRXSN	-	SRRS
AcPIN2	**350**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	-	-
AcPIN3	**362**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
AcPIN5	**62**	PRTSN	-	-	-	-
MgPIN1-1	**311**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
MgPIN1-2	**279**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
MgPIN1A	**292**	TPRXS(S/N)	TPRXS(S/N)	-	RXAS	SRRS
MgPIN2	**365**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	-	-
MgPIN3-1	**340**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
MgPIN3-2	**351**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
MgPIN6	**218**	TPRXS(S/N)	-	TPRXS(S/N)	-	SVSS
AtPIN1	**330**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
AtPIN2	**355**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	-	-
AtPIN3	**348**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
AtPIN4	**324**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
AtPIN7	**327**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
AtPIN6	**278**	TPRXS(S/N)	-	TPRXS(S/N)	-	SVSS
PvPINA-1	**293**	TPRXS(S/N)	TPRXS(S/N)	SPRXSN	-	SRRS
PvPIN1-1	**302**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
PvPIN1-2	**316**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
PvPIN1-3	**196**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
PvPIN2	**337**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	-	-
PvPIN3-1	**368**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
PvPIN3-2	**343**	TPRXS(S/N)	TPRXS(S/N)	TPRXS(S/N)	RXSNF	SRRS
PvPIN6	**233**	TPRXS(S/N)	-	TPRXS(S/N)	-	SVSS

The co-evolution of AGC3 and D6 kinases corresponds to an increase in plant body complexity and growth responses

We have shown that AGC kinases and their phosphorylation motifs in the PIN HL are conserved across land plants. The question is now if there is any correlation between complexity of organ growth or tropisms and complexity of AGC kinases, PINs and 'S' motifs. There is a clear difference between the bryophyte *P. patens*, the lycophyte *S. moellendorffi* and flowering plants. Their AGC3 and D6 kinases group separately from those of flowering plants and their long PINs and phosphorylation motifs are markedly different as well. *P. patens* is a primitive prevascular plant and it has simple roots, rhizoids, which are made up of tip growing cells, which do display polarity, but do not have to function within a more complex tissue (Schaefer and Zrÿd, 2001). The gametophyte of *P. patens* has a more complex leaf structure and a role in organ patterning might be played by AGC3, D6 kinases and PINs. *S. moellendorffii* is part of a clade of plant life (lycophytes) that does possess a root with developed vasculature and collumella, similar to higher plants, while also possessing a feathered leaf structure with regulated branching (Banks, 2009). It also has significantly more PINs than *P. patens,* however the number of kinases is roughly the same, so here an increase in complexity would be correlated with an increase in PINs.

A good example of a process dependent upon the dynamic regulation of auxin flow in higher plants is gravitropism. Research into lower plants such as *P. patens, S. moellendorfii, Chara sp., Funaria* and *Ceratodon* has shown that at least some parts of these plant forms can sense gravity and that this is dependent on the sedimentation of amyloplasts (Banks, 2009; Braun, 1997; Jenkins et al., 1986; Kern et al., 2001; Kuznetsov et al., 1999; Liu and Sun, 1994; Swuchow et al., 1995). However, in most lower plants, gravitropism functions in single tip growing cells such as caulonemata, protonemata and rhizoids. Statoliths in the tip of growing cells sediment, causing growth of the cell in the direction of the gravity vector (Sievers et al., 1996). This mechanism has similarities (statolith sedimentation) and differences (gravitropism regulation in cells vs multicellular tissues) to that in higher plants. *S. moellendorffii* is one of the earliest organisms with multicellular tissues showing growth in response to gravity. Different from higher plants the statoliths are not present in the collumella, but in the endodermis layer (periblem) of the root elongation zone 0,13 to 0,16 mm shootward of the root quiescent center. The fact that removal of the collumella does not inhibit the gravitropic response of *S. moellendorfii*, indicates that the collumella is not involved in gravity sensing (Haberlandt, 1914). Since *S. moellendorffii* also possesses PINs and AGC3 kinases, we can speculate that gravitropism in this organism operates through similar mechanisms as in higher land plants. The mechanism of gravitropism requires the concerted action of at least four types of PINs (PIN1, 2, 3, 4) in *A. thaliana*, so the need for more PINs in *S. moellendorffii* compared to *P. patens* may have been a consequence of the formation of a true root and the associated gravitropic growth.

In flowering plants, the AGC3 and D6 kinase phosphorylation motifs in PINs are more conserved than in *P. patens* and *S. moellendorffii* and there are more (diverse) AGC3 and D6 kinases. This could be seen as a selective pressure on the proliferation of this kind of dynamic regulation of polar auxin transport. On the other hand, in *P. patens*, PINs are plasma membrane targeted and a *Pppina Pppinb* double mutant shows defective leaf initiation and development (Viaene et al., 2014). Furthermore, disrupting auxin transport through NPA application disturbs the development of the bryophyte *M. polymorpha* (Flores-Sandoval et al., 2015). Thus in principle, mosses have the same tools of polar auxin transport, but they have less of them and there is more scope for variety. Flowering plants generally have more complex tissue patterns and tropisms (in different tissues), which needs more complex regulatory modules, such as four different AGC3 kinases instead of two, and six long PINs instead of three (*A. thaliana* vs *P. patens*). However, the basic regulatory mechanism (phosphorylation motifs, kinases) is more conserved and probably under higher selective pressure.

Conclusions

Phosphorylation of long PIN proteins at their central HL has a profound influence on PIN polarity and trafficking. Phosphorylation at conserved TPRXS(S/N) motifs by AGC3 kinases controls PIN polarity and these motifs are subsequently highly conserved among PINs throughout the plant lineage. AGC3 kinases that phosphorylate these HLs appeared at the same time as true PIN transporters. AGC3 kinases share a last common ancestor with the D6PKs. The D6PKs can also phosphorylate PINs, at different and overlapping residues compared to the AGC3 kinases. Although phosphorylation specific antibodies suggest that there is little difference in specificity for the PIN1 HL between D6PK and PID (Weller et al., 2017), gain and loss of function mutants of phosphorylation sites S1,2 and 3 mirror mutant and overexpression effects of *PID* and not of *D6PK*. A rough model would be that AGC3s determine plasma membrane polarity, while D6PKs determine transport activity. There is evidence that PID phosphorylation also leads to transport activation (Zourelidou et al., 2014), however this has not been shown *in planta*. Further research into this area is needed to say with certainty if this is true. The motifs that are phosphorylated by AGC3s and D6PKs evolved at the same time as these kinases, however PINs in early land plants have clearly more variation in the composition or occurrence of these motifs.

Comparing the variety in PINs and phosphorylation motifs from *P. patens* and *S. moellendorffii* with those of higher plants shows that with increasing developmental complexity comes an increase in PIN types, but also a higher degree of conservation. The stem eudicot *A. coerulea* has a relatively simple (but complete) set of PINs and AGC3 kinases. This set is expanded upon in the rest of the dicot phylogeny. It is likely that there

are more important phosphorylation sites in PINs than the ones described in this review, and more research into this topic will therefore certainly yield new signaling routes that alter plant growth and development by modulating PIN polarity or activity .

Short Summary of Thesis

The research presented in this thesis focuses on some of the topics discussed in this review. **Chapter 2** describes the results of a functional analysis of AGC3-4 and shows the different functionality of this kinase compared to its close homologs PID, WAG1 and WAG2. *AGC3-4* overexpression did not result in the typical root meristem collapse phenotype observed for *PID, WAG1* or *WAG2* overexpression and evidence is provided that AGC3-4's effect on PIN polairty is opposite to that of the other three AGC3 kinases. **Chapter 3** is a cell-biological analysis of the role of AGC3-mediated phosphorylation on vacuolar targeting of PIN1 and PIN2 and shows that this phosphorylation controls PM abundance as well as polarity in a phosphorylation-dose dependent manner. **Chapter 4** concerns the process of *Arabidopsis* fruit dehiscence and the role of *PID* and *PIN3* in this process. This chapter shows that variations in PIN3 plasma membrane abundance in the valve margin of developing *Arabidopsis* fruits are tightly correlated with auxin response maxima and cell-divisions, and that they time the development of the dehiscence layer. It is the transcriptional regulation of the *PID* gene by the transcription factor INDEHISCENT that controls the amount of PIN3 on the plasma membrane and thereby the auxin maxima in the valve margin. **Chapter 5** is about a group of scaffolding proteins called MAB4/MELs that are involved in PIN polarity and auxin-related developmental processes. In this chapter we show that MAB4/MELs interact with both PID, PIN1 and PIN2 and that this interaction leads to stabilization of PINs in microdomains at the PM. This stabilization is measured by a reduction in endocytosis and vacuolar targeting. **Chapter 6** describes the finding that nuclear PID can reduce vacuolar targeting of PIN1, independent of phosphorylation at the TPRXS motifs. This leads to an investigation in the nuclear role of AGC3 kinases and the interaction with and phosphorylation of the key photomorphogenesis regulators COP9-SIGNALOSOME subunit CSN8 and COP1.

Method of phylogenetic analysis

Representatives of lower non-flowering plants are the moss *Physcomitrella patens* (*Pp*) and the lycophyte *Selaginella moellendorffii* (*Sm*) (Banks et al., 2011; Rensing et al., 2008). The monocot model organisms *Brachypodium distachion* (*Bd*) and *O. sativa* (Os), basal eudicot *Aquilegia coerulea* (*Ac,* Ranunculaceae) and non rosid *Mimulus guttatus* (*Mg*) were used to provide a broad evolutionary scope overview of flowering plants (Hellsten et al.,

2013; Vogel et al., 2010). In water dwelling green algae, the only kinase that possesses AGCVIII characteristics is a *PHOT2-like* gene, and the *Volvox carteri PHOT2* was used as an example of an ancestral AGCVIII kinase. A first selection of kinase sequences was made by performing a protein-protein BLAST analysis with PID, WAG1, WAG2, AGC3-4 and D6PK protein sequences on the phytozome (phytozome.net) website and subsequently downloaded from there. Putative D6PK and AGC3 kinases were identified by constructing individual phylogenies of the sequences found with BLAST and the *Arabidopsis thaliana* AGCVIII kinases. Clades grouping with non-D6PK AGC1 kinases and with AGC2 kinases were excluded. An alignment was made using the proteins sequences from the first conserved part of the kinase domain ((K/R)PH(K/R) until the last conserved part before the C-terminal part (PPX(V/I/L). Potential PIN proteins were identified using a Protein BLAST analysis with *A. thaliana* PIN genes and selecting the candidates with conserved PIN transmembrane domains. PINs containing short hydrophilic loops were excluded from the HL motif analyses, but included into the phylogenetic analysis. For Figures 1, S1 and S2 MEGA was used to construct both alignments and cladograms (http://www.megasoftware.net/). Alignments were made with ClustalW and manual corrections, cladogram with neighbor joining and maximum likelihood phylogeny. For Figures S3 and S4 CLC-BIO genomics workbench was used to construct the alignment and cladograms (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/).

CLC-BIO Alignment parameters: Gap open cost: 5, gap extension cost 0.5, no penalty for extra gaps was applied. Standard neighbor joining algorithms combined with a maximum likelihood algorithm were used to construct the cladograms.

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Supplemental data

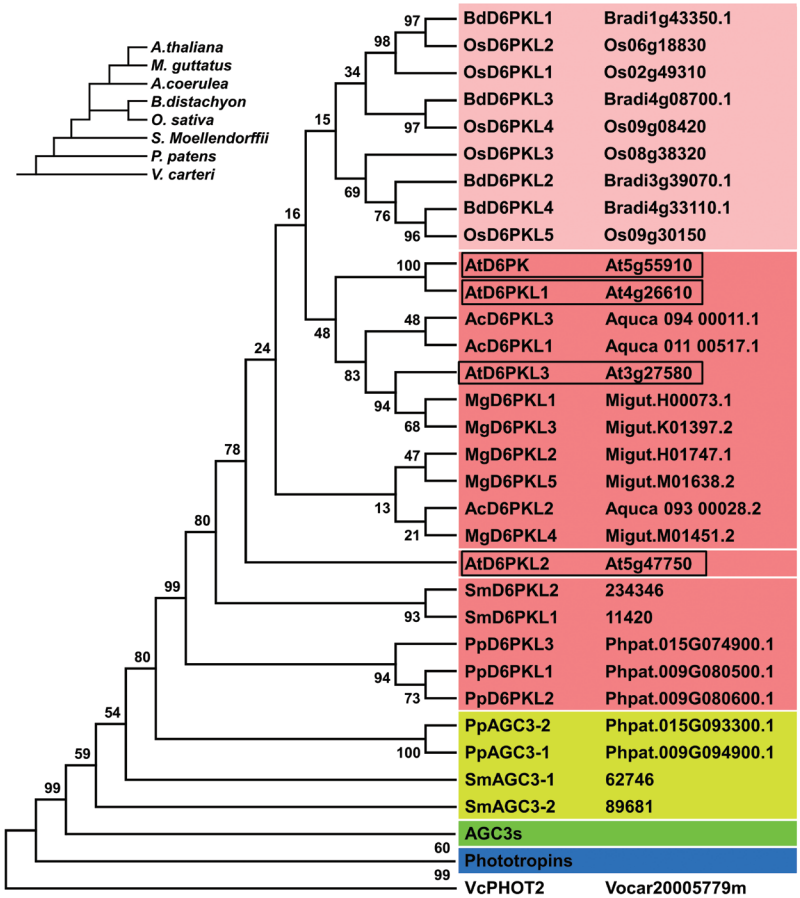

Figure S1: Phylogenetic analysis of the D6-like AGC1 kinases, continued from Fig. 1.
Maximum likelihood phylogenetic analysis of the phototropins, AGC3 and D6 kinase domains, focusing on the D6 kinases. Bootstrap values are depicted at each node (1000 repeats). The length of the horizontal branches does not represent the evolutionary distance. The phylogenetic analysis focusing on AGC3s and phototropins is shown in **Fig. 1** and **Fig. S2**, respectively.

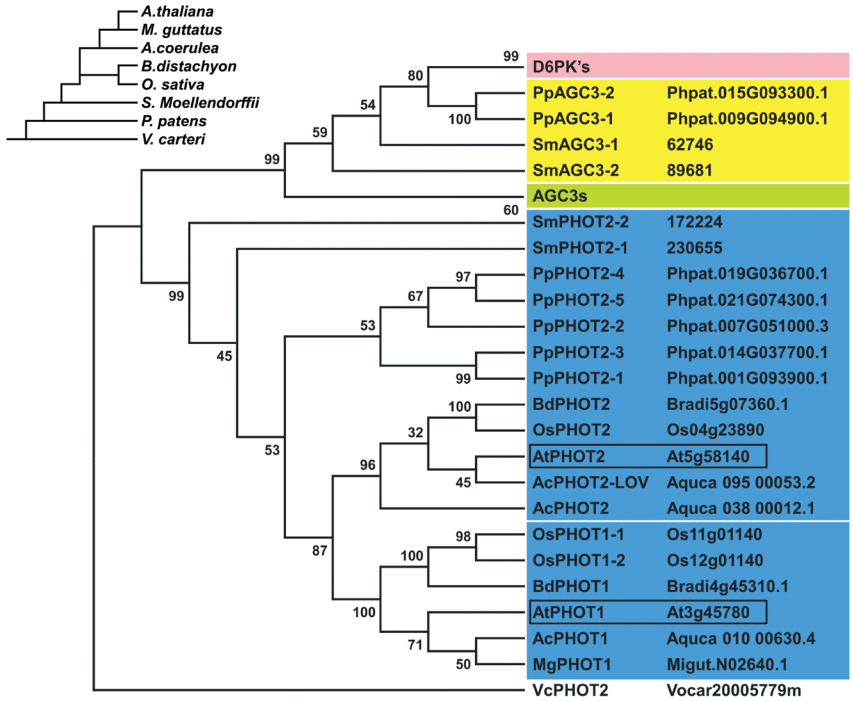

Figure S2: Phylogenetic tree of the PHOT-kinases, continued from Fig. 1.
Maximum likelihood phylogeny of the phototropins, AGC3 and D6PK kinase domains, focusing on the AGC3 kinases. Bootstrap values are depicted at each node (1000 repeats). The length of the horizontal branches does not represent the evolutionary distance. A phylogenetic analysis focusing on D6PKs and AGC3s is shown in **Fig. S1** and **Fig. 1**, respectively.

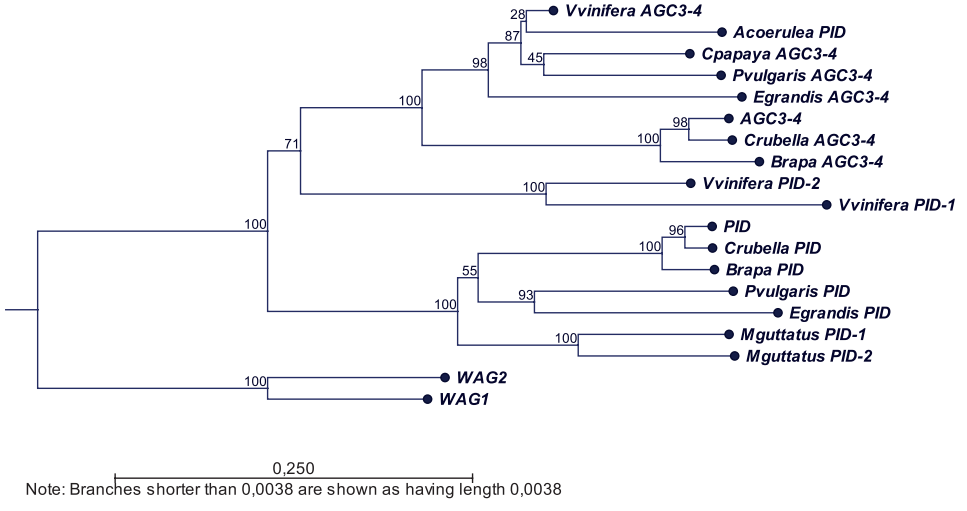

Figure S3: Phylogenetic tree showing examples of AGC3-4-type kinases found in land plants, compared to their PID-like counterparts.
Maximum likelihood phylogeny of selected AGC3 kinases obtained by BLAST algorithm using AtPID and AtAGC3-4.

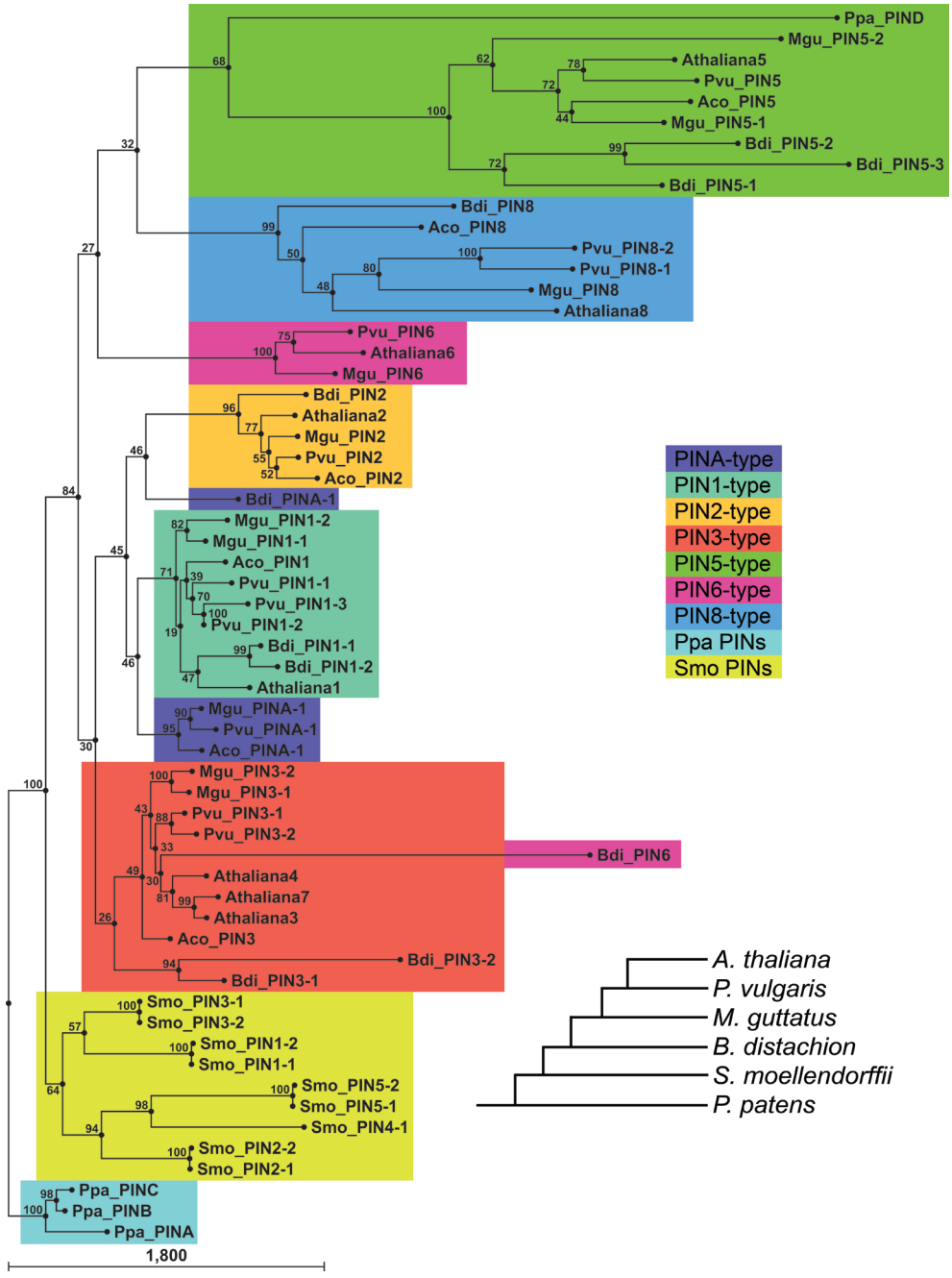

Figure S4: Phylogenetic tree of the PIN proteins.

A whole sequence alignment (supplemental files) was used to construct this tree, of which the evolutionary most ancient one (*P. patens* (Ppa) PINs) were set as an outgroup. PINs are grouped and colour-coded according to the PIN-type. The evolutionary relationships between the organisms used is depicted in the small tree in the bottom right. Bootstrap values are depicted at each node (1000 repeats) and the scale bar represents molecular distance. For a detailed analysis of *O. sativa* PINs see: Balzan et al., 2014.