Molecular mechanisms of the cytokinin-regulated endomembrane trafficking to coordinate plant organogenesis

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Abstract

Plants maintain the capacity to develop new organs e.g. lateral roots post-embryonically throughout their whole life and thereby flexibly adapt to ever-changing environmental conditions. Plant hormones auxin and cytokinin are the main regulators of the lateral root organogenesis. Additionally to their solo activities, the interaction between auxin and cytokinin plays crucial role in fine-tuning of lateral root development and growth. In particular, cytokinin modulates auxin distribution within the developing lateral root by affecting the endomembrane trafficking of auxin transporter PIN1 and promoting its vacuolar degradation (Marhavý et al., 2011, 2014). This effect is independent of transcription and translation. Therefore, it suggest novel, non-canonical cytokinin activity occuring possibly on the posttranslational level. Impact of cytokinin and other plant hormones on auxin transporters (including PIN1) on the posttranslational level is described in detail in the introduction part of this thesis in a form of a review (Semeradova et al., 2020).

To gain insights into the molecular machinery underlying cytokinin effect on the endomembrane trafficking in the plant cell, in particular on the PIN1 degradation, we conducted two large proteomic screens: 1) Identification of cytokinin binding proteins using chemical proteomics. 2) Monitoring of proteomic and phosphoproteomic changes upon cytokinin treatment. In the first screen, we identified DYNAMIN RELATED PROTEIN 2A (DRP2A). We found that DRP2A plays a role in cytokinin regulated processes during the plant growth and that cytokinin treatment promotes destabilization of DRP2A protein. However, the role of DRP2A in the PIN1 degradation remains to be elucidated. In the second screen, we found VACUOLAR PROTEIN SORTING 9A (VPS9A). VPS9a plays crutial role in plant's response to cytokin and in cytokinin mediated PIN1 degradation.

Alltogether, we identified proteins, which bind to cytokinin and proteins that in response to cytokinin exhibit significantly changed abundance or phosphorylation pattern. By combining information from these two screens, we can pave our way towards understanding of non-canonical cytokinin effects.

About the Author

Hana Semerádová completed a BSc in Molecular Biology and Biochemistry in 2013 and a MSc in Experimental Plant Biology in 2015 at the Charles University in Prague. She joined IST in September 2015. Her main research question is how hormonal regulation of the vesicular trafficking shapes the architecture of the root system in plants. In particular, she focuses on the non-canonical effects of the hormone cytokinin on protein phosphorylation. She co-authored two publications in *Nature Communications* and published a review on hormonal cross-talk in *Plant Communications*.

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List of Publications Appearing in Thesis

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All Roads Lead to Auxin: Post-translational Regulation of Auxin Transport by Multiple Hormonal Pathways

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Summary: Plant growth and development is orchestrated by complex network of mutually interacting hormonal pathways. Fine-tuning of auxin distribution by hormones appears as an important cross-talk mechanism that enables rapid adaptation of plant growth to fluctuating environmental conditions. The review summarizes recent advances in the hormonal cross-talk research with particular focus on the post-translational mechanisms of the auxin transport control.

Abstract

Auxin is a key hormonal regulator, which governs plant growth and development in concert with other hormonal pathways. The unique feature of auxin is its polar, cell-to-cell transport that leads to formation of local auxin maxima and gradients, which coordinate initiation and patterning of plant organs. The molecular machinery mediating polar auxin transport is one of the important points of interaction with other hormones. Multiple hormonal pathways converge at the regulation of auxin transport and form a regulatory network that integrates various developmental and environmental inputs to steer plant development. In our review, we discuss the most recent advances in understanding the mechanisms that underlie regulation of polar auxin transport by multiple hormonal pathways. Specifically, we focus on the post-translational mechanisms that contribute to fine-tuning of abundance and polarity of auxin transporters at the plasma membrane and thereby enable rapid modification of the auxin flow to coordinate plant growth and development.

Key words: plant hormones, polar auxin transport (PAT), post-translational regulation, trafficking, PINs, abiotic stress

Introduction

Plant hormones including auxin, cytokinin, gibberellins, jasmonates, strigolactones, salicylic acid, ethylene, brassinosteroids and abscisic acid are essential endogenous regulators involved in virtually all aspects of plant growth and development. As signaling molecules, they act at very low concentrations, and through specific signaling pathways they contribute to coordination of various processes, including embryogenesis, seed germination, primary and lateral root growth, and adaptive responses to various biotic and abiotic stresses. Regulatory input of a single hormone is a result of orchestrated activities of pathways controlling its metabolism, transport, perception and signal transduction. Interactions with other hormonal pathways represent an important additional level of control contributing to fine-tuning of the hormone activity (reviewed in Vanstraelen and Benková, 2012). Hormones interconnected through various mechanisms of cross-talk including transcriptional (Zemlyanskaya et al., 2018; Zubo and Schaller, 2020), post-transcriptional (Liu et al., 2007; Liu et al., 2009) or post-translational (Hill, 2015) regulations of gene activities fine-tune cellular responses and coordinate growth and developmental processes during plants' entire lifespan.

Amongst plant hormones, auxin stands out for its dominating function in morpho- and organogenic processes, including embryo patterning, postembryonic initiation and formation of plant organs as well as regulation of tropic responses (Adamowski and Friml, 2015). A key regulatory feature of auxin action is its graded distribution, established and tightly controlled through the polar auxin transport (PAT) machinery, consisting of auxin influx and efflux transporters such as AUX1/LIKE AUX1 (AUX1/LAX), PIN formed (PINs) and ABC/PGP families (Grebe et al., 2002; Benková et al., 2003; Adamowski and Friml, 2015; Singh et al., 2018; Sauer and Kleine-Vehn, 2019; Swarup and Bhosale, 2019). A number of studies have pointed at the PAT as an important point of convergence with other hormonal pathways (Dello Ioio et al., 2008; Shkolnik-Inbar and Bar-Zvi, 2010; Bao et al., 2004; Crawford et al., 2010). Intriguingly, besides transcriptional regulation of genes encoding components of the PAT machinery by various hormones (Vieten et al., 2005; Dello Ioio et al., 2008; Ruzicka et al., 2009; Sun et al., 2009; Lewis et al., 2011; Šimášková et al., 2015; Rowe et al., 2016), a rapid modulation of activity of auxin transporters at post-translational level appears as an alternative, highly biologically relevant mode of the hormonal cross-talk. Several plant hormones and signaling molecules such as cytokinin, gibberellin, jasmonate, salicylic acid, brassinosteroids, abscisic

acid or nitric oxide have been shown to execute part of their regulatory functions by targeting pathways mediating delivery of auxin transporters to the plasma membrane (PM), their recycling between the PM and endomembrane compartments or re-directing for lytic degradation to vacuoles. Thereby hormones can rapidly alter the rate, amount or direction of auxin transported through tissues and organs and thus to coordinate plant growth and development in ever changing environmental conditions.

In our review, we will discuss recent advances in the hormonal cross-talk research with particular focus on the post-translational mechanisms that enable rapid fine-tuning of the PAT and play a role in the regulation of plant growth and development.

Auxin gradients formed by polar auxin transport

To accomplish its regulatory functions, auxin has to be delivered from sites of its production, such as the shoot apical meristem and leaf primordia, to target tissues (Vernoux et al., 2010). While long-distance transport enables fast relocation of auxin via phloem vasculature (Friml, 2003), short-distance polar cell-to-cell transport facilitated by auxin transporters has a unique regulatory function. It contributes to the formation of local auxin maxima and gradients, which have instructive function in organ initiation, tissue patterning or tropic responses (Chandler, 2009; Vanneste and Friml, 2009; Overvoorde et al., 2010). Several gene families have been identified for their ability to transport auxin into cells (influx) and out of cells, (efflux), as well as to coordinate intracellular movement of auxin (Abualia et al., 2018). Among them AUX1/LAX influx, PIN and ABC/PGP efflux carriers are major families of transporters involved in the PAT. Their abundance at the PM, polarity and capacity to transport auxin determine rate and directionality of the intercellular auxin flow and thereby define pattern of auxin distribution (reviewed by Adamowski and Friml, 2015).

In *Arabidopsis thaliana*, influx of auxin into cells is facilitated mainly by *AUX1/LAX* transporters belonging to the auxin amino acid permease (AAAP) family of proton-driven transporters (Bennett et al., 1996). The *AUX1/LAX* family encompasses four highly homologous genes (*AUX1*, *LAX1*, *LAX2*, and *LAX3*), which encode transmembrane proteins (Carrier et al., 2008; Yang and Murphy, 2009) involved in numerous developmental processes, including embryogenesis, seed germination, vascular development, root development, leaf morphogenesis, apical hook development and many others (reviewed in Swarup and

Bhosale, 2019; Swarup and Péret, 2012). The amount and polarity of AUX1/LAX proteins at the PM is tightly controlled and thereby distribution of auxin essential for proper growth and development of plants coordinated (Swarup et al., 2004; Kleine-Vehn et al., 2006; Péret et al., 2012; Jonsson et al., 2017; Liu et al., 2017a). For example, in roots, asymmetric localization of AUX1 at the apical PM of protophloem cells facilitates flow of auxin in the acropetal (rootwards) direction, while the basal localization of AUX1 in the lateral root cap and epidermal cells drives basipetal (shootwards) stream of auxin (Swarup et al., 2001). In root columella cells, increased proportion of AUX1 in the cytosol hints at very dynamic regulation of PM targeting and turnover of AUX1. Overall, the flexible subcellular localization and polarity of AUX1 across root tissues allows rapid control of auxin flow and thereby regulation of root growth in response to gravistimulation or other environmental inputs (Swarup et al., 2001).

Two distinct classes of transporters mediate auxin efflux. The ATP-binding cassette transporter (ABCs) family are non-polar transporters uniformly distributed along the PM (reviewed in Fukui and Hayashi, 2018; Geisler et al., 2017). Although ABCB1, ABCB4 and ABCB19 have been characterized as a non-polar auxin efflux transporters, recent studies have shown that some homologs, including ABCB14 and ABCB15, might exhibit polar membrane localization and thus contribute to directionality of auxin flow (reviewed by Cho and Cho, 2013; Geisler et al., 2017). Polarly localized transporters, PINs, are components of the PAT machinery with a major impact on the directionality of auxin flow in plant tissues and organs (Okada et al., 1991; Friml et al., 2002; Benková et al., 2003). Eight members of the PIN family are transmembrane proteins localizing either to the PM (PIN1, PIN2, PIN3, PIN4 and PIN7), the ER (PIN5 and PIN8), or exhibit dual ER and the PM localization (PIN6) (Zhou and Luo, 2018). Typically, PINs located in the PM contain long hydrophilic loop, which separates multiple transmembrane domains, whereas ER-located PINs are characterized by a short hydrophilic loop. The ability of PINs to transport auxin has been demonstrated in single-cell-based plant systems (Petrášek et al., 2006; Barbez et al., 2013), but also in the heterologous systems, including mammalian cells or *Xenopus* oocytes (Petrášek et al., 2006; Zourelidou et al., 2014). Developmental and physiological roles of PINs have been widely studied and their specific functions in the regulation of various developmental process including embryogenesis,

initiation, positioning and formation of new organs as well as tropic responses have been demonstrated (Benková et al., 2003; Billou et al., 2005; Zhang et al., 2019)

Importantly, several studies suggest that PINs and ABCBs interact and function both independently and interdependently to control the PAT *in planta* (Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Titapiwatanakun et al., 2009).

Subcellular trafficking of the auxin transporters

Due to the essential impact of PIN transporters on the rate and directionality of auxin flow, the mechanisms that control and determine their localization at the PM and their transport activity has become one of the major focuses in plant cell biology. Various cell biology, genetic and molecular biology approaches have been implemented to dissect molecular pathways involved in the regulation of PIN subcellular trafficking and polarity establishment with major focus on PIN1 and PIN2. Several recent studies have demonstrated that polarity and abundance of PINs at the PM are controlled by multiple cell type and PIN protein-specific cues, and both the PM abundance and polarity of PINs can flexibly change in response to varying endogenous and environmental signals (Ganguly et al., 2012; Ganguly et al., 2014; Habets and Offringa, 2014; Zwiewka et al., 2019a).

A constant cycling of PIN1 and PIN2 between the PM and endosomal compartments has been revealed using a brefeldin A (BFA), an inhibitor of the subclass of ADP-ribosylation factor guanine-nucleotide exchange factors (ARF-GEFs), which act as essential regulators of the vesicle trafficking (Geldner et al., 2001; Geldner et al., 2003; Adamowski and Friml, 2015; Naramoto, 2017). BFA treatment leads to aggregation of endosomes as well as endosome-resident PIN proteins, forming a subcellular structure called “BFA body” or “BFA compartments” (Geldner et al., 2001; Geldner et al., 2003). The constitutive endocytosis and recycling of PIN proteins depends on a complex subcellular trafficking machinery. Genetic and pharmacological perturbations of endocytosis exhibit dramatic effects on BFA compartmentation of PIN proteins. In particular, this has been reported for the coat protein clathrin, putative clathrin uncoating factors AUXILIN-LIKEs, GNOM and other BFA sensitive ARF-GEFs, the ARF-GTPase-activating protein VASCULAR NETWORK DEFECTIVE3, and the small GTPase Rab1b (Geldner et al., 2001; Geldner et al., 2003; Kitakura et al., 2011; Feraru et al., 2012; Adamowski et al., 2018; Kania et al., 2018; Mishev et al., 2018; Dejonghe et al.,

2019). Notably, clathrin-mediated endocytosis, together with *de novo* protein synthesis, is essential for PIN2 polarity re-establishment post cytokinesis (Glanc et al., 2018). Downstream of endocytosis, the early endosomal trafficking of PINs is controlled by another ARF-GEF, the BFA-visualized endocytic trafficking defective1 (BEN1), and the Sec1/Munc18 family protein BEN2 (Tanaka et al., 2009; Tanaka et al., 2013). Moreover, membrane lipids compositions are emerging as essential regulators for PIN trafficking and polarity. For instance, PI4P 5-kinases PIP5K1 and PIP5K2, which catalyse the production of phosphatidylinositol-4,5-bisphosphate [$PI(4,5)_2$] at the PM, regulate general endocytosis process, thereby playing a major role in PIN trafficking and localization (Mei et al., 2012; Ischebeck et al., 2013; Tejos et al., 2014; Marhava et al., 2020). In addition, phosphatidylserine (PS) binds directly to ROP6 (Rho of Plants 6, a small GTPase) and regulates the dynamics of its nanoclustering at the PM, participating in endocytosis of PIN2 (Plater et al., 2019). Recently, aminophospholipid ATPase3 (ALA3), a phospholipid flippase, has been identified as a novel regulatory factor, which modulates the distribution of phospholipids at PM and together with GNOM and BIG3 ARF GEFs controls PIN trafficking and polarity (Zhang et al., 2020a).

Unlike PINs, molecular factors and pathways involved in the regulation of trafficking and polar membrane localization of AUX1/LAX are less characterized. Similarly to PINs, also AUX1 undergoes a constant and dynamic recycling from the PM through recycling endosomes, however, it utilizes a distinct GNOM independent pathway (Kleine-Vehn et al., 2006; Fan et al., 2015). Recently, using apical hook as a model system it has been shown that AUX1 trafficking to the PM is mediated by ECHIDNA, ARF1, and BIG proteins (Jonsson et al., 2017). Furthermore, a role of RopGEF1, a guanine nucleotide exchange factor and activator of Rho GTPases of plants (ROPs), and ARF-GTPase-activating proteins (GAPs) for proper trafficking of AUX1 to the PM development has been recognised (Du and Chong, 2011; Liu et al., 2017a).

Posttranslational modification of the auxin transporters

Posttranslational modifications of the auxin transporters have been recognized as an important mechanism underlying control of their polar distribution at the PM and transport activity. Several protein kinase families including AGCIII kinases, the Ca^{2+}/Calmodulin-Dependent Protein Kinase-Related Kinases (CRKs) and mitogen-activated protein (MAP)

kinases (MPKs) have been identified as an important regulators of phosphorylation status of auxin transporters (reviewed by Armengot et al., 2016; Löfke et al., 2013a; Zhou and Luo, 2018). The AGCVIII protein-serine/threonine kinases, including PINOID (PID) and closely related WAVY ROOT GROWTH 1 (WAG1) and WAG2 control phosphorylation of PIN proteins and thereby facilitate their trafficking to the specific polar membrane domains (Friml et al., 2004). In addition to PINs, ABCB1 has also been recognized among targets of PID (Henrichs et al., 2012). Another members of the AGCIII kinase family, D6 PROTEIN KINASE (D6PK) and related proteins D6PK-like (D6PKL), were demonstrated to phosphorylate PIN proteins and thereby regulate their auxin transport activity (Zourelidou et al., 2009). Regulation of PIN2 phosphorylation status by CRK5, a member of CRK family, has been found to control root gravitropic response (Rigó et al., 2013). Furthermore, several environmentally regulated mitogen-activated protein kinases including MAP kinase kinase 7 (MKK7)-MPK6 cascade and MPK4 contribute to regulation of PIN phosphorylation and thus might play a role in rapid fine-tuning of auxin transport in response to external stimuli (Dory et al., 2018).

The PID-mediated phosphorylation of PINs is counteracted by phosphatases such as PP6-type phosphatase holoenzyme complex formed by PP2AA proteins (RCN1/PP2AA1, PP2AA2, PP2AA3) and FyPP1/3, SAL (Michniewicz et al., 2007; Dai et al., 2012), as well as type-one protein phosphatase TOPP4 (Guo et al., 2015). In addition to phosphorylation, ubiquitination has been recognized as another developmentally important posttranslational modification that determines turnover of PIN2 during root gravity response (Abas et al., 2006).

Auxin feedback on its own transport

Early hypotheses and models of the PAT considered feedback of auxin on its own transport as a potential mechanism for establishment and maintenance of directionality and rate of auxin distribution (Sachs, 1969, 1975, 1981). Together these ideas merged into a canalization hypothesis that describes a fascinating ability of auxin to focus and polarize its own flux, which consequently results in vasculature formation. Later works provided important experimental support (Sauer et al., 2006; Mazur et al., 2020a; Mazur et al., 2020b), and effects of auxin flux and concentrations on localization of its own transporters and vice versa were key assumptions for mathematical models to successfully capture and simulate this process (Grieneisen et al., 2007; van Berkel et al., 2013; Bennett et al., 2014).

In agreement with feedback on its own transport, auxin has been found to transcriptionally (Vieten et al., 2005), and post-translationally regulate components of the PAT including PINs (Paciorek et al., 2005; reviewed in Doyle et al., 2015; Prát et al., 2018). A model was proposed in which auxin promotes its own polar transport by inhibiting clathrin-mediated endocytosis of PINs through a pathway mediated by Auxin Binding Protein 1 (ABP1) (Paciorek et al., 2005; Robert et al., 2010). However, the role of ABP1 as a receptor to perceive extracellular auxin levels and the exact cellular effects of NAA, a synthetic auxin analogue widely used in these works, were challenged by multiple studies, so the role of auxin feedback on PIN endocytosis is an open question (Gao et al., 2015; Jásik et al., 2016; Paponov et al., 2019). A recent study shows that auxin exhibits a dramatic effect on lipid distribution at the PM, which further stabilizes ROP6 clusters at nanodomain and inhibits PIN2 endocytosis (Platre et al., 2019). Furthermore, a similar auxin-induced clustering phenomenon was also observed for TRANSMEMBRANE KINASE 1 (TMK1) (Pan et al., 2019), a proposed auxin co-receptor which was described to form a complex with ABP1 (Xu et al., 2014). Notably, auxin-induced ROP6 clustering was blocked by *tmk1 tmk4* mutations, suggesting involvement of this receptor kinase. However, the underlying mechanism, through which auxin is perceived by TMK1 and how it regulates lipid dynamics, awaits further characterization. Intriguingly, besides inhibition of PIN endocytosis by higher concentration of auxin, reduced levels of auxin promote lytic degradation of PIN2, thus reinforcing an asymmetry of auxin distribution during root gravity response (Sieberer et al., 2000; Abas et al., 2006). Furthermore, increased accumulation of auxin at the lower side of root bending in response to gravistimulus might trigger lytic degradation of PIN2 in a $SCF^{TIR1/AFB}$ -dependent manner. The high auxin-driven lytic degradation of PIN2 takes place in the later stages of gravitropic response, and it might prevent the root from further bending (Baster et al., 2013). Importantly, these findings indicate that PIN2 resides on the PM at the auxin concentration optimum and any deviation from this optimum might lead to the PIN2 degradation and hence attenuation of auxin transport. Notably, auxin regulates PIN subcellular (re)localization through the canonical TIR1/AFB signaling pathway in distinct developmental processes, including vascular development (Prát et al., 2018; Verna et al., 2019; Mazur et al., 2020a), and hypocotyl gravitropism (Rakusová et al., 2016; Han et al., 2020), the above-mentioned studies highlight

the importance of dynamic changes of auxin fluxes and its self-regulatory abilities in regulation of various developmental processes and flexible adaptation of plant growth to environmental stimuli.

Hormonal regulation of subcellular trafficking of auxin transporters as a mechanism to control the auxin gradient formation

A number of recent studies have shown that various environmental and endogenous stimuli including plant hormones can interfere with recycling of PINs between the PMs and endomembrane compartments, or trigger their re-targeting for lytic degradation to vacuoles and thus modulate rate and directionality of auxin flow in plant tissues and organs. In the following paragraphs, we will review and discuss current insights into mechanisms that underlie these rapid modes of hormone interactions with the PAT.

Cytokinins promote lytic degradation of PINs in roots

Cytokinins are N^6 substituted adenine derivatives that jointly with auxin control basic cellular processes such as cell division, and differentiation (Skoog and Miller, 1957; Dello Ioio et al., 2008; Kieber and Schaller, 2018). Cytokinin signaling is mediated through a multistep phosphorelay pathway with histidine kinase acting as a receptor, represented in *Arabidopsis* by a small family of three histidine kinases (AHK2, AHK3 and CRE1/AHK4). Cytokinins, after binding the receptor, trigger a cascade of auto- and trans-phosphorylation events to activate signaling components, including HISTIDIN-CONTAINING PHOSPHOTRANSFER (AHP) and downstream acting type-B response regulators (type-B ARR), which trigger transcriptional responses (Keshishian and Rashotte, 2015; Osugi and Sakakibara, 2015; Kieber and Schaller, 2018). Studies focused on the cytokinin regulated plant development have revealed that a number of processes involve cytokinin interaction with the PAT (e.g., root and shoot apical meristem activity maintenance, lateral root organogenesis, vasculature differentiation or phylotaxis (Dello Ioio et al., 2008; Ruzicka et al., 2009; Zhao et al., 2010; Bishopp et al., 2011; Pernisova et al., 2016; Waldie and Leyser, 2018). Interestingly, besides transcriptional regulation of the PAT machinery components (Dello Ioio et al., 2008; Ruzicka et al., 2009; Šimášková et al., 2015; Pernisova et al., 2016; Street et al., 2016), several recent works have pointed at a post-translational control of PINs (Marhavý et al., 2011; Zhang et al., 2011; Waldie and Leyser, 2018). In roots, cytokinin has been found to interfere with endomembrane

trafficking of PIN1 and to promote its re-targeting for lytic degradation to vacuoles, thus reducing PIN1 abundance at the PM (Marhavý et al., 2011; Marhavý et al., 2014). Consistently, in the type-A *arr* mutant, which lacks multiple negative regulators of the cytokinin response, the post-translational down-regulation of several PIN proteins including PIN1 has been demonstrated (Zhang et al., 2011). The cytokinin mediated targeting of PIN1 to the vacuole is dependent on the intact actin network and regulatory components of the BFA-sensitive trafficking pathway, including BEN1/BIG5/MIN7, an ARF-GEF from the BIG subfamily, and BEN2/VPS45, a member of SEC1/MUNC18 family, both shown to be involved in control of PIN1 endocytosis (Tanaka et al., 2009; Tanaka et al., 2013). Intriguingly, cytokinin does not trigger bulk flow of proteins to vacuoles, but exhibits selectivity for proteins and their polar membrane localization. PIN1 located at the basal PM of cells in the root provasculature is more sensitive to the cytokinin-triggered lytic degradation when compared to the PIN7 homologue (also basally located), or AUX1, or PIN2 at apical PM of epidermal cells (Marhavý et al., 2011; Marhavý et al., 2014). Furthermore, reduced sensitivity of the phospho-mimetic when compared to loss of phosphorylation allele of PIN1 to cytokinin triggered lytic degradation suggests that the PIN phosphorylation status might affect responsiveness of PIN proteins to the hormone (Marhavý et al., 2014). Although the cytokinin effect on PIN1 trafficking is rapid and independent of transcription and *de novo* protein synthesis, it requires components of the canonical cytokinin signaling, including cytokinin receptor CRE1/AHK4 and some of type-B ARR (Marhavý et al., 2011). So far it is unclear, whether cytokinin through AHK4 interferes with trafficking pathway mediating PIN1 recycling to the PM and as a consequence the protein is re-directed to vacuoles or AHK4 mediated signaling targets molecular factors controlling phosphorylation of PIN1 and thereby interferes with its sorting.

A rapid fine-tuning of the PAT machinery through a post-translational regulation of its major components might be important in processes such as maintenance of root apical meristem size or lateral root organogenesis. For example, cytokinin-promoted depletion of PIN1 located at transversal membranes of cells in lateral root primordia might act as a polarizing cue that specify re-direction of auxin flow towards the tip of newly forming primordia and promote their outgrowth (Bielach et al., 2012; Marhavý et al., 2014). Cytokinins have also been found to post-translationally regulate levels of PIN proteins in shoots. However unlike in roots, cytokinins in shoots promote accumulation of PIN3, PIN4 and PIN7 at the PM, thereby

coordinating bud outgrowth and branching (Waldie and Leyser, 2018). Collectively, these studies suggest that cytokinins might regulate trafficking of PINs in a developmental context-dependent manner and thus contribute to regulation of various plant organogenic processes.

Ethylene acts through AUX1 trafficking in apical hook

Ethylene is a gaseous hormone known to regulate various plant growth and developmental processes, in particular fruit ripening, organ abscission, senescence and adaptive responses to biotic and abiotic stresses (Bleecker and Kende, 2000; Dubois et al., 2018). Ethylene is perceived by a group of partially redundant receptors, ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE INSENSITIVE4 (EIN4), which show similarity to bacterial two-component histidine kinases (Hua and Meyerowitz, 1998; Hall et al., 2007). Ethylene-bound receptors inhibit CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) kinase activity towards EIN2. As a result, the C-terminal part of EIN2 is cleaved and translocated to the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). There, it stabilizes ETHYLENE INSENSITIVE 3 (EIN3) and presumably other transcription factors, which initiate ethylene response (Chao et al., 1997; Alonso and Ecker, 2001; Guo and Ecker, 2003; Stepanova and Alonso, 2009).

In roots, ethylene has been found to modulate expression of several components of the PAT machinery, including AUX1, and several members of the PIN family (Růžička et al., 2007; Lewis et al., 2011; Méndez-Bravo et al., 2019). Furthermore, several studies highlighted a role of ethylene-mediated regulation of PAT in apical hook development by transcriptional regulation of genes encoding for auxin transporters (Vandenbussche et al., 2010; Žádníkova et al., 2010; Žádníková et al., 2016). Interestingly, fluorescence recovery after photobleaching (FRAP) analysis of the AUX1-YFP revealed faster fluorescence recovery in cells at the inner side of the apical hook formed in the presence of ethylene. These results suggest that ethylene-regulated turnover of AUX1 might be part of a mechanism that coordinates the apical hook development (Vandenbussche et al., 2010). Recently, Jonsson et al. (2017) has provided important molecular insights into the pathway controlling trafficking of AUX1 during the apical hook development. Applying genetic and cell biological approaches, the role of the ADP-ribosylation factor1 (ARF1)-GTPase and its activators ARF-guanine-exchange factors (GEFs) of the Brefeldin A-inhibited GEF (BIG) family in the secretion of the AUX1 influx carrier

to the plasma membrane from the TGN has been demonstrated. Defects in BIG or ARF1 severely affected sensitivity of the apical hook to ethylene (Jonsson et al., 2017).

Jasmonates (JAs) affect membrane localization and trafficking of PINs

Jasmonates (JAs), including Jasmonic acid (JA) and its derivatives e.g. methylester jasmonate (MeJA), are group of lipid-derived plant hormones. They play an active role in the plant interaction with the environment, particularly in responses to abiotic and biotic stresses, as well as in regulation of various developmental processes (reviewed in Ahmad et al., 2016; Dar et al., 2015; Wasternack and Song, 2017). JA signaling is mediated through CORONATINE INSENSITIVE 1 (COI1) receptor, an F-box protein, component of a SCF^{COI1}E3 ubiquitin ligase complex. In presence of JA, the receptor promotes ubiquitination and proteosomal degradation of transcriptional repressors, thereby activating transcription of JAs-responsive genes (reviewed in Wasternack and Song, 2017). Although cross-talk of JAs with other hormones has been primarily linked with salicylic acid in plant responses to pathogen attack (reviewed in Thaler et al., 2012), a number of recent studies demonstrate an active interaction of JAs with auxin pathway and the PAT.

MeJA promotes biosynthesis of auxin through stimulation of the expression of *ANTHRANILATE SYNTHASE α1* (*ASA1*), encoding a rate-limiting enzyme in biosynthesis of the auxin precursor tryptophan (Trp) (Sun et al., 2009). In addition to the role in the fine-tuning of endogenous levels of auxin, MeJA has been found to modulate the subcellular trafficking and the PM localization of PIN2 in a concentration-dependent manner (Sun et al., 2011). Whereas low levels of MeJA attenuate accumulation of PIN2 in BFA-induced endomembrane compartments, indicating that JAs interfere with PIN2 endocytosis, higher MeJA concentrations reduce abundance of PIN2 at the PM. Although distinct, both high and low concentration-dependent effects of MeJA on PIN2 require functional jasmonate receptor COI1 (Yan et al., 2009; Sun et al., 2011).

The inhibitory effect of low MeJA on PIN2 endocytosis is dramatically attenuated in *asa1* mutant when compared to wild-type control, and is fully recovered by exogenous auxin application. This suggests that MeJA at low concentrations through transcriptional activation of the *ASA1* gene stimulates biosynthesis of auxin, which in turn might inhibit PIN2 endocytosis. This is in line with study of Paciorek et al., 2005 demonstrating the auxin

inhibitory effect on PIN endocytosis. On the contrary, the depletion of PIN2 at the PM triggered by high concentrations of MeJA is enhanced in *asa1* background. As at high MeJA levels no dramatic alterations of the *PIN2* transcription can be detected, posttranslational regulations have been hypothesized to underlie these MeJA effects on PIN2. In addition to PIN2, MeJA promoted a weak depletion of PIN1, but not AUX1, which points at a selectivity of MeJA towards certain cargo and/or sorting pathway (Sun et al., 2011).

A reduced gravity response observed in roots treated with high concentrations of MeJA suggests that the hormone, through modulation of the PIN2 trafficking, might contribute to fine-tuning of the auxin flow and thereby to steer bending of root. Despite both JA and auxin receptors are involved in the JA regulated PIN2 subcellular trafficking, an underlying molecular mechanism does not require *de novo* protein synthesis. Hence, the nature of this non-conventional receptor mediated signaling, that acts independently of transcription remains to be dissected (Sun et al., 2011).

Salicylic Acid (SA) interferes with endocytosis

Salicylic acid (SA) is a phenolic signaling compound coordinating plant responses to pathogens, as well as many physiological and developmental aspects of plant life (reviewed in Khan et al., 2015; Rivas-San Vicente and Plasencia, 2011). SA signaling acts through a set of NPR (NONEXPRESSER OF PATHOGENESIS RELATED GENES) receptors, which regulate the expression of pathogenesis-related genes and other targets upon SA binding (Cao et al., 1994; Fu et al., 2012; Ding et al., 2018).

The canonical SA signaling cascade steers plant processes via specific transcriptional output, albeit a number of observations have pointed at a role of SA in the regulation of clathrin-mediated endocytosis from the PM (Du et al., 2013; Rong et al., 2016; Wang et al., 2016). For example, exogenous application of SA interfered with an uptake of the endomembrane marker FM4-64 and negatively affected incidence of clathrin light and heavy chains and ADAPTOR PROTEIN2 (AP-2) at the PM (Du et al., 2013; Wang et al., 2016). Furthermore, the accumulation of an early endosomes/TGN markers ARF-1 and VHAa1 in BFA bodies remained unaffected, which supported a conclusion that SA suppresses endocytosis of proteins from the PM rather than interfering with exocytosis or endosomal dynamics. Intriguingly, the SA modulation of the endomembrane trafficking was found to be

independent of the NPR-mediated transduction cascade (Du et al., 2013; Rong et al., 2016), suggesting the existence of a novel SA regulatory pathway. In line with the SA effects on endocytic machinery, PIN proteins have been found to sensitively react to alterations in SA concentrations. In particular, the internalization of PIN1 and PIN2 in BFA-endosomal compartments was severely attenuated in roots of the *cpr1* and *cpr5* mutants (CONSTITUTIVE EXPRESSOR OF PATHOGENESIS RELATED GENES; Bowling et al., 1997, 1994) with high endogenous levels of SA. Consistently, exogenous application of SA attenuated accumulation of PIN proteins in BFA bodies, suggesting that part of SA regulatory effects on plant growth might involve modulation of the PAT.

Recently, important molecular insights into mechanisms underlying SA mediated regulation of the PAT has been revealed (Tan et al., 2020). SA through direct biding attenuates activity of the PP2A, the phosphatase involved in de-phosphorylation of PIN (Michniewicz et al., 2007), and thereby enhances phosphorylation of PIN2. Consequently, hyperphosphorylation of PIN2 after prolonged SA treatment results in increased internalization and reduced polar membrane localisation of the auxin transporter (Tan et al., 2020). All together, these findings suggest that along with driving the response to pathogens, SA may be able to steer plant growth by targeting the PAT.

Strigolactones (SLs) promote PIN depletion from the PM in shoot

Strigolactones (SLs) are class of carotenoid-derived plant hormones with a special importance for shoot branching (Brewer et al., 2013; Lumba et al., 2017). Strigolactones are recognized by the D14 receptor, which, after hormone binding, triggers MAX2-dependent degradation of a small family of HSP101-like proteins (in Arabidopsis SMXL6, SMXL7 and SMXL8) and activate downstream responses (Stirnberg et al., 2002; Stirnberg et al., 2007; Jiang et al., 2013; Zhou et al., 2013; Soundappan et al., 2015). Regulation of the shoot branching by SLs is tightly linked with auxin and an inhibitory effect of the auxin transport from shoot to root on the outgrowth of shoot branches. It has been proposed that the auxin moving in the main stem indirectly prevents bud activity by reducing ability of the axillary buds to establish their own flow of auxin connected with the main auxin stream in the stem (reviewed in Leyser, 2009). The interaction of SLs with the PAT has been recognized as one of the important mechanisms underlying SLs-regulated shoot branching. GR24, a synthetic SL,

has been found to induce a rapid depletion of PIN1 from the PM by stimulation of its endocytosis (Shinohara et al., 2013). This SLs-triggered reduction of the PIN1 abundance at the PM is not affected by cycloheximide, an inhibitor of proteosynthesis, but is sensitive to A23, an inhibitor of clathrin mediated endocytosis, indicating that a posttranslational clathrin-dependent mechanism might be involved in the SLs regulated PIN1 trafficking (Shinohara et al., 2013). Loss of the MAX2 function interfered with the SL effect on the PIN1 endocytosis, pointing to the importance of the SL signaling in this process. The PM localization of another membrane protein, aquaporin PIP1, is not affected by SLs, suggesting a protein specificity of the SLs-action. Based on these observations, it has been proposed that SLs regulation of the PAT interferes with establishment of the canalized auxin flow from buds into the main stem and, as a consequence, branching is reduced (Shinohara et al., 2013). Originally PIN1 dependent transport of auxin has been primarily associated with SLs-regulated shoot branching. Currently more complex model of the SLs-PAT cross-talk has been proposed, which, in addition to PIN1-mediated high-conductance polar auxin transport, recognizes contribution of the connective less polar auxin transport controlled by PIN3, PIN4 and PIN7 in this developmental process (Bennett et al., 2016). However, whether subcellular trafficking of PIN3, PIN4 and PIN7 is also regulated by SLs, similarly to PIN1, remains to be further studied.

Gibberellic Acid (GA) promotes PM localization of PINs

Gibberellic acid (GA) is well-established endogenous regulator of various developmental processes, including seed germination, dormancy, flower development, and elongation growth of plant organs (Ueguchi-Tanaka et al., 2007; Hedden and Sponsel, 2015). GA signal is perceived by a soluble nuclear protein GID1, which, in the presence of GA, binds the DELLA transcriptional repressors and targets them to the proteasome for degradation. As a result, expression of GA-responsive genes is activated (Achard and Genschik, 2009; Daviere and Achard, 2013). GA and auxin pathways are intertwined at many levels. Auxin promotes GA biosynthesis and signaling responses (Fu and Harberd, 2003; Weiss and Ori, 2007), whereas GA modulates accumulation of PINs at the PM, thereby fine-tuning the PAT (Willige et al., 2011; Löfke et al., 2013b). The role of GA interaction with the PIN-mediated auxin transport has been demonstrated in regulation of the root response to gravistimulation. GAs,

similarly to auxin, accumulate asymmetrically at the lower side of gravi-stimulated roots (Löfke et al., 2013b). The local, gravi-response driven formation of the GAs maximum in epidermal cells coincides with increased abundance of PIN2 at the PM, whereas reduced levels of GA at the opposite side of the roots correlate with a lower amount of PIN2 at the PM and enhanced vacuolar degradation (Löfke et al., 2013b). A recent study addressing mechanisms underlying the interaction between GA and PIN2-dependent auxin transport revealed that GAs coordinate subcellular trafficking of the PM proteins (including PINs) in a concentration-dependent manner (Salanenka et al., 2018). Whereas at low concentrations, GAs promote vacuolar delivery and lytic degradation of multiple cargos, including PIN proteins, high concentrations of GA enhance their recycling to the PM. Hence, GA might act as a hormonal modulator of balance between vacuolar trafficking and exocytosis. Albeit a role of DELLA signaling pathway repressors in the GA regulated PIN trafficking has been detected, protein biosynthesis is not required, hinting at a posttranslational nature of mechanism underlying this GAs activity. Further cell biology and genetic approaches have pointed at several molecular factors involved in the GA-mediated regulation of PIN2 trafficking. This included microtubule (MT) cytoskeleton, components of the retromer complex such as Sorting Nexin 1 (SNX1) and a microtubule (MT)-associated protein (the Cytoplasmic Linker-Associated Protein (CLASP), which has been proposed to control tethering of endosomal vesicles to MTs via direct interaction with SNX1 (Ambrose et al., 2013). In light of these findings an alternative mechanism assuming involvement of the tubulin-folding factors Prefoldins (PFDs) has been proposed. In non-plant organisms PFDs can control MT folding and dynamics (Le Bot et al., 2003; Lundin et al., 2008) while in plants their interaction with DELLAs has been detected (Locascio et al., 2013). The PDF function in DELLA mediated regulation of MTs and subcellular trafficking is supported by observation of *pfd* mutants, which were found to be insensitive to GA and to exhibit reduced abundance of PIN2 at the PM (Salanenka et al., 2018).

Hence, in addition to the canonical GA transduction cascade that coordinates plant growth through transcriptional regulation of target genes, a novel, non-transcriptional regulatory pathway mediating GA signal has been identified. Through this pathway GAs can rapidly modulate the final destiny of PIN2, but presumably also other PM proteins, either to be recycled to the PM or to be degraded in the vacuole. So far this mode of GAs interaction

with the PAT has been mainly implicated in regulation of the root gravity response, although future research might provide further insights into role of this cross-talk in other developmental processes.

Brassinosteroids interfere with PIN degradation

Brassinosteroids (BR) are a class of steroidal plant hormones that play role in a broad spectrum of growth and developmental processes, such as cell division and elongation, vascular-differentiation, root development, regulation of flowering and in plant adaptation to biotic and abiotic stresses (Fridman and Savaldi-Goldstein, 2013; Wei and Li, 2016). BRs perception is driven by leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) which, together with its co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), initiates a signaling phosphorylation cascade. The BR signal leads to a proteasomal degradation of the main inhibitor of the pathway, a kinase BRASSINOSTEROID INSENSITIVE2 (BIN2). Concomitantly, BIN2 interacting partners, transcription factors BRASSINAZOLE RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1)/BZR2, are dephosphorylated and translocated to the nucleus, where they regulate expression of BR-responsive genes (reviewed in Clouse, 2011).

The cross-talk between BR and auxin at the level of hormone metabolism and signaling has been described (Peres et al., 2019). Several studies have reported BR effects on the PIN dependent PAT, and indicated that some of the BR mediated regulations might occur at the posttranslational level (Hacham et al., 2012; Keicher et al., 2017). For example, levels of PIN2 proteins were significantly reduced in *bri1* receptor mutant, or after treatment with the inhibitor of BR biosynthesis (BRZ), although the corresponding changes at the transcript level could not be consistently detected (Hacham et al., 2012). Furthermore, studies on 14-3-3 proteins linked with regulation of BR signaling supported a role of BR pathway in the regulation of PIN trafficking. Five of twelve isoforms of 14-3-3 including 14-3-3ε (epsilon) members were identified as BZR1 interacting partners in a yeast-two hybrid screen (Gampala et al., 2007), and the function of several of the identified isomers as negative regulators of the BR signaling cascade has been demonstrated. In absence of BRs, 14-3-3 proteins interact with the phosphorylated form of BZR1, thus preventing their translocation to the nucleus, which is required for activation of downstream transcriptional responses (Gampala et al.,

2007). Intriguingly, the interference with the expression of *14-3-3* genes from the ε group resulted in auxin-related phenotypes, such as absence of lateral roots, wavy main root and inability to form an apical hook. On the cellular level, the downregulation of the 14-3-3 activity affected expression of PIN1 and PIN2. Reduced expression of the 14-3-3 correlated with ectopic expression of PIN1 and PIN2 at the root tip and enhanced accumulation of both PIN1 and PIN2 at the lateral PM of endodermal and cortex cells. This indicated that activity of the 14-3-3 of ε subgroup might be involved in the regulation of PIN trafficking, which was further supported by monitoring of endomembrane trafficking in roots with attenuated activity of the 14-3-3. The absence of the 14-3-3 ε group members interfered with two trafficking pathways – from the TGN to vacuoles and to the PM, thus causing a higher accumulation of PIN2 in endosomal vesicles (Keicher et al., 2017). However, although plausible, there is not yet evidence that 14-3-3ε proteins regulate subcellular trafficking through their interaction with BR signaling pathway, and therefore other BR-independent mechanisms cannot be fully ruled out.

In the recent study aiming at identification of signals and mechanisms controlling subcellular trafficking and abundance of PIN2, BR was recognized as a strong hormonal antagonist of the endocytic sorting of PIN2 destined for degradation (Retzer et al., 2019). BR through canonical brassinosteroid signaling pathway, but independently of *de novo* protein synthesis, interfered with endocytosis and targeting of ubiquitinated PIN2 to vacuoles. Retzer et al., (2019) propose that the BR effect on PIN2 sorting might play a role during root response to gravity. Experimental data reveal correlation between asymmetric BR signaling, with maximum at lower side of gravi-stimulated roots, accumulation of PIN2 at the PM and BR effect on root gravity response. Together with mathematical modeling these findings point at a role of BR as a hormonal factor that in concert with auxin determines the rate of gravity-induced root curvature.

Also study focused on the BR role in regulation of the root gravity response supported posttranslational mechanism of BR cross-talk with the PAT machinery. Li et al. (2005) found a correlation between expansion of the PIN2 domain into the proximal root elongation zone and accumulation of ROP2, a member of the RAC/ROP GTPase family involved in the cortical F-actin formation (Fu et al., 2002). Overexpression of ROP2 as well as its dominant/negative forms affected both PIN2 expression and root gravity response. Based on these observations,

a model for the BR regulated PAT has been proposed, in which ROP2 stimulated by BR modulates the localization of PIN2 through the assembly/reassembly of F-actins, and thereby mediates the BR effect of root gravity response (Li et al., 2005).

Abscisic acid (ABA) attenuates PIN endocytosis

Abscisic acid (ABA) is a plant hormone primarily involved in the regulation of plant adaptive responses to various types of abiotic stresses. It acts as an endogenous regulator of stomatal guard cell closure during drought stress, increases heat tolerance through facilitated accumulation of osmo-protectant solutes and mediates adaptation of root system to salt or drought stresses. In addition to these stress tolerance roles, it controls early phases of seed maturation and germination (reviewed in Cutler et al., 2010; Finkelstein, 2013; Moriwaki et al., 2013). ABA is perceived by intracellular PYR/PYL/RCAR ABA receptors (PYLs). The hormone binding promotes receptor interaction with type 2C protein phosphatases (PP2Cs), and thus prevents dephosphorylation of class III SNF-1-related protein kinase 2 (SnRK2s). The released SnRK2s through phosphorylation of downstream signaling components, including basic leucine zipper (bZIP) transcription factors (AREBs/ABFs) and S-type anion channels (e.g. slow anion channel 1, SLAC1) induce ABA responses (Fujii et al., 2009; Geiger et al., 2009; Umezawa et al., 2009; Melcher et al., 2010; Brandt et al., 2012; Finkelstein, 2013).

Besides well-established cross-talk of ABA with gibberellic acid (GA) in regulation of seed development, root growth and adaptation to abiotic stresses (Liu and Hou, 2018), interaction of ABA with auxin pathway through regulation of the PAT has been revealed (Xu et al., 2013). Exogenous ABA treatment and salt stress or osmotic stress, which are typically associated with an increase of endogenous ABA levels, upregulate levels of PIN2 but reduce AUX1, PIN1, and PIN4 (Rowe et al., 2016).

Several studies focused on the mechanism mediating the ABA effects on the primary root growth and branching showed that ABA interacts with pathways controlling subcellular trafficking of PIN proteins and their abundance at the PM (Yang et al., 2014; Zhu et al., 2019). The ABA has been found to decrease accumulation of PIN2 in BFA bodies and to attenuate re-targeting of PIN2 for lytic degradation to vacuoles in epidermal cells at the upper side of roots after the gravi-stimulation. The ABA mediated regulation of PROTEIN PHOSPHATASE 2A

(PP2A) activity and thereby phosphorylation status of PIN2 was found to underlie the effects of ABA on PIN2 trafficking (Michniewicz et al., 2007; Li et al., 2020).

Potential regulatory component of ABA sensitive PIN trafficking has been identified by profiling of ABA responsive transcriptome. *HEATSHOCK PROTEIN 22* (*sHSP22*) emerged as a gene whose expression is regulated by both ABA and auxin (Li et al., 2018). Interestingly, the induction of *sHSP22* expression by auxin is dependent on ABI1, a key component of the ABA signal transduction pathway, hinting at cross-talk between auxin and ABA signaling. Importantly, overexpression of sHSP22 decreases the levels of PIN1 and other homologous proteins (including PIN3, PIN4, and PIN7) in a transcription-independent manner. Reduction of PIN1 at the PM in *sHSP22ox* line correlates with its rapid accumulation in BFA bodies, suggesting that sHSP22 might affect subcellular trafficking of PIN1.

In maize (*Zea mays*), increased levels of ABA or salt stress also led to alterations in the accumulation and polar localization of ZmPIN1 in lateral root primordia (Lu et al., 2019). The observed changes in ZmPIN1 localization correlated with defects in auxin distribution and severe defects in lateral root primordia growth. However, whether it is regulation on transcription level or an interference of ABA with trafficking of the maize PIN1 remains to be elucidated.

In addition, several recent studies with a focus on the ABA signaling have provided important hints on potential mechanisms mediating the ABA effect on the PIN subcellular trafficking. For example, ABA has been found to enhance degradation of Rop GEF1 and 2, which act as an upstream regulators of Rop GTPases including ROP2 and ROP6 (Zhao et al., 2015; Li et al., 2016). Intriguingly, ROP2 as well as ROP6 are implicated in establishment of PIN1 and PIN2 polarity through control of cytoskeleton dynamics (Chen et al., 2012; Nagawa et al., 2012). Furthermore, a recently reported effect of ABA on exocyst offers a viable scenario of mechanism behind the ABA controlled trafficking of PIN (Drdová et al., 2013; Seo et al., 2016). Although plausible, whether and how the outlined pathways mediate the ABA effect on PIN subcellular trafficking awaits further experimental work.

Nitric Oxide (NO) affects PIN internalization

Nitric Oxide (NO) is a small gaseous molecule acting as a key signaling molecule with a wide range of biological functions across kingdoms (Wendehenne et al., 2004). In plants,

NO participates in regulation of stomata closure, cell death, and root gravitropism, as well as adaptive responses to various biotic and abiotic stresses (Durner et al., 1998; Neill et al., 2002; Neill et al., 2003; Romero-Puertas et al., 2004; Hu et al., 2005; Ye et al., 2012; Begara-Morales et al., 2019; Sánchez-Vicente et al., 2019). At the molecular level, NO regulates biological processes through S-nitrosylation, a posttranslational modification of proteins analogous to phosphorylation (Hess et al., 2005). S-nitrosylation impacts the conformation, activity or localization of the target proteins. The level of protein S-nitrosylation is dynamic and governed by NO cellular levels and de-nitrosylation catalyzed by S-nitrosoglutathione reductase (GSNOR) (Liu et al., 2001; Feechan et al., 2005) and thioredoxin (Benhar et al., 2009; Tada et al., 2009; Sengupta and Holmgren, 2012). GSNOR is the key enzyme controlling S-nitrosoglutathione (GSNO) levels, and loss of its function leads to increased cellular levels of S-nitrosylated proteins (Liu et al., 2001; Liu et al., 2004; Feechan et al., 2005).

A number of studies in plants have demonstrated that NO-regulated processes might involve interaction with auxin signaling and the PAT. An increase of NO either by exogenous NO donor treatment or in an NO-overproducing mutant (*nox1*) (He et al., 2004) results in decreased PIN1-GFP signal (Fernández-Marcos et al., 2011). Likewise, in mutants lacking GSNOR1 the levels of endogenous PIN1, PIN2 and their homologues PIN3, PIN4 and PIN7 were significantly reduced when compared to wild-type. While no corresponding changes in transcription of *PIN* genes could be detected, it has been proposed that NO might affect PINs at the posttranslational level (Shi et al., 2015). This notion has been further supported by monitoring of PINs subcellular trafficking in plants with altered levels of NO. Ni et al. (2017) used the vesicle trafficking inhibitor BFA to demonstrate effects of NO on internalization of PIN2.

The NO-mediated regulation of PIN2 trafficking appears to play an important regulatory role in root response to gravity. Monitoring of NO in gravistimulated roots revealed asymmetric distribution and accumulation of this signaling molecule in epidermal cells at the lower side of roots. Importantly, overall reduction of NO levels in roots using the NO scavenger cPTIO attenuated asymmetric distribution of both NO and PIN2. Consequently, roots with reduced levels of NO exhibited defects in responses to gravistimulation (París et al., 2018).

Altogether these studies demonstrate that regulation of the PAT through modulation of PIN trafficking might be an important part of mechanisms underlying NO action in plants. However, detailed molecular mechanisms need to be further investigated.

Modulation of polar auxin transport in response to environmental stresses

Over the last decades, it has become evident that hormones have an important regulatory role in plant adaptation and defence mechanisms and act as internal mediators of the interaction between plants and their surrounding environment. Auxin and the PAT play a major role in plant adaptive responses to environmental stresses as key factors in regulation of growth and development.

Genome-wide analyses of transcriptomes performed in rice (*Oryza sativa*) and maize (*Zea mais*) after various abiotic stresses like drought, salt, and cold revealed alterations in expression of major components of the PAT including PIN, PILS, LAX and ABCB auxin transporters (Yue et al., 2015; Chai and Subudhi, 2016).

In addition to transcriptional regulation of individual auxin transporters in plants exposed to abiotic stresses, several recent studies pointed at impacts of abiotic stress on the subcellular trafficking and accumulation of auxin transporters at the PM. Rapid modulation of the PAT has a significant impact on direction and amount of auxin distributed in plant tissues and consequently on flexible adaptation of plant growth and development to stress. A typical example of such an adaptive response is a rapid bending of roots away from high-salt containing environments, which is known as a halotropism (Rosquete and Kleine-Vehn, 2013). The halotropic bending of roots is a result of tuning the PAT that leads to asymmetric redistribution of auxin at the root tip (Galvan-Ampudia et al., 2013; van den Berg et al., 2016; Korver et al., 2020). It has been shown that on the side of root that faces a high-salinity environment, clathrin mediated endocytosis (CME) of PIN2 is increased. Consequently, as result of the reduced amount of PIN2 at the PM in epidermal cells at the salt exposed side of the root, auxin at the root tip is asymmetrically redistributed, which steers the root away from the salty surroundings (Galvan-Ampudia et al., 2013). Phospholipases PLDs and phosphatidic acid (PA), a signaling molecule formed by the action of PLD, have been identified as important molecular players in regulation of auxin transport mediated through AUX1 and PIN2 in response to salt stress (Li and Xue, 2007; Testerink and Munnik, 2011; Galvan-Ampudia et al.,

2013; Korver et al., 2020). Salt-induced stimulation of PLD activity increases the clathrin-mediated endocytosis of PIN2 at the side of the root facing the higher salt concentration, suggesting that PA controls the polar distribution of PIN and auxin polarity during halotropism in plants (Galvan-Ampudia et al., 2013). Several lines of genetic and biochemical evidences suggested that PLD-derived PA might be involved in the PAT through regulation of PIN phosphorylation. Interaction of PA with PINOID and D6PK, kinases from the AGCVIII family that control PIN phosphorylation status (Barbosa et al., 2018), provides a possible link between lipid responses and the PAT (Zegzouti et al., 2006; Barbosa et al., 2016; Simon et al., 2016; Wang et al., 2019). Interestingly, RCN1, one of the PP2A regulatory subunits that is required for dephosphorylation and proper targeting of PIN2 (Michniewicz et al., 2007) was also identified in a screen for PA-binding proteins (Testerink et al., 2004) and hints at other possible mechanism underlying adjustment of the PAT to stresses.

An excessive accumulation of metals in soil also poses a challenge for plant growth and development. High amounts of metals such as cadmium, coper or iron were found to affect transcription of the PAT components (Hu et al., 2013; Yuan et al., 2013; Li et al., 2015). It is noteworthy that in roots exposed to high levels of nickel, a rapid decrease in PIN2-GFP signal was not accompanied with a concordant drop in gene transcription. Under high-nickel stress, PIN2 exhibited less pronounced polar localization at the PM and increased accumulation inside of the epidermal cells. Changes in PIN2 subcellular localisation correlated with root growth defects and attenuated response to gravity stimulus (Lešková et al., 2020).

The PAT is also affected in roots exposed to cold stress. Detailed analyses revealed that cold stress dramatically decreases amount of PIN2 recycling from the PM into BFA bodies and attenuates PIN3 re-localization to the lower side of columella cells upon gravitropic stimulus. The observed changes in subcellular trafficking of PIN2 and PIN3 correlate with attenuated root response to gravistimulation in cold treated plants (Shibasaki et al., 2009).

The studies discussed above convincingly demonstrate that plant adaptation to various stresses might rely also on rapid adjustment of the PAT. However, underlying molecular pathways, including perception and transduction of the signals to adequate responses, await further investigation. Factors such as Ca^{2+} and reactive oxygen species (ROS) as well as hormonal pathways including ABA, ethylene and JA need to be integrated to obtain a full picture of dynamic regulation of the PAT in plants challenged by stresses (Vanneste and

Friml, 2013; Julkowska and Testerink, 2015; Tognetti et al., 2017; Li et al., 2019; Zwiewka et al., 2019b; Zhang et al., 2020b).

Conclusion and perspectives

The PAT is one of the core mechanisms determining auxin distribution and formation of auxin gradients that have instructive functions in plant morpho- and organogenesis. In the course of a plant's lifespan, whether as part of the developmental program or in response to environmental factors, a rate, a capacity and a directionality of auxin flux can be rapidly modulated, thereby allowing for flexible developmental adaptations. Hormones act as essential endogenous translators of these developmental and exogenous signals, and their interaction with the PAT might have evolved as an effective feedback mechanism to fine-tune growth and developmental processes. Albeit transcriptional regulation of the PAT components is an efficient way to adjust the rate and amount of auxin transported in tissues and organs, the non-transcriptional mechanisms that target trafficking, turnover or polarity of auxin transporters provide another regulatory level that additionally enables rapid modulation of the auxin flow directionality.

Nearly all classes of hormones have been demonstrated to impinge on the PAT; however, investigation of the underlying molecular pathways is still only beginning. In light of recent findings, there are some aspects of hormone - PAT interactions that deserve to be highlighted, and potentially taken into consideration in the future studies (Table 1). Hormonal effects on PAT exhibit striking differences in terms of protein specificity. Some, such as GA and SA, interfere with a subcellular transport of a larger spectrum of plasma membrane proteins, hinting at their interaction with more generic regulators of protein sorting machinery. Others, including CK, JA or SL, exhibit a higher level of selectivity, presumably as a result of their impact on specialized pathways or steps in sorting of specific proteins.

Individual hormones seem to target distinct steps of subcellular trafficking, often depending on their concentrations. For example, auxin at high concentration and SA attenuate PIN endocytosis, thereby promoting their accumulation at the PM. Conversely, SLs deplete PIN1 from the PM, by promotion its endocytosis. In addition, auxin, low concentrations of GA, as well as CK re-direct some PIN family members for degradation to vacuoles, while in contrast, BR blocks vacuolar sorting of PIN2 (Figure 1).

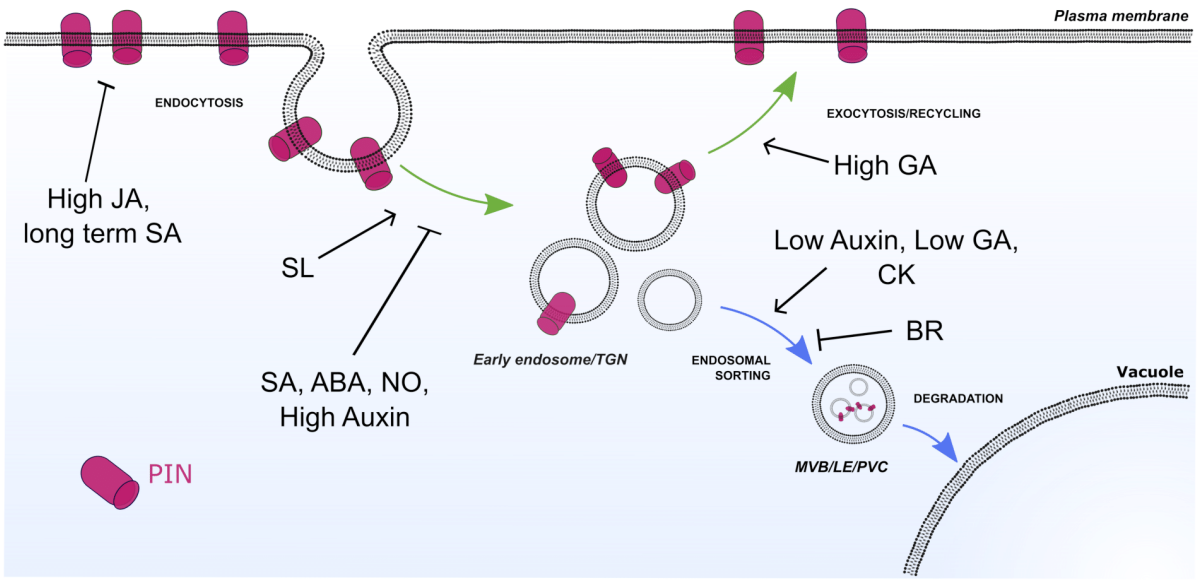

Figure 1. Hormonal regulation of PIN trafficking *Polar auxin transporters (e.g. PINs) undergo constant re-cycling between plasma membrane and endosomal compartments (green arrows). In response to developmental or environmental signals levels of PINs can be downregulated by their re-direction for lytic degradation to vacuoles (blue arrows). Plant hormones interfere with distinct steps of the PIN trafficking pathway, thereby contributing to fine-tuning of auxin transport and regulation of plant growth and development. ABA - Abscisic acid, BR - Brassinosteroids, CK - Cytokinins, GA - Gibberellic acid, JA - Jasmonic acid, NO - Nitric oxide, SA - Salicylic acid, SL -Strigolactones, LE - Late endosome, MVB - Multivesicular body, PVC - Prevacuolar compartment, TGN - Trans-Golgi network.*

So far it is unclear whether alterations of subcellular trafficking is a consequence of a direct, hormone-triggered posttranslational modifications of auxin transporters (e.g. phosphorylation, sumoylation, ubiquitination), or indirect interference with transport and sorting machineries, thereby affecting cellular movements of PIN proteins. Another intriguing question is the role of canonical hormonal signaling pathways, typically acting through transcriptional regulatory outputs. Although in nearly all hormone – PAT interactions, receptors and/or downstream components of transduction cascades are involved, this contrasts with transcription /proteosynthesis-independent nature of the above discussed hormone-PAT cross-talks. Could this mean that in parallel to well-established hormone signaling pathways there are other, so far unknown signal transduction cascades to be discovered?

With an increasing number of confirmed molecular interactions and circuits that determine and fine-tune the PAT, modelling and mathematical simulations might offer important tools

to provide novel insights into the dynamics of the PAT (Prusinkiewicz et al., 2009; Voß et al., 2014; Allen and Ptashnyk, 2020). Several models have been developed to gain a better understanding of the hormonal regulation of the PAT in the context of various developmental processes, such as root growth (Di Mambro et al., 2017), apical hook development (Žádníková et al., 2016), and vasculature differentiation (De Rybel et al., 2014; Mellor et al., 2019). Typically, the models are focused on specific hormonal pathways such as cytokinin, ethylene or gibberellin converging at the regulation of the PAT and auxin signaling (Moore et al., 2015; Muraro et al., 2016; Liu et al., 2017b). Nevertheless, a complex, all-embracing model of hormonal effects on the PAT remains a challenge for future research.

	Auxin transporter	Canonical receptor	Other molecular factors involved	Dependence of hormonal effect on transcription (chemical used)	Dependence of hormonal effect on translation (chemical used)	References
Auxin	PIN1, PIN2,PIN4	TIR1 independent (PIN endocytosis), TIR1 dependent (PIN lytic degradation)	BIG, clathrin, ROP6, TMK1	No (cordycepin)	No (CHX)	(Paciorek et al., 2005; Abas et al., 2006; Robert et al., 2010; Baster et al., 2013; Xu et al., 2014)
Cytokinin	PIN1, PIN3, PIN4, PIN7	CRE1/AHK4 dependent	BEN1, BEN2	No (cordycepin)	No (CHX)	(Marhavý et al., 2011; Zhang et al., 2011; Marhavý et al., 2014; Waldie and Leyser, 2018)
Ethylene	AUX1	N.A.	BIG3, ARF1	N.A.	Yes (CHX)	(Jonsson et al., 2017)
Jasmonates	PIN1, PIN2	COI1 dependent	ASA1, AXR1, TIR1, AFB1,2,3	N.A.	No (CHX)	(Sun et al., 2011)
Salicylic acid	PIN1, PIN2	NPR1,2,3 independent	CHC2, AP-2, PP2A	No (cordycepin)	No (CHX)	(Du et al., 2013; Tan et al., 2020)
Strigolactones	PIN1	MAX2 dependent	clathrin	N.A.	No (CHX)	(Shinohara et al., 2013)
Gibberellic acid	PIN1, PIN2, PIN3, PIN4, PIN7	DELLA dependent	SNX1, CLASP, PFDs, KTN	N.A.	No (CHX)	(Willige et al., 2011; Löfke et al., 2013; Salanenka et al., 2018)
Brassinosteroids	PIN1, PIN2, PIN4	BRI1 dependent	14-3-3, ROP2, GSK3/Shaggy-type kinases	N.A.	No (CHX)	(Li et al., 2005; Keicher et al., 2017; Retzer et al., 2019)
Abscisic acid	PIN1, PIN2, PIN3, PIN4, PIN7	PYLs dependent	PP2A, ABI1	N.A.	N.A.	(Yang et al., 2014; Li et al., 2018; Zhu et al., 2019; Li et al., 2020)
Nitric oxide	PIN2	N.A.	GSNOR1	N.A.	No (CHX)	(Ni et al., 2017; París et al., 2018)

Table 1. Posttranslational regulation of auxin transporters by plant hormones *Summary of hormonal effects on auxin transporters. Canonical receptors and molecular factors involved indicated. For detailed information please see the review. CHX – Cycloheximide; N.A. – Not Available; PM – plasma membrane.*

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References

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wiřniewska, J., Moulinier–Anzola, J. C., Sieberer, T., Friml, J., and Luschnig, C. (2006). Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nat. Cell Biol.* 8:249–256.

Abualia, R., Benkova, E., and Lacombe, B. (2018). Transporters and Mechanisms of Hormone Transport in Arabidopsis. *Adv. Bot. Res.* 87:115–138.

Achard, P., and Genschik, P. (2009). Releasing the brakes of plant growth: How GAs shutdown della proteins. *J. Exp. Bot.* 60:1085–1092.

Adamowski, M., and Friml, J. (2015). PIN-Dependent Auxin Transport: Action, Regulation, and Evolution. *Plant Cell Online* 27:20–32.

Adamowski, M., Narasimhan, M., Kania, U., Glanc, M., De Jaeger, G., and Friml, J. (2018). A functional study of AUXILIN-LIKE1 and 2, two putative clathrin uncoating factors in arabidopsis. *Plant Cell* 30:700–716.

Ahmad, P., Rasool, S., Gul, A., Sheikh, S. a., Akram, N. a., Ashraf, M., Kazi, a. M., and Gucel, S. (2016). Jasmonates: Multifunctional Roles in Stress Tolerance. *Front. Plant Sci.* 7.

Allen, H. R., and Ptashnyk, M. (2020). Mathematical Modelling of Auxin Transport in Plant Tissues: Flux Meets Signalling and Growth. *Bull. Math. Biol.* 82:1–35.

Alonso, J. M., and Ecker, J. R. (2001). The ethylene pathway: a paradigm for plant hormone signaling and interaction. *Sci. STKE* 2001:1–11.

Ambrose, C., Ruan, Y., Gardiner, J., Tamblyn, L. M., Catching, A., Kirik, V., Marc, J., Overall, R., and Wasteneys, G. O. (2013). CLASP Interacts with Sorting Nexin 1 to Link Microtubules and Auxin Transport via PIN2 Recycling in Arabidopsis thaliana. *Dev. Cell* 24:649–659.

Armengot, L., Marquès-Bueno, M. M., and Jaillais, Y. (2016). Regulation of polar auxin transport by protein and lipid kinases. *J. Exp. Bot.* Advance Access published 2016, doi:10.1093/jxb/erw216.

Bandyopadhyay, A., Blakeslee, J. J., Lee, O. R., Mravec, J., Sauer, M., Titapiwatanakun, B., Makam, S. N., Bouchard, R., Geisler, M., Martinoia, E., et al. (2007). Interactions of PIN and PGP auxin transport mechanisms. *Biochem. Soc. Trans.* 35:137–141.

Barbez, E., Laňková, M., Pařezová, M., Maizel, A., Zažímalová, E., Petrášek, J., Friml, J., and

Kleine-Vehn, J. (2013). Single-cell-based system to monitor carrier driven cellular auxin homeostasis. *BMC Plant Biol.* 13.

Barbosa, I. C. R., Shikata, H., Zourelidou, M., Heilmann, M., Heilmann, I., and Schwechheimer, C. (2016). Phospholipid composition and a polybasic motif determine D6 PROTEIN KINASE polar association with the plasma membrane and tropic responses. *Dev.* 143:4687–4700.

Barbosa, I. C. R., Hammes, U. Z., and Schwechheimer, C. (2018). Activation and Polarity Control of PIN-FORMED Auxin Transporters by Phosphorylation. *Trends Plant Sci.* 23:523–538.

Baster, P., Robert, S., Kleine-Vehn, J., Vanneste, S., Kania, U., Grunewald, W., De Rybel, B., Beeckman, T., and Friml, J. (2013). SCFTIR1/AFB-auxin signalling regulates PIN vacuolar trafficking and auxin fluxes during root gravitropism. *EMBO J.* 32:260–274.

Begara-Morales, J. C., Chaki, M., Valderrama, R., Mata-Pérez, C., Padilla, M. N., Barroso, J. B., and Brouquisse, R. (2019). The function of S-nitrosothiols during abiotic stress in plants. *J. Exp. Bot.* 70:4429–4439.

Benhar, M., Forrester, M. T., and Stamler, J. S. (2009). Protein denitrosylation: Enzymatic mechanisms and cellular functions. *Nat. Rev. Mol. Cell Biol.* 10:721–732.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. *Cell* 115:591–602.

Bennett, M. J., Marchant, A., Green, H. G., May, S. T., Sally, P., Millner, P. A., Walker, A. R., Schulz, B., Feldmann, K. A., Bennett, M. J., et al. (1996). Arabidopsis AUX1 Gene : A Permease-Like Regulator of Root Gravitropism Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/2891528 JSTOR is a not-for-profit service that helps scholars , researchers 273:948–950.

Bennett, T., Hines, G., and Leyser, O. (2014). Canalization: What the flux? *Trends Genet.* 30:41–48.

Bennett, T., Hines, G., van Rongen, M., Waldie, T., Sawchuk, M. G., Scarpella, E., Ljung, K., and Leyser, O. (2016). Connective Auxin Transport in the Shoot Facilitates Communication between Shoot Apices. *PLoS Biol.* 14:1–33.

Bielach, a., Podlesakova, K., Marhavy, P., Duclercq, J., Cuesta, C., Muller, B., Grunewald, W., Tarkowski, P., and Benkova, E. (2012). Spatiotemporal Regulation of Lateral Root Organogenesis in Arabidopsis by Cytokinin. *Plant Cell* 24:3967–3981.

Billou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Frimi, J., Heldstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature* 433:39–44.

Bishopp, A., Lehesranta, S., Vate, A., Help, H., El-showk, S., and Scheres, B. (2011). Report Phloem-Transported Cytokinin Regulates Polar Auxin Transport and Maintains Vascular Pattern in the Root Meristem. *Curr. Biol.* Advance Access published 2011, doi:10.1016/j.cub.2011.04.049.

Blakeslee, J. J., Bandyopadhyay, A., Ok, R. L., Mravec, J., Titapiwatanakun, B., Sauer, M., Makam, S. N., Cheng, Y., Bouchard, R., Adamec, J., et al. (2007). Interactions among PIN-FORMED and P-glycoprotein auxin transporters in Arabidopsis. *Plant Cell* 19:131–147.

Bleecker, A. B., and Kende, H. (2000). Ethylene: A Gaseous Signal Molecule in Plants. *Annu. Rev. Cell Dev. Biol.* 16:1–18.

Bowling, S. A., Clarke, J. D., Liu, Y., Klessig, D. F., and Dong, X. (1997). The cpr5 mutant of arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9:1573–1584.

Bowling et al., 1994 (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. *Plant Cell* 6:1845–1857.

Brandt, B., Brodsky, D. E., Xue, S., Negi, J., Iba, K., Kangasjärvi, J., Ghassemian, M., Stephan, A. B., Hu, H., and Schroeder, J. I. (2012). Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. *Proc. Natl. Acad. Sci. U. S. A.* 109:10593–10598.

Brewer, P. B., Koltai, H., and Beveridge, C. a. (2013). Diverse roles of strigolactones in plant development. *Mol. Plant* 6:18–28.

Cao, H., Bowling, S. a., Gordon, a. S., and Dong, X. (1994). Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. *Plant Cell* 6:1583.

Carrier, D. J., Bakar, N. T. A., Swarup, R., Callaghan, R., Napier, R. M., Bennett, M. J., and Kerr, I. D. (2008). The binding of auxin to the arabidopsis auxin influx transporter AUX1. *Plant Physiol.* 148:529–535.

Chai, C., and Subudhi, P. K. (2016). Comprehensive Analysis and Expression Profiling of the OsLAX and OsABCB Auxin Transporter Gene Families in Rice (Oryza sativa) under Phytohormone Stimuli and Abiotic Stresses. *Front. Plant Sci.* 7:1–13.

Chandler, J. W. (2009). Local auxin production: A small contribution to a big field. *BioEssays* 31:60–70.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J. R. (1997). Activation of the ethylene gas response pathway in arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* 89:1133–1144.

Chen, X., Naramoto, S., Robert, S., Tejos, R., Löfke, C., Lin, D., Yang, Z., and Friml, J. (2012). ABP1 and ROP6 GTPase signaling regulate clathrin-mediated endocytosis in Arabidopsis roots. *Curr. Biol.* 22:1326–1332.

Cho, M., and Cho, H. T. (2013). The function of ABCB transporters in auxin transport. *Plant Signal. Behav.* 8:6–9.

Clouse, S. D. (2011). Brassinosteroid signal transduction: From receptor kinase activation to transcriptional networks regulating plant development. *Plant Cell* 23:1219–1230.

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R., and Abrams, S. R. (2010). *Abscisic Acid:*

Emergence of a Core Signaling Network.

Dai, M., Zhang, C., Kania, U., Chen, F., Xue, Q., McCray, T., Li, G., Qin, G., Wakeley, M., Terzaghi, W., et al. (2012). A PP6-type phosphatase holoenzyme directly regulates PIN phosphorylation and auxin efflux in Arabidopsis. *Plant Cell* 24:2497–2514.

Dar, T. A., Uddin, M., Khan, M. M. a., Hakeem, K. R., and Jaleel, H. (2015). Jasmonates counter plant stress: A review. *Environ. Exp. Bot.* 115:49–57.

Daviere, J.-M., and Achard, P. (2013). Gibberellin signaling in plants. *Development* 140:1147–1151.

De Rybel, B., Adibi, M., Breda, A. S., Wendrich, J. R., Smit, M. E., Novák, O., Yamaguchi, N., Yoshida, S., Van Isterdael, G., Palovaara, J., et al. (2014). Integration of growth and patterning during vascular tissue formation in Arabidopsis. *Science (80-.).* 345.

Dejonghe, W., Sharma, I., Denoo, B., De Munck, S., Lu, Q., Mishev, K., Bulut, H., Mylle, E., De Rycke, R., Vasileva, M., et al. (2019). Disruption of endocytosis through chemical inhibition of clathrin heavy chain function. *Nat. Chem. Biol.* 15:641–649.

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., Aoyama, T., Costantino, P., and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. *Science (80-.).* 322:1380–1384.

Di Mambro, R., De Ruvo, M., Pacifici, E., Salvi, E., Sozzani, R., Benfey, P. N., Busch, W., Novak, O., Ljung, K., Di Paola, L., et al. (2017). Auxin minimum triggers the developmental switch from cell division to cell differentiation in the *Arabidopsis* root. *Proc. Natl. Acad. Sci.* 114:E7641–E7649.

Ding, Y., Sun, T., Ao, K., Peng, Y., Zhang, Y., Li, X., and Zhang, Y. (2018). Opposite Roles of Salicylic Acid Receptors NPR1 and NPR3/NPR4 in Transcriptional Regulation of Plant Immunity. *Cell* 173:1454-1467.e10.

Dory, M., Hatzimasoura, E., Kállai, B. M., Nagy, S. K., Jäger, K., Darula, Z., Nádai, T. V., Mészáros, T., López-Juez, E., Barnabás, B., et al. (2018). Coevolving MAPK and PID phosphosites indicate an ancient environmental control of PIN auxin transporters in land plants. *FEBS Lett.* 592:89–102.

Doyle, S. M., Vain, T., and Robert, S. (2015). Small molecules unravel complex interplay between auxin biology and endomembrane trafficking. *J. Exp. Bot.* 66:4971–4982.

Drdová, E. J., Synek, L., Pečenková, T., Hála, M., Kulich, I., Fowler, J. E., Murphy, A. S., and Žárský, V. (2013). The exocyst complex contributes to PIN auxin efflux carrier recycling and polar auxin transport in Arabidopsis. *Plant J.* 73:709–719.

Du, C., and Chong, K. (2011). ARF-GTPase activating protein mediates auxin influx carrier AUX1 early endosome trafficking to regulate auxin dependent plant development. *Plant Signal. Behav.* 6:1644–1646.

Du, Y., Tejos, R., Beck, M., Himschoot, E., Li, H., Robatzek, S., Vanneste, S., and Friml, J. (2013). Salicylic acid interferes with clathrin-mediated endocytic protein trafficking. *Proc. Natl. Acad. Sci.* 110:7946–7951.

Dubois, M., Van den Broeck, L., and Inzé, D. (2018). The Pivotal Role of Ethylene in Plant Growth. *Trends Plant Sci.* 23:311–323.

Durner, J., Wendehenne, D., and Klessig, D. F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl. Acad. Sci. U. S. A.* 95:10328–10333.

Fan, L., Li, R., Pan, J., Ding, Z., and Lin, J. (2015). Endocytosis and its regulation in plants. *Trends Plant Sci.* 20:388–397.

Feechan, A., Kwon, E., Yun, B. W., Wang, Y., Pallas, J. A., and Loake, G. J. (2005). A central role for S-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. U. S. A.* 102:8054–8059.

Feraru, E., Feraru, M. I., Asaoka, R., Paciorek, T., De Rycke, R., Tanaka, H., Nakano, a., and Friml, J. (2012). BEX5/RabA1b Regulates trans-Golgi Network-to-Plasma Membrane Protein Trafficking in Arabidopsis. *Plant Cell* 24:3074–3086.

Fernández-Marcos, M., Sanz, L., Lewis, D. R., Muday, G. K., and Lorenzo, O. (2011). Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport. *Proc. Natl. Acad. Sci. U. S. A.* 108:18506–18511.

Finkelstein, R. (2013). Abscisic Acid Synthesis and Response. *Arab. B.* 11:e0166.

Fridman, Y., and Savaldi-Goldstein, S. (2013). Brassinosteroids in growth control: How, when and where. *Plant Sci.* 209:24–31.

Friml, J. (2003). Auxin transport - Shaping the plant. *Curr. Opin. Plant Biol.* Advance Access published 2003, doi:10.1016/S1369526602000031.

Friml, J., Benková, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jürgens, G., et al. (2002). AtPIN4 mediates sink-driven auxin gradients and root patterning in Arabidopsis. *Cell* 108:661–673.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P. B. F., Ljung, K., Sandberg, G., et al. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science* 306:862–865.

Fu, X., and Harberd, N. P. (2003). Auxin promotes Arabidopsis root growth by modulating gibberellin response. *Nature* 421:740–743.

Fu, Y., Li, H., and Yang, Z. (2002). The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during arabidopsis organogenesis. *Plant Cell* 14:777–794.

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., Mohan, R., Spoel, S. H., Tada, Y., Zheng, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486:228–232.

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S. Y., Cutler, S. R., Sheen, J., Rodriguez, P. L., and Zhu, J. K. (2009). In vitro reconstitution of an abscisic acid signalling pathway. *Nature* 462:660–664.

Fukui, K., and Hayashi, K. I. (2018). Manipulation and sensing of auxin metabolism, transport and signaling. *Plant Cell Physiol.* 59:1500–1510.

Galvan-Ampudia, C. S., Julkowska, M. M., Darwish, E., Gandullo, J., Korver, R. A., Brunoud, G., Haring, M. A., Munnik, T., Vernoux, T., and Testerink, C. (2013). Halotropism is a response of plant roots to avoid a saline environment. *Curr. Biol.* 23:2044–2050.

Gampala, S. S., Kim, T. W., He, J. X., Tang, W., Deng, Z., Bai, M. Y., Guan, S., Lalonde, S., Sun, Y., Gendron, J. M., et al. (2007). An Essential Role for 14-3-3 Proteins in Brassinosteroid Signal Transduction in Arabidopsis. *Dev. Cell* 13:177–189.

Ganguly, A., Lee, S. H., and Cho, H. T. (2012). Functional identification of the phosphorylation sites of Arabidopsis PIN-FORMED3 for its subcellular localization and biological role. *Plant J.* 71:810–823.

Ganguly, A., Park, M., Kesawat, M. S., and Cho, H. T. (2014). Functional analysis of the hydrophilic loop in intracellular trafficking of Arabidopsis PIN-FORMED proteins. *Plant Cell* 26:1570–1585.

Gao, Y., Zhang, Y., Zhang, D., Dai, X., Estelle, M., and Zhao, Y. (2015). Auxin binding protein 1 (ABP1) is not required for either auxin signaling or Arabidopsis development. *Proc. Natl. Acad. Sci. U. S. A.* 112:2275–2280.

Geiger, D., Scherzer, S., Mumm, P., Stange, A., Marten, I., Bauer, H., Ache, P., Matschi, S., Liese, A., Al-Rasheid, K. A. S., et al. (2009). Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proc. Natl. Acad. Sci. U. S. A.* 106:21425–21430.

Geisler, M., Aryal, B., Di Donato, M., and Hao, P. (2017). A critical view on ABC transporters and their interacting partners in auxin transport. *Plant Cell Physiol.* 58:1601–1604.

Geldner, N., Friml, J., Stierhof, Y. D., Jürgens, G., and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* 413:425–428.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., and Jürgens, G. (2003). The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* 112:219–230.

Glanc, M., Fendrych, M., and Friml, J. (2018). Mechanistic framework for cell-intrinsic re-establishment of PIN2 polarity after cell division. *Nat. Plants* 4:1082–1088.

Grebe, M., Friml, J., Swarup, R., Sandberg, G., Terlou, M., Palme, K., Bennett, M. J., and Scheres, B. (2002). Cell polarity signaling in Arabidopsis involves a BFA-sensitive auxin influx pathway. *Curr. Biol.* 12:329–334.

Grieneisen, V. A., Xu, J., Marée, A. F. M., Hogeweg, P., and Scheres, B. (2007). Auxin transport is sufficient to generate a maximum and gradient guiding root growth. *Nature* 449:1008–1013.

Guo, H., and Ecker, J. R. (2003). Plant responses to ethylene gas are mediated by SCFEBF1/EBF2- dependent proteolysis of EIN3 transcription factor. *Cell* 115:667–677.

Guo, X., Qin, Q., Yan, J., Niu, Y., Huang, B., Guan, L., Li, Y., Ren, D., Li, J., and Hou, S. (2015). Type-one protein phosphatase4 regulates pavement cell interdigitation by modulating PIN-FORMED1 polarity and trafficking in arabidopsis. *Plant Physiol.* 167:1058–1075.

Habets, M. E. J., and Offringa, R. (2014). PIN-driven polar auxin transport in plant developmental plasticity: A key target for environmental and endogenous signals. *New Phytol.* 203:362–377.

Hacham, Y., Sela, A., Friedlander, L., and Savaldi-Goldstein, S. (2012). BRI1 activity in the root meristem involves post-transcriptional regulation of PIN auxin efflux carriers. *Plant Signal. Behav.* 7.

Hall, B. P., Shakeel, S. N., and Schaller, G. E. (2007). Ethylene receptors: Ethylene perception and signal transduction. *J. Plant Growth Regul.* 26:118–130.

Han, H., Rakusova, H., Verstraeten, I., Zhang, Y., and Friml, J. (2020). SCFTIR1/AFB auxin signaling for bending termination during shoot gravitropism. *Plant Physiol.* 1:pp.00212.2020.

He, Y., Tang, R. H., Hao, Y., Stevens, R. D., Cook, C. W., Ahn, S. M., Jing, L., Yang, Z., Chen, L., Guo, F., et al. (2004). Nitric oxide represses the Arabidopsis floral transition. *Science (80-.).* 305:1968–1971.

Hedden, P., and Sponsel, V. (2015). A Century of Gibberellin Research. *J. Plant Growth Regul.* 34:740–760.

Henrichs, S., Wang, B., Fukao, Y., Zhu, J., Charrier, L., Bailly, A., Oehring, S. C., Linnert, M., Weiwad, M., Endler, A., et al. (2012). Regulation of ABCB1/PGP1-catalysed auxin transport by linker phosphorylation. *EMBO J.* 31:2965–2980.

Hess, D. T., Matsumoto, A., Kim, S. O., Marshall, H. E., and Stamler, J. S. (2005). Protein S-nitrosylation: Purview and parameters. *Nat. Rev. Mol. Cell Biol.* 6:150–166.

Hill, K. (2015). Post-translational modifications of hormone-responsive transcription factors: The next level of regulation. *J. Exp. Bot.* 66:4933–4945.

Hu, X., Neill, S. J., Tang, Z., and Cai, W. (2005). Nitric Oxide Mediates Gravitropic Bending in Soybean Roots. *Plant Physiol.* 137:663–670.

Hu, Y. F., Zhou, G., Na, X. F., Yang, L., Nan, W. Bin, Liu, X., Zhang, Y. Q., Li, J. L., and Bi, Y. R. (2013). Cadmium interferes with maintenance of auxin homeostasis in Arabidopsis seedlings. *J. Plant Physiol.* 170:965–975.

Hua, J., and Meyerowitz, E. M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. *Cell* 94:261–271.

Ischebeck, T., Werner, S., Krishnamoorthy, P., Lerche, J., Meijón, M., Stenzel, I., Löfke, C., Wiessner, T., Im, Y. J., Perera, I. Y., et al. (2013). Phosphatidylinositol 4,5-bisphosphate influences PIN polarization by controlling clathrin-mediated membrane trafficking in Arabidopsis. *Plant Cell* 25:4894–4911.

Jásik, J., Bokor, B., Stuchlík, S., Mičieta, K., Turňa, J., and Schmelzer, E. (2016). Effects of auxins on PIN-FORMED2 (PIN2) dynamics are not mediated by inhibiting PIN2

endocytosis. *Plant Physiol.* 172:1019–1031.

Jiang, L., Liu, X., Xiong, G., Liu, H., Chen, F., Wang, L., Meng, X., Liu, G., Yu, H., Yuan, Y., et al. (2013). DWARF 53 acts as a repressor of strigolactone signalling in rice. *Nature* 504:401–405.

Jonsson, K., Boutté, Y., Singh, R. K., Gendre, D., and Bhalerao, R. P. (2017). Ethylene Regulates Differential Growth via BIG ARF-GEF-Dependent Post-Golgi Secretory Trafficking in Arabidopsis. *Plant Cell* 29:1039–1052.

Ju, C., Yoon, G. M., Shemansky, J. M., Lin, D. Y., Ying, Z. I., Chang, J., Garrett, W. M., Kessenbrock, M., Groth, G., Tucker, M. L., et al. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. *Proc. Natl. Acad. Sci.* 109:19486–19491.

Julkowska, M. M., and Testerink, C. (2015). Tuning plant signaling and growth to survive salt. *Trends Plant Sci.* 20:586–594.

Kania, U., Nodzyński, T., Lu, Q., Hicks, G. R., Nerinckx, W., Mishev, K., Peurois, F., Cherfils, J., De Rycke, R., Grones, P., et al. (2018). The inhibitor endosidin 4 targets sec7 domain-type arf gtpase exchange factors and interferes with subcellular trafficking in eukaryotes[open]. *Plant Cell* 30:2553–2572.

Keicher, J., Jaspert, N., Weckermann, K., Möller, C., Throm, C., Kintzi, A., and Oecking, C. (2017). Arabidopsis 14-3-3 epsilon members contribute to polarity of PIN auxin carrier and auxin transport-related development. *Elife* 6:1–21.

Keshishian, E. A., and Rashotte, A. M. (2015). Plant cytokinin signalling. *Essays Biochem.* 58:13–27.

Khan, M. I. R., Fatma, M., Per, T. S., Anjum, N. a., and Khan, N. a. (2015). Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. *Front. Plant Sci.* 6:1–17.

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:1–7.

Kitakura, S., Vanneste, S., Robert, S., Löfke, C., Teichmann, T., Tanaka, H., and Friml, J. (2011). Clathrin mediates endocytosis and polar distribution of PIN auxin transporters in Arabidopsis. *Plant Cell* 23:1920–1931.

Kleine-Vehn, J., Dhonukshe, P., Swarup, R., Bennett, M., and Friml, J. (2006). Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. *Plant Cell* 18:3171–3181.

Korver, R. A., van den Berg, T., Meyer, A. J., Galvan-Ampudia, C. S., ten Tusscher, K. H. W. J., and Testerink, C. (2020). Halotropism requires phospholipase Dζ1-mediated modulation of cellular polarity of auxin transport carriers. *Plant Cell Environ.* 43:143–158.

Le Bot, Nathalie, Miao-Chih Tsai, Robert K. Andrews, and J. A. (2003). TAC-1, a Regulator of Microtubule Length in the C. elegans Embryo. *Curr. Biol.* 6742:10–12.

Lešková, A., Zvarï K, M., Araya, T., and Giehl, R. F. H. (2020). Nickel Toxicity Targets Cell Wall-Related Processes and PIN2-Mediated Auxin Transport to Inhibit Root Elongation and Gravitropic Responses in Arabidopsis. *Plant Cell Physiol.* 61:519–535.

Lewis, D. R., Negi, S., Sukumar, P., and Muday, G. K. (2011). Ethylene inhibits lateral root development, increases IAA transport and expression of PIN3 and PIN7 auxin efflux carriers. *Development* 138:3485–3495.

Leyser, O. (2009). The control of shoot branching: An example of plant information processing. *Plant, Cell Environ.* 32:694–703.

Li, G., and Xue, H. W. (2007). Arabidopsis PLDζ2 regulates vesicle trafficking and is required for auxin response. *Plant Cell* 19:281–295.

Li, L., Xu, J., Xu, Z. H., and Xue, H. W. (2005). Brassinosteroids stimulate plant tropisms through modulation of polar auxin transport in Brassica and Arabidopsis. *Plant Cell* 17:2738–2753.

Li, G., Song, H., Li, B., Kronzucker, H. J., and Shi, W. (2015). Auxin resistant1 and PIN-FORMED2 protect lateral root formation in arabidopsis under iron stress. *Plant Physiol.* 169:2608–2623.

Li, Z., Waadt, R., and Schroeder, J. I. (2016). Release of GTP Exchange Factor Mediated Down-Regulation of Abscisic Acid Signal Transduction through ABA-Induced Rapid Degradation of RopGEFs. *PLoS Biol.* 14:1–27.

Li, Y., Li, Y., Liu, Y., Wu, Y., and Xie, Q. (2018). The sHSP22 heat shock protein requires the ABI1 protein phosphatase to modulate polar auxin transport and downstream responses. *Plant Physiol.* 176:2406–2425.

Li, T., Yan, A., Bhatia, N., Altinok, A., Afik, E., Durand-Smet, P., Tarr, P. T., Schroeder, J. I., Heisler, M. G., and Meyerowitz, E. M. (2019). Calcium signals are necessary to establish auxin transporter polarity in a plant stem cell niche. *Nat. Commun.* 10:1–9.

Li, Y., Wang, Y., Tan, S., Li, Z., Yuan, Z., Glanc, M., Domjan, D., Wang, K., Xuan, W., Guo, Y., et al. (2020). Root Growth Adaptation is Mediated by PYLs ABA Receptor-PP2A Protein Phosphatase Complex. *Adv. Sci.* 7.

Liu, X., and Hou, X. (2018). Antagonistic regulation of ABA and GA in metabolism and signaling pathways. *Front. Plant Sci.* 9:1–7.

Liu, S. X., Kawai, K., Tyurin, V. A., Tyurina, Y. Y., Borisenko, G. G., Fabisiak, J. P., Quinn, P. J., Pitt, B. R., and Kagan, V. E. (2001). Nitric oxide-dependent pro-oxidant and pro-apoptotic effect of metallothioneins in HL-60 cells challenged with cupric nitrilotriacetate. *Biochem. J.* 354:397–406.

Liu, L., Yan, Y., Zeng, M., Zhang, J., Hanes, M. A., Ahearn, G., McMahon, T. J., Dickfeld, T., Marshall, H. E., Que, L. G., et al. (2004). Essential Roles of S-Nitrosothiols in Vascular Homeostasis and Endotoxic Shock. *Cell* 116:617–628.

Liu, P. P., Montgomery, T. A., Fahlgren, N., Kasschau, K. D., Nonogaki, H., and Carrington, J. C. (2007). Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for

seed germination and post-germination stages. *Plant J.* 52:133–146.

Liu, Q., Zhang, Y. C., Wang, C. Y., Luo, Y. C., Huang, Q. J., Chen, S. Y., Zhou, H., Qu, L. H., and Chen, Y. Q. (2009). Expression analysis of phytohormone-regulated microRNAs in rice, implying their regulation roles in plant hormone signaling. *FEBS Lett.* 583:723–728.

Liu, Y., Dong, Q., Kita, D., Huang, J. B., Liu, G., Wu, X., Zhu, X., Cheung, A. Y., Wu, H. M., and Tao, L. Z. (2017a). RopGEF1 plays a critical role in polar auxin transport in early development. *Plant Physiol.* 175:157–171.

Liu, J., Moore, S., Chen, C., and Lindsey, K. (2017b). Crosstalk Complexities between Auxin, Cytokinin, and Ethylene in Arabidopsis Root Development: From Experiments to Systems Modeling, and Back Again. *Mol. Plant* 10:1480–1496.

Locascio, A., Blázquez, M. A., and Alabadí, D. (2013). Dynamic regulation of cortical microtubule organization through prefoldin-DELLA interaction. *Curr. Biol.* 23:804–809.

Löfke, C., Luschnig, C., and Kleine-Vehn, J. (2013a). Posttranslational modification and trafficking of PIN auxin efflux carriers. *Mech. Dev.* 130:82–94.

Löfke, C., Zwiewka, M., Heilmann, I., Van Montagu, M. C. E., Teichmann, T., and Friml, J. (2013b). Asymmetric gibberellin signaling regulates vacuolar trafficking of PIN auxin transporters during root gravitropism. *Proc. Natl. Acad. Sci. U. S. A.* 110:3627–32.

Lu, C., Chen, M. X., Liu, R., Zhang, L., Hou, X., Liu, S., Ding, X., Jiang, Y., Xu, J., Zhang, J., et al. (2019). Abscisic acid regulates auxin distribution to mediate maize lateral root development under salt stress. *Front. Plant Sci.* 10:1–16.

Lumba, S., Holbrook-Smith, D., and McCourt, P. (2017). The perception of strigolactones in vascular plants. *Nat. Chem. Biol.* 13:599–606.

Lundin, V. F., Srayko, M., Hyman, A. A., and Leroux, M. R. (2008). Efficient chaperone-mediated tubulin biogenesis is essential for cell division and cell migration in C. elegans. *Dev. Biol.* 313:320–334.

Marhava, P., Aliaga Fandino, A. C., Koh, S. W. H., Jelínková, A., Kolb, M., Janacek, D. P., Breda, A. S., Cattaneo, P., Hammes, U. Z., Petrášek, J., et al. (2020). Plasma Membrane Domain Patterning and Self-Reinforcing Polarity in Arabidopsis. *Dev. Cell* 52:223-235.e5.

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., et al. (2011). Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis. *Dev. Cell* 21:796–804.

Marhavý, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J., Schwechheimer, C., Murphy, A., and Benková, E. (2014). Cytokinin Controls Polarity of PIN1-Dependent Auxin Transport during Lateral Root Organogenesis. *Curr. Biol.* 24:1031–1037.

Mazur, E., Kulik, I., Hajný, J., and Friml, J. (2020a). Auxin Canalization and Vascular Tissue Formation by TIR1/AFB-Mediated Auxin Signaling in Arabidopsis. *New Phytol.* Advance

Access published 2020, doi:10.1111/nph.16446.

Mazur, E., Gallei, M., Adamowski, M., Han, H., Robert, H. S., and Friml, J. (2020b). Clathrin-mediated trafficking and PIN trafficking are required for auxin canalization and vascular tissue formation in Arabidopsis. *Plant Sci.* 293.

Mei, Y., Jia, W. J., Chu, Y. J., and Xue, H. W. (2012). Arabidopsis phosphatidylinositol monophosphate 5-kinase 2 is involved in root gravitropism through regulation of polar auxin transport by affecting the cycling of PIN proteins. *Cell Res.* 22:581–597.

Melcher, K., Zhou, X. E., and Xu, H. E. (2010). Thirsty plants and beyond: Structural mechanisms of abscisic acid perception and signaling. *Curr. Opin. Struct. Biol.* 20:722–729.

Mellor, N., Vaughan-Hirsch, J., Kumpers, B. M. C., Help-Rinta-Rahko, H., Miyashima, S., Mahonen, A. P., Campilho, A., King, J. R., and Bishopp, A. (2019). A core mechanism for specifying root vascular patterning can replicate the anatomical variation seen in diverse plant species. *Dev.* 146:1–10.

Méndez-Bravo, A., Ruiz-Herrera, L. F., Cruz-Ramírez, A., Guzman, P., Martínez-Trujillo, M., Ortiz-Castro, R., and López-Bucio, J. (2019). CONSTITUTIVE TRIPLE RESPONSE1 and PIN2 act in a coordinate manner to support the indeterminate root growth and meristem cell proliferating activity in Arabidopsis seedlings. *Plant Sci.* 280:175–186.

Michniewicz, M., Zago, M. K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M. G., Ohno, C., Zhang, J., Huang, F., et al. (2007). Antagonistic Regulation of PIN Phosphorylation by PP2A and PINOID Directs Auxin Flux. *Cell* 130:1044–1056.

Mishev, K., Lu, Q., Denoo, B., Peurois, F., Dejonghe, W., Hullaert, J., De Rycke, R., Boeren, S., Bretou, M., De Munck, S., et al. (2018). Nonselective chemical inhibition of sec7 domain-containing arf gtpase exchange factors. *Plant Cell* 30:2573–2593.

Moore, S., Zhang, X., Mudge, A., Rowe, J. H., Topping, J. F., Liu, J., and Lindsey, K. (2015). Spatiotemporal modelling of hormonal crosstalk explains the level and patterning of hormones and gene expression in Arabidopsis thaliana wild-type and mutant roots. *New Phytol.* 207:1110–1122.

Moriwaki, T., Miyazawa, Y., Kobayashi, A., and Takahashi, H. (2013). Molecular mechanisms of hydrotropism in seedling roots of Arabidopsis Thaliana (Brassicaceae). *Am. J. Bot.* 100:25–34.

Muraro, D., Larrieu, A., Lucas, M., Chopard, J., Byrne, H., Godin, C., and King, J. (2016). A multi-scale model of the interplay between cell signalling and hormone transport in specifying the root meristem of Arabidopsis thaliana. *J. Theor. Biol.* 404:182–205.

Nagawa, S., Xu, T., Lin, D., Dhonukshe, P., Zhang, X., Friml, J., Scheres, B., Fu, Y., and Yang, Z. (2012). ROP GTPase-dependent actin microfilaments promote PIN1 polarization by localized inhibition of clathrin-dependent endocytosis. *PLoS Biol.* 10.

Naramoto, S. (2017). Polar transport in plants mediated by membrane transporters: focus on mechanisms of polar auxin transport. *Curr. Opin. Plant Biol.* 40:8–14.

Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D., and Hancock, J. T. (2002). Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot.* 53:1237–1247.

Neill, S. J., Desikan, R., and Hancock, J. T. (2003). Nitric oxide signalling in plants. *New Phytol.* 159:11–35.

Okada, K., Ueda, J., Komaki, M. K., Bell, C. J., and Shimura, Y. (1991). Requirement of the auxin polar transport system in early stages of Arabidopsis floral bud formation. *Plant Cell* 3:677–684.

Osugi, A., and Sakakibara, H. (2015). Q&A: How do plants respond to cytokinins and what is their importance? *BMC Biol.* 13:102.

Overvoorde, P., Fukaki, H., and Beeckman, T. (2010). Auxin control of root development. *Cold Spring Harb. Perspect. Biol.* 2:1–17.

Paciorek, T., Zažímalová, E., Ruthardt, N., Petrášek, J., Stierhof, Y.-D., Kleine-Vehn, J., Morris, D. a., Emans, N., Jürgens, G., Geldner, N., et al. (2005). Auxin inhibits endocytosis and promotes its own efflux from cells. *Nature* 435:1251–1256.

Pan, X., Fang, L., Liu, J., Senay-Aras, B., Lin, W., Zheng, S., Zhang, T., Manor, U., Chen, W., and Yang, Z. (2019). Auxin-induced nanoclustering of membrane signaling complexes underlies cell polarity establishment in Arabidopsis. *bioRxiv* Advance Access published 2019, doi:10.1101/734665.

Paponov, I. A., Friz, T., Budnyk, V., Teale, W., Wüst, F., Paponov, M., Al-Babili, S., and Palme, K. (2019). Natural auxin does not inhibit brefeldin a induced PIN1 and PIN2 internalization in root cells. *Front. Plant Sci.* 10:1–7.

París, R., Vazquez, M. M., Graziano, M., Terrile, M. C., Miller, N. D., Spalding, E. P., Otegui, M. S., and Casalongué, C. a. (2018). Distribution of Endogenous NO Regulates Early Gravitropic Response and PIN2 Localization in Arabidopsis Roots. *Front. Plant Sci.* 9:1–11.

Peres, A. L. G. L., Soares, J. S., Tavares, R. G., Righetto, G., Zullo, M. A. T., Mandava, N. B., and Menossi, M. (2019). Brassinosteroids, the sixth class of phytohormones: A molecular view from the discovery to hormonal interactions in plant development and stress adaptation. *Int. J. Mol. Sci.* 20.

Péret, B., Swarup, K., Ferguson, A., Seth, M., Yang, Y., Dhondt, S., James, N., Casimiro, I., Perry, P., Syed, A., et al. (2012). AUX/LAX genes encode a family of auxin influx transporters that perform distinct functions during arabidopsis development. *Plant Cell* 24:2874–2885.

Pernisova, M., Prat, T., Grones, P., Harustiakova, D., Matonohova, M., Spichal, L., Nodzynski, T., Friml, J., and Hejatko, J. (2016). Cytokinins influence root gravitropism via differential regulation of auxin transporter expression and localization in Arabidopsis. *New Phytol.* 212:497–509.

Petrášek, J., Mravec, J., Bouchard, R., Blakeslee, J. J., Abas, M., Seifertová, D., Wiśniewska, J., Tadele, Z., Kubeš, M., Čovanová, M., et al. (2006). PIN Proteins Perform a Rate-Limiting Function in Cellular Auxin Efflux. *Science (80-.).* 312:914 LP – 918.

Platre, M. P., Bayle, V., Armengot, L., Bareille, J., del Mar Marquès-Bueno, M., Creff, A., Maneta-Peyret, L., Fiche, J. B., Nollmann, M., Miège, C., et al. (2019). Developmental control of plant Rho GTPase nano-organization by the lipid phosphatidylserine. *Science (80-.).* 364:57–62.

Prát, T., Hajný, J., Grunewald, W., Vasileva, M., Molnár, G., Tejos, R., Schmid, M., Sauer, M., and Friml, J. (2018). WRKY23 is a component of the transcriptional network mediating auxin feedback on PIN polarity. *PLoS Genet.* 14:1–18.

Prusinkiewicz, P., Crawford, S., Smith, R. S., Ljung, K., Bennett, T., Ongaro, V., and Leyser, O. (2009). Control of bud activation by an auxin transport switch. *Proc. Natl. Acad. Sci. U. S. A.* 106:17431–17436.

Qiao, H., Shen, Z., Huang, S. -s. C., Schmitz, R. J., Urich, M. a., Briggs, S. P., and Ecker, J. R. (2012). Processing and Subcellular Trafficking of ER-Tethered EIN2 Control Response to Ethylene Gas. *Science (80-.).* 338:390–393.

Rakusová, H., Abbas, M., Han, H., Song, S., Robert, H. S., and Friml, J. (2016). Termination of Shoot Gravitropic Responses by Auxin Feedback on PIN3 Polarity. *Curr. Biol.* 26:3026–3032.

Retzer, K., Akhmanova, M., Konstantinova, N., Malínská, K., Leitner, J., Petrášek, J., and Luschnig, C. (2019). Brassinosteroid signaling delimits root gravitropism via sorting of the Arabidopsis PIN2 auxin transporter. *Nat. Commun.* 10:5516.

Rigó, G., Ayaydin, F., Tietz, O., Zsigmond, L., Kovács, H., Páy, A., Salchert, K., Darula, Z., Medzihradszky, K. F., Szabados, L., et al. (2013). Inactivation of plasma membrane-localized CDPK-RELATED KINASE5 decelerates PIN2 exocytosis and root gravitropic response in Arabidopsis. *Plant Cell* 25:1592–1608.

Rivas-San Vicente, M., and Plasencia, J. (2011). Salicylic acid beyond defence: Its role in plant growth and development. *J. Exp. Bot.* 62:3321–3338.

Robert, S., Kleine-Vehn, J., Barbez, E., Sauer, M., Paciorek, T., Baster, P., Vanneste, S., Zhang, J., Simon, S., Čovanová, M., et al. (2010). ABP1 mediates auxin inhibition of clathrin-dependent endocytosis in arabidopsis. *Cell* 143:111–121.

Romero-Puertas, M. C., Perazzolli, M., Zago, E. D., and Delledonne, M. (2004). Nitric oxide signalling functions in plant-pathogen interactions. *Cell. Microbiol.* 6:795–803.

Rong, D., Luo, N., Mollet, J. C., Liu, X., and Yang, Z. (2016). Salicylic Acid Regulates Pollen Tip Growth through an NPR3/NPR4-Independent Pathway. *Mol. Plant* 9:1478–1491.

Rosquete, M. R., and Kleine-Vehn, J. (2013). Halotropism: Turning down the salty date. *Curr. Biol.* 23:927–929.

Rowe, J. H., Topping, J. F., Liu, J., and Lindsey, K. (2016). Abscisic acid regulates root growth under osmotic stress conditions via an interacting hormonal network with cytokinin, ethylene and auxin. *New Phytol.* 211:225–239.

Ruzicka, K., Simásková, M., Duclercq, J., Petrásek, J., Zazímalová, E., Simon, S., Friml, J., Van Montagu, M. C. E., and Benková, E. (2009). Cytokinin regulates root meristem activity

via modulation of the polar auxin transport. *Proc. Natl. Acad. Sci. U. S. A.* 106:4284–4289.

Růžička, K., Ljung, K., Vanneste, S., Podhorská, R., Beeckman, T., Friml, J., and Benková, E. (2007). Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. *Plant Cell* 19:2197–2212.

Sachs, T. (1975). The induction of transport channels by auxin. *Planta* 127:201–206.

Sachs, T. (1981). The Control of the Patterned Differentiation of Vascular Tissues. In (ed. Woolhouse, H. W.), pp. 151–262. Academic Press.

SACHS, T. (1969). Polarity and the Induction of Organized Vascular Tissues. *Ann. Bot.* 33:263–275.

Salanenka, Y., Verstraeten, I., Löfke, C., Tabata, K., Naramoto, S., Glanc, M., and Friml, J. (2018). Gibberellin DELLA signaling targets the retromer complex to redirect protein trafficking to the plasma membrane. *Proc. Natl. Acad. Sci.* 115:3716–3721.

Sánchez-Vicente, I., Fernández-Espinosa, M. G., Lorenzo, O., and Brouquisse, R. (2019). Nitric oxide molecular targets: Reprogramming plant development upon stress. *J. Exp. Bot.* 70:4441–4460.

Sauer, M., and Kleine-Vehn, J. (2019). PIN-FORMED and PIN-LIKES auxin transport facilitators. *Development* 146:dev168088.

Sauer, M., Balla, J., Luschnig, C., Wiśniewska, J., Reinöhl, V., Friml, J., and Benková, E. (2006). Canalization of auxin flow by Aux/IAA-ARF-dependent feedback regulation of PIN polarity. *Genes Dev.* 20:2902–2911.

Sengupta, R., and Holmgren, A. (2012). The role of thioredoxin in the regulation of cellular processes by S-nitrosylation. *Biochim. Biophys. Acta - Gen. Subj.* 1820:689–700.

Seo, D. H., Ahn, M. Y., Park, K. Y., Kim, E. Y., and Kim, W. T. (2016). The N-terminal und motif of the arabidopsis U-box E3 ligase pub18 is critical for the negative regulation of aba-mediated stomatal movement and determines its ubiquitination specificity for exocyst subunit Exo70B1. *Plant Cell* 28:2952–2973.

Shi, Y. F., Wang, D. L., Wang, C., Culler, A. H., Kreiser, M. A., Suresh, J., Cohen, J. D., Pan, J., Baker, B., and Liu, J. Z. (2015). Loss of GSNOR1 function leads to compromised auxin signaling and polar auxin transport. *Mol. Plant* 8:1350–1365.

Shibasaki, K., Uemura, M., Tsurumi, S., and Rahman, A. (2009). Auxin response in arabidopsis under cold stress: Underlying molecular mechanisms. *Plant Cell* 21:3823–3838.

Shinohara, N., Taylor, C., and Leyser, O. (2013). Strigolactone Can Promote or Inhibit Shoot Branching by Triggering Rapid Depletion of the Auxin Efflux Protein PIN1 from the Plasma Membrane. *PLoS Biol.* 11.

Sieberer, T., Seifert, G. J., Hauser, M. T., Grisafi, P., Fink, G. R., and Luschnig, C. (2000). Post-transcriptional control of the Arabidopsis auxin efflux carrier EIR1 requires AXR1. *Curr. Biol.* 10:1595–1598.

Šimášková, M., O'Brien, J. A., Khan, M., Van Noorden, G., Ötvös, K., Vieten, A., De Clercq, I., Van Haperen, J. M. A., Cuesta, C., Hoyerová, K., et al. (2015). Cytokinin response factors regulate PIN-FORMED auxin transporters. *Nat. Commun.* 6:8717.

Simon, M. L. A., Platre, M. P., Marquès-Bueno, M. M., Armengot, L., Stanislas, T., Bayle, V., Caillaud, M. C., and Jaillais, Y. (2016). A PtdIns(4)P-driven electrostatic field controls cell membrane identity and signalling in plants. *Nat. Plants* 2:1–10.

Singh, G., Retzer, K., Vosolsobě, S., and Napier, R. (2018). Advances in understanding the mechanism of action of the auxin permease aux1. *Int. J. Mol. Sci.* 19:9–11.

SKOOG, F., and MILLER, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. Exp. Biol.* 11:118–130.

Soundappan, I., Bennett, T., Morffy, N., Liang, Y., Stanga, J. P., Abbas, A., Leyser, O., and Nelsona, D. C. (2015). SMAX1-LIKE/D53 family members enable distinct MAX2-dependent responses to strigolactones and karrikins in arabidopsis. *Plant Cell* 27:3143–3159.

Stepanova, A. N., and Alonso, J. M. (2009). Ethylene signaling and response: where different regulatory modules meet. *Curr. Opin. Plant Biol.* 12:548–555.

Stirnberg, P., van De Sande, K., and Leyser, H. M. O. (2002). MAX1 and MAX2 control shoot lateral branching in Arabidopsis. *Development* 129:1131–41.

Stirnberg, P., Furner, I. J., and Ottoline Leyser, H. M. (2007). MAX2 participates in an SCF complex which acts locally at the node to suppress shoot branching. *Plant J.* 50:80–94.

Street, I. H., Mathews, D. E., Yamburkenko, M. V., Sorooshzadeh, A., John, R. T., Swarup, R., Bennett, M. J., Kieber, J. J., and Schaller, G. E. (2016). Cytokinin acts through the auxin influx carrier AUX1 to regulate cell elongation in the root. *Development* 143:3982–3993.

Sun, J., Xu, Y., Ye, S., Jiang, H., Chen, Q., Liu, F., Zhou, W., Chen, R., Li, X., Tietz, O., et al. (2009). Arabidopsis ASA1 Is Important for Jasmonate-Mediated Regulation of Auxin Biosynthesis and Transport during Lateral Root Formation . *Plant Cell* 21:1495–1511.

Sun, J., Chen, Q., Qi, L., Jiang, H., Li, S., Xu, Y., Liu, F., Zhou, W., Pan, J., Li, X., et al. (2011). Jasmonate modulates endocytosis and plasma membrane accumulation of the arabidopsis pin2 protein. *New Phytol.* 191:360–375.

Swarup, R., and Bhosale, R. (2019). Developmental Roles of AUX1/LAX Auxin Influx Carriers in Plants. *Front. Plant Sci.* 10:1–14.

Swarup, R., and Péret, B. (2012). AUX/LAX family of auxin influx carriers-An overview. *Front. Plant Sci.* 3:1–11.

Swarup, R., Swarup, R., Marchant, A., Marchant, A., Ljung, K., Ljung, K., Sandberg, G., Sandberg, G., Palme, K., Palme, K., et al. (2001). Localization of the auxin permease AUX1suggests two functionally distinct hormone transport pathways operate in the Arabidopsis root apex Ranjan. *Genes Dev.* Advance Access published 2001, doi:10.1101/gad.210501.2648.

Swarup, R., Kargul, J., Marchant, A., Zadik, D., Rahman, A., Mills, R., Yemm, A., May, S., Williams, L., Millner, P., et al. (2004). Structure-function analysis of the presumptive Arabidopsis auxin permease AUX1. *Plant Cell* 16:3069–3083.

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2009). Plant Immunity Requires Conformational Charges of NPR1 via S-Nitrosylation and Thioredoxins. *Science (80-.).* 321:952–957.

Tan, S., Abas, M., Verstraeten, I., Glanc, M., Molnár, G., Hajný, J., Lasák, P., Petřík, I., Russinova, E., Petrášek, J., et al. (2020). Salicylic Acid Targets Protein Phosphatase 2A to Attenuate Growth in Plants. *Curr. Biol.* 30:381-395.e8.

Tanaka, H., Kitakura, S., De Rycke, R., De Groodt, R., and Friml, J. (2009). Fluorescence Imaging-Based Screen Identifies ARF GEF Component of Early Endosomal Trafficking. *Curr. Biol.* 19:391–397.

Tanaka, H., Kitakura, S., Rakusová, H., Uemura, T., Feraru, M. I., de Rycke, R., Robert, S., Kakimoto, T., and Friml, J. (2013). Cell Polarity and Patterning by PIN Trafficking through Early Endosomal Compartments in Arabidopsis thaliana. *PLoS Genet.* 9.

Tejos, R., Sauer, M., Vanneste, S., Palacios-Gomez, M., Li, H., Heilmann, M., van Wijk, R., Vermeer, J. E. M., Heilmann, I., Munnik, T., et al. (2014). Bipolar plasma membrane distribution of phosphoinositides and their requirement for auxin-mediated cell polarity and patterning in Arabidopsis. *Plant Cell* 26:2114–2128.

Testerink, C., and Munnik, T. (2011). Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. *J. Exp. Bot.* 62:2349–2361.

Testerink, C., Dekker, H. L., Lim, Z. Y., Johns, M. K., Holmes, A. B., De Koster, C. G., Ktistakis, N. T., and Munnik, T. (2004). Isolation and identification of phosphatidic acid targets from plants. *Plant J.* 39:527–536.

Thaler, J. S., Humphrey, P. T., and Whiteman, N. K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends Plant Sci.* 17:260–270.

Titapiwatanakun, B., Blakeslee, J. J., Bandyopadhyay, A., Yang, H., Mravec, J., Sauer, M., Cheng, Y., Adamec, J., Nagashima, A., Geisler, M., et al. (2009). ABCB19/PGP19 stabilises PIN1 in membrane microdomains in Arabidopsis. *Plant J.* 57:27–44.

Tognetti, V. B., Bielach, A., and Hrtyan, M. (2017). Redox regulation at the site of primary growth: Auxin, cytokinin and ROS crosstalk. *Plant Cell Environ.* 40:2586–2605.

Ueguchi-Tanaka, M., Nakajima, M., Motoyuki, A., and Matsuoka, M. (2007). Gibberellin Receptor and Its Role in Gibberellin Signaling in Plants. *Annu. Rev. Plant Biol.* 58:183–198.

Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., and Shinozaki, K. (2009). Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 106:17588–17593.

van Berkel, K., de Boer, R. J., Scheres, B., and ten Tusscher, K. (2013). Polar auxin trsansport:

Models and mechanisms. *Dev.* 140:2253–2268.

van den Berg, T., Korver, R. A., Testerink, C., and ten Tusscher, K. H. W. J. (2016). Modeling halotropism: A key role for root tip architecture and reflux loop remodeling in redistributing auxin. *Dev.* 143:3350–3362.

Vandenbussche, F., Petrášek, J., Žádníková, P., Hoyerová, K., Pešek, B., Raz, V., Swarup, R., Bennett, M., Zažímolavá, E., Benkova, E., et al. (2010). The auxin influx carriers AUX1 and LAX3 are involved in auxin-ethylene interactions during apical hook development in Arabidopsis thaliana seedlings. *Development* 137:597–606.

Vanneste, S., and Friml, J. (2009). Auxin: A Trigger for Change in Plant Development. *Cell* 136:1005–1016.

Vanneste, S., and Friml, J. (2013). Calcium: The missing link in auxin action. *Plants* 2:650–675.

Vanstraelen, M., and Benková, E. (2012). Hormonal Interactions in the Regulation of Plant Development. *Annu. Rev. Cell Dev. Biol.* 28:463–487.

Verna, C., Ravichandran, S. J., Sawchuk, M., Linh, N. M., and Scarpella, E. (2019). Coordination of Tissue Cell Polarity by Auxin Transport and Signaling. *Elife* 1:1–30.

Vernoux, T., Besnard, F., and Traas, J. (2010). Auxin at the shoot apical meristem. *Cold Spring Harb. Perspect. Biol.* 2:1–15.

Vieten, A., Vanneste, S., Wiśniewska, J., Benková, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J. (2005). Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* 132:4521–4531.

Voß, U., Bishopp, A., Farcot, E., and Bennett, M. J. (2014). Modelling hormonal response and development. *Trends Plant Sci.* 19:311–319.

Waldie, T., and Leyser, O. (2018). Cytokinin targets auxin transport to promote shoot branching. *Plant Physiol.* 177:803–818.

Wang, C., Hu, T., Yan, X., Meng, T., Wang, Y., Wang, Q., Zhang, X., Gu, Y., Sánchez-Rodríguez, C., Gadeyne, A., et al. (2016). Differential regulation of clathrin and its adaptor proteins during membrane recruitment for endocytosis. *Plant Physiol.* 171:215–229.

Wang, P., Shen, L., Guo, J., Jing, W., Qu, Y., Li, W., Bi, R., Xuan, W., Zhang, Q., and Zhang, W. (2019). Phosphatidic acid directly regulates pinoid-dependent phosphorylation and activation of the pin-formed2 auxin efflux transporter in response to salt stress. *Plant Cell* 31:250–271.

Wasternack, C., and Song, S. (2017). Jasmonates: Biosynthesis, metabolism, and signaling by proteins activating and repressing transcription. *J. Exp. Bot.* 68:1303–1321.

Wei, Z., and Li, J. (2016). Brassinosteroids Regulate Root Growth, Development, and Symbiosis. *Mol. Plant* 9:86–100.

Weiss, D., and Ori, N. (2007). Mechanisms of cross talk between gibberellin and other hormones. *Plant Physiol.* 144:1240–1246.

Wen, X., Zhang, C., Ji, Y., Zhao, Q., He, W., An, F., Jiang, L., and Guo, H. (2012). Activation of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus. *Cell Res.* 22:1613–1616.

Wendehenne, D., Durner, J., and Klessig, D. F. (2004). Nitric oxide: A new player in plant signalling and defence responses. *Curr. Opin. Plant Biol.* 7:449–455.

Willige, B. C., Isono, E., Richter, R., Zourelidou, M., and Schwechheimer, C. (2011). Gibberellin Regulates PIN-FORMED Abundance and Is Required for Auxin Transport–Dependent Growth and Development in Arabidopsis thaliana . *Plant Cell* 23:2184–2195.

Xu, W., Jia, L., Shi, W., Liang, J., Zhou, F., Li, Q., and Zhang, J. (2013). Abscisic acid accumulation modulates auxin transport in the root tip to enhance proton secretion for maintaining root growth under moderate water stress. *New Phytol.* 197:139–150.

Xu, T., Dai, N., Chen, J., Nagawa, S., Cao, M., Li, H., Zhou, Z., Chen, X., Rycke, R. De, Rakusová, H., et al. (2014). Cell Surface ABP1-TMK Auxin-Sensing Complex Activates ROP GTPase Signaling. *Science (80-.).* 343:1025–1029.

Yan, J., Zhang, C., Gu, M., Bai, Z., Zhang, W., Qi, T., Cheng, Z., Peng, W., Luo, H., Nan, F., et al. (2009). The arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. *Plant Cell* 21:2220–2236.

Yang, H., and Murphy, A. S. (2009). Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe. *Plant J.* 59:179–191.

Yang, L., Zhang, J., He, J., Qin, Y., Hua, D., Duan, Y., Chen, Z., and Gong, Z. (2014). ABA-Mediated ROS in Mitochondria Regulate Root Meristem Activity by Controlling PLETHORA Expression in Arabidopsis. *PLoS Genet.* 10.

Ye, Y., Li, Z., and Xing, D. (2012). Sorting out the role of nitric oxide in cadmium-induced Arabidopsis thaliana programmed cell death. *Plant Signal. Behav.* 7:20–22.

Yuan, H. M., Xu, H. H., Liu, W. C., and Lu, Y. T. (2013). Copper regulates primary root elongation through PIN1-mediated auxin redistribution. *Plant Cell Physiol.* 54:766–778.

Yue, R., Tie, S., Sun, T., Zhang, L., Yang, Y., Qi, J., Yan, S., Han, X., Wang, H., and Shen, C. (2015). Genome-wide identification and expression profiling analysis of ZmPIN,ZmPILS,ZmLAX and ZmABCB auxin transporter gene families in maize (Zea mays L.) under various abiotic stresses. *PLoS One* 10:1–23.

Žádníkova, P., Petrášek, J., Marhavý, P., Raz, V., Vandenbussche, F., Ding, Z., Schwarzerová, K., Morita, M. T., Tasaka, M., Hejatko, J., et al. (2010). Role of PIN-mediated auxin efflux in apical hook development of Arabidopsis thaliana. *Development* 137:607–617.

Žádníková, P., Wabnik, K., Abuzeineh, A., Gallemi, M., Van Der Straeten, D., Smith, R. S., Inzé, D., Friml, J., Prusinkiewicz, P., and Benková, E. (2016). A model of differential growth-guided apical hook formation in plants. *Plant Cell* 28:2464–2477.

Zegzouti, H., Li, W., Lorenz, T. C., Xie, M., Payne, C. T., Smith, K., Glenny, S., Payne, G. S., and

Christensen, S. K. (2006). Structural and functional insights into the regulation of Arabidopsis AGC VIIIa kinases. *J. Biol. Chem.* 281:35520–35530.

Zemlyanskaya, E. V., Omelyanchuk, N. a., Ubogoeva, E. V., and Mironova, V. V. (2018). Deciphering auxin-ethylene crosstalk at a systems level. *Int. J. Mol. Sci.* 19:1–15.

Zhang, W., To, J. P. C., Cheng, C. Y., Eric Schaller, G., and Kieber, J. J. (2011). Type-A response regulators are required for proper root apical meristem function through post-transcriptional regulation of PIN auxin efflux carriers. *Plant J.* 68:1–10.

Zhang, Y., Xiao, G., Wang, X., Zhang, X., and Friml, J. (2019). Evolution of fast root gravitropism in seed plants. *Nat. Commun.* 10:4–13.

Zhang, X., Adamowski, M., Marhava, P., Tan, S., Zhang, Y., Rodriguez, L., Zwiewka, M., Pukyšová, V., Sánchez, A. S., Raxwal, V. K., et al. (2020a). Arabidopsis Flippases Cooperate with ARF GTPase Exchange Factors to Regulate the Trafficking and Polarity of PIN Auxin Transporters. *Plant Cell* 12:tpc.00869.2019.

Zhang, X. P., Ma, C. X., Sun, L. R., and Hao, F. S. (2020b). Roles and mechanisms of Ca in regulating primary root growth of plants. *Plant Signal. Behav.* 00.

Zhao, Z., Andersen, S. U., Ljung, K., Dolezal, K., Miotk, A., Schultheiss, S. J., and Lohmann, J. U. (2010). Hormonal control of the shoot stem-cell niche. *Nature* 465:1089–1092.

Zhao, S., Wu, Y., He, Y., Wang, Y., Xiao, J., Li, L., Wang, Y., Chen, X., Xiong, W., and Wu, Y. (2015). RopGEF2 is involved in ABA-suppression of seed germination and post-germination growth of Arabidopsis. *Plant J.* 84:886–899.

Zhou, J. J., and Luo, J. (2018). The PIN-FORMED auxin efflux carriers in plants. *Int. J. Mol. Sci.* 19:1–21.

Zhou, F., Lin, Q., Zhu, L., Ren, Y., Zhou, K., Shabek, N., Wu, F., Mao, H., Dong, W., Gan, L., et al. (2013). D14-SCF D3 -dependent degradation of D53 regulates strigolactone signalling. *Nature* 504:406–410.

Zhu, Q., Gallemí, M., Pospíšil, J., Žádníková, P., Strnad, M., and Benková, E. (2019). Root gravity response module guides differential growth determining both root bending and apical hook formation. *Development* Advance Access published 2019, doi:10.1242/dev.175919.

Zourelidou, M., Müller, I., Willige, B. C., Nill, C., Jikumaru, Y., Li, H., and Schwechheimer, C. (2009). The polarly localized D6 PROTEIN KINASE is required for efficient auxin transport in Arabidopsis thaliana. *Development* 136:627–636.

Zourelidou, M., Absmanner, B., Weller, B., Barbosa, I. C. R., Willige, B. C., Fastner, A., Streit, V., Port, S. A., Colcombet, J., van Bentem, S. de la F., et al. (2014). Auxin efflux by PIN-FORMED proteins is activated by two different protein kinases, D6 PROTEIN KINASE and PINOID. *Elife* 2014:1–25.

Zubo, Y. O., and Schaller, G. E. (2020). Role of the cytokinin-activated type-B response regulators in hormone crosstalk. *Plants* 9.

Zwiewka, M., Bilanovičová, V., Seifu, Y. W., and Nodzyński, T. (2019a). The Nuts and Bolts of

PIN Auxin Efflux Carriers. *Front. Plant Sci.* 10.

Zwiewka, M., Bielach, A., Tamizhselvan, P., Madhavan, S., Ryad, E. E., Tan, S., Hrtyan, M., Dobrev, P., Vanková, R., Friml, J., et al. (2019b). Root Adaptation to H2O2-Induced Oxidative Stress by ARF-GEF BEN1- and Cytoskeleton-Mediated PIN2 Trafficking. *Plant Cell Physiol.* 60:255–273.

Molecular Mechanisms of the Cytokinin-Regulated Endomembrane Trafficking to Coordinate Plant Organogenesis

Molecular Mechanisms of the Cytokinin-Regulated Endomembrane Trafficking to Coordinate Plant Organogenesis

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Plants maintain the capacity to develop new organs post-embryonically throughout their whole life and thereby flexibly adapt their growth and development to ever-changing environmental conditions. They build a convoluted root system that enables effective exploitation of the soil nutrients by recurrent initiation and development of new lateral organs. The lateral root organogenesis is under control of complex regulatory networks including plant hormones. Auxin and cytokinin are one of the key hormonal regulators of this developmental process. Whereas auxin acts as a positive regulator of the lateral root initiation and development, cytokinin suppresses both phases of the lateral root organogenesis (Cavallari et al., 2021). These opposing activities must be tightly coordinated. Therefore, auxin and cytokinin balance their activities through mutual regulation of their metabolic (Jones et al., 2010) and signaling pathways (Müller & Sheen, 2008; Zhao et al., 2010). Additionally, cytokinin-mediated regulation of the auxin transport has been identified as an important cross-talk intersection. In particular, cytokinin was found to influence cell-to-cell auxin transport by modification of the expression of several auxin transport components and thus to modulate auxin distribution within the root. Dissection of the molecular mechanisms revealed that cytokinin fine-tunes transcription of several homologs of PIN auxin efflux carrier family (Dello Ioio et al., 2008; Ruzicka et al., 2009; Šimášková et al., 2015,Pernisova et al., 2016)

Importantly, cytokinin was also shown to interfere with endocytic trafficking of PIN1 and redirect it for lytic degradation into vacuoles (Marhavý et al., 2011, 2014). The cytokinin-mediated regulation of PIN1 trafficking was shown to be independent of transcription and translation. This observation suggests existence of a conceptually novel cytokinin activity mode, very different from known signal transduction cascade, typically results in the downstream response on the level of transcription. However, the molecular mechanism mediating such cytokinin activity is unknown.

Vesicular trafficking as a platform for hormonal cross-talk

Cell-biological studies revealed that PINs, auxin efflux transporters, undergo constitutive cycles of endocytosis and recycling back to the plasma membrane (Niko Geldner et al., 2001, 2003). The abundance of PIN proteins at the plasma membrane determines the efficiency and the direction of auxin flow, thereby regulates the availability of the hormone in cells and tissues (Adamowski & Friml, 2015; Naramoto, 2017). The trafficking of PIN proteins depends on the complex subcellular trafficking machinery including the coat protein clathrin (Dhonukshe et al., 2007; Kitakura et al., 2011), ADP-ribosylation factor guanine-nucleotide exchange factors (ARF-GEFs) (Geldner et al., 2001; Kleine-Vehn et al., 2008), ARF-GTPase-activating protein VASCULAR NETWORK DEFECTIVE3 (Naramoto et al., 2010), the related ARF-GEF GNOM-LIKE1 (Teh & Moore, 2007), and small GTPase RabA1b (Feraru et al., 2012). The early endosomal trafficking of PINs is controlled by another ARF-GEF, BFA-visualized endocytic trafficking defective1 (BEN1), and the Sec1/Munc18 family protein BEN2 (Tanaka et al., 2009, 2013).

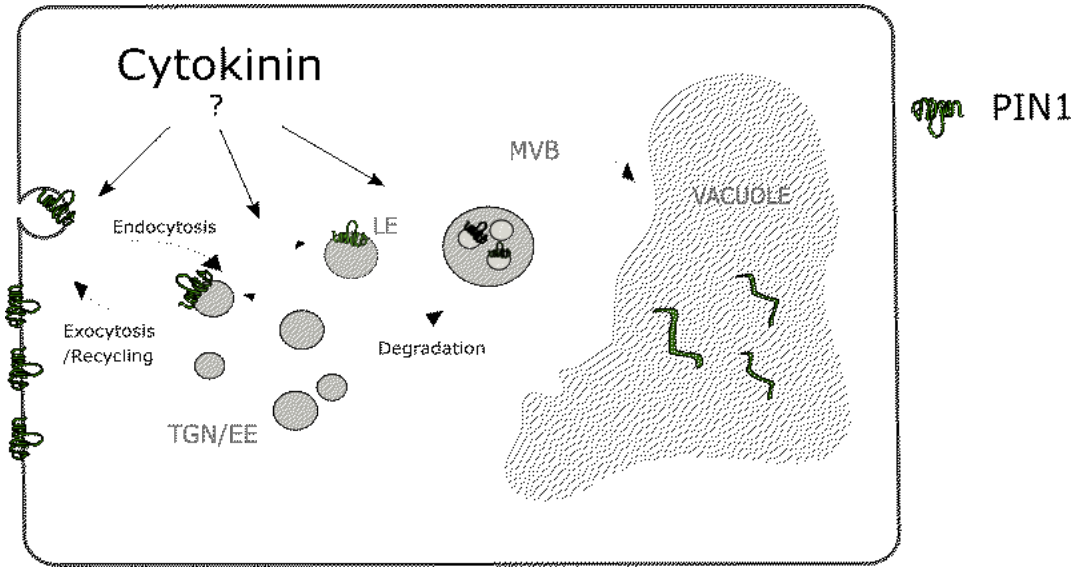

Figure 1. Model of the plant cell depicting various endomembrane trafficking processes of PIN1 that might be the target of cytokinin action. *TGN/EE = trans-Golgi network, LE = late endosome, MVB = multivesicular bodies*

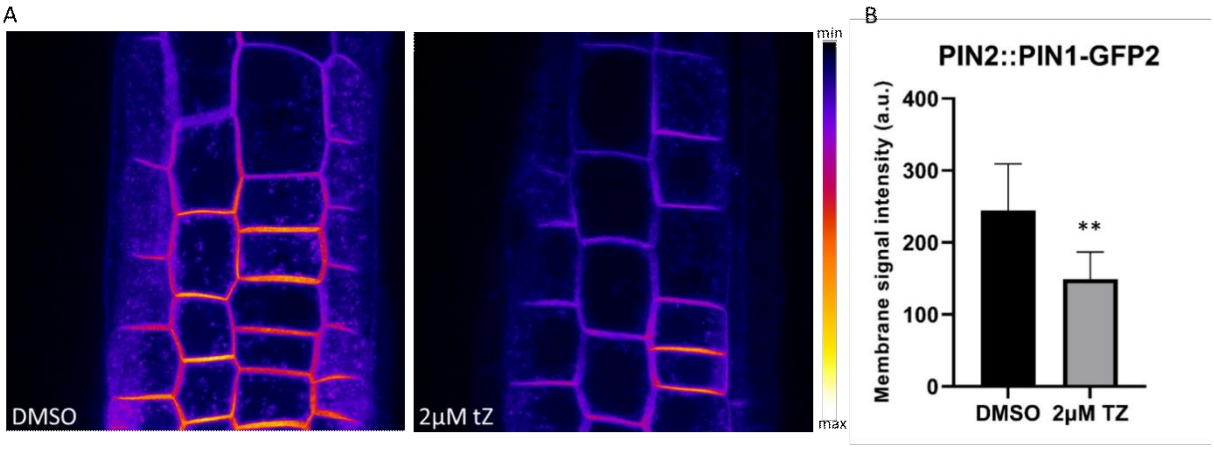

Figure 2. PIN1 degradation upon cytokinin treatment *(A) Confocal image of epidermal cells of 5-days-old seedlings expressing PIN2::PIN1-GFP2 after treatment with mock (DMSO) or cytokinin (trans-Zeatin). (B) Quantification of membrane signal in atrichoblasts. The bars represent mean ± s.d., ** = p < 0.01, by Student's t-test, n > 5.*

The regulation of the PIN trafficking provides an important platform for hormonal cross-talk. All major plant hormones, including auxin, strigolactones, gibberellins, salicylic acid, jasmonic acid, brassinosteroids, and cytokinin, affect various growth and developmental processes through modulation of the PIN intracellular trafficking (Semeradova et al., 2020).

In my thesis, I focus on obtaining molecular insights into cytokinin-regulated PIN1 trafficking, which is a developmentally important convergence point of cytokinin and auxin pathways in control of plant postembryonic organogenesis (Figure1, Marhavý et al., 2011, 2014).

Results

Cytokinin treatment triggers depletion of PIN1 from the plasma membrane

We monitored the impact of cytokinin on the subcellular trafficking of PIN1 protein and other membrane markers. For an optimal imaging set-up, we chose to observe the PIN1 expressed in epidermal cells of the primary root meristem (driven by PIN2 promoter, Wisniewska et al., 2006), which have the major advantage of accessibility for microscopy. 5-days-old plants were transferred on the media supplemented with DMSO/2µM cytokinin (trans-Zeatin, tZ) and incubated for 6 hours. We can observe that the PIN1-GFP signal is depleted from the membrane after cytokinin treatment compared to mock (Figure 2, Marhavý et al., 2011, 2014).

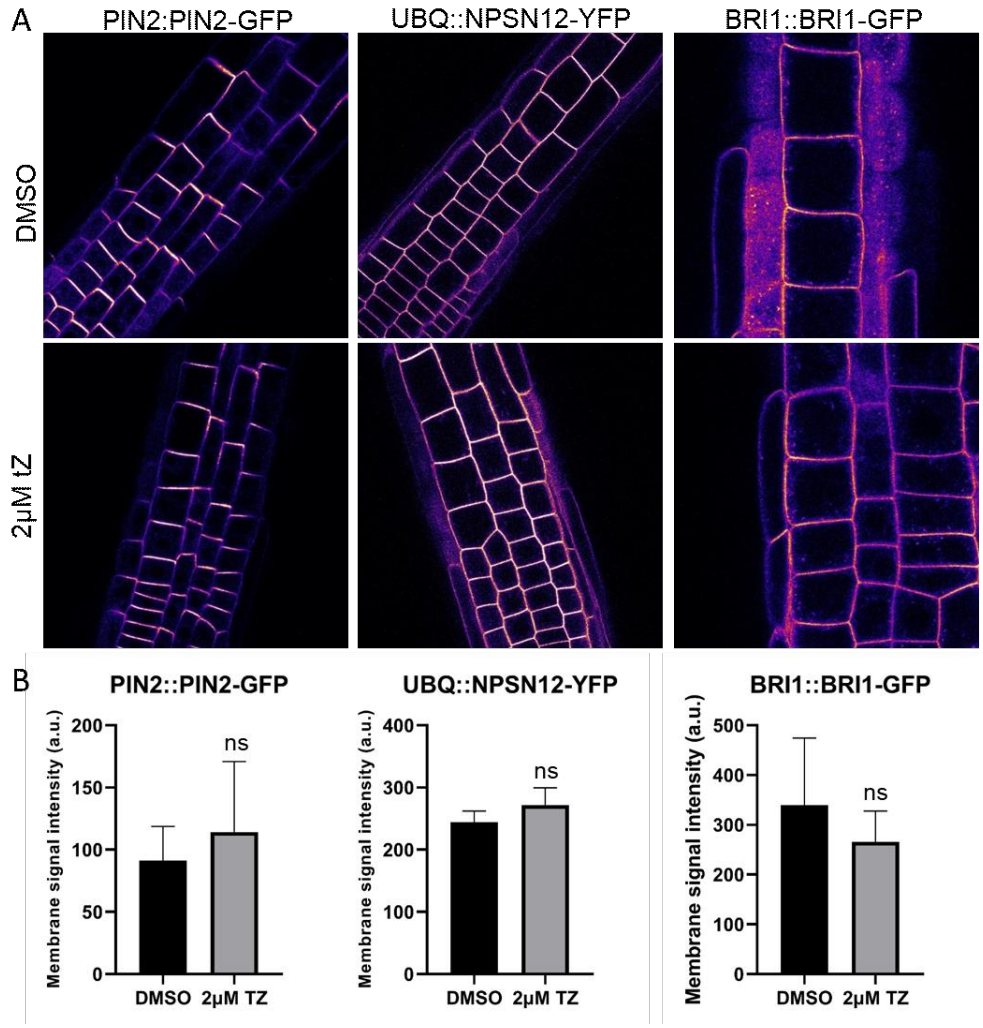

Figure3. Cytokinin treatment of plasma membrane markers *(A) Confocal images of epidermal cells expressing various membrane markers after treatment with mock (DMSO) or cytokinin (Trans-Zeatin) for 6 hours (B) Quantification of the membrane signal. The bars represent mean ± s.d., n.s. = p > 0.05 by Student's t-test, n > 5.*

To confirm the specificity of the cytokinin effect, we observed other membrane markers such as PIN2-GFP (Xu & Scheres, 2005), NOVELE PLANT SNARE W131-YFP (Geldner et al., 2009) or the brassinosteroid receptor BRI1-GFP (Geldner et al., 2007). None of them showed a significant decrease of the membrane signal upon cytokinin treatment (Figure 3).

Search for molecular players involved in the cytokinin effect on endomembrane trafficking

The main objective of this thesis is to gain insights into the molecular machinery underlying cytokinin effect on the endomembrane trafficking in the plant cell, in particular on the PIN1

degradation. In order to find new players involved in this effect, we conducted two large proteomic screens:

1) Identification of Cytokinin Binding Proteins using Chemical Proteomics (Chapter 1)
2) Monitoring of Proteomic and Phosphoproteomic Changes in *Arabidopsis thaliana* Roots upon Treatment with Cytokinin (Chapter 2, Chapter 3)

The experimental design, execution, and results of these screens are described in detail in further chapters of this thesis.

Methods

Plant material

Plant material used in this study: PIN2::PIN1-GFP2 (Wisniewska et al., 2006), PIN2::PIN2-GFP ((Xu & Scheres, 2005), UBQ::NPSN12-YFP (W131, Geldner et al., 2009), BRI1::BRI1-GFP (Geldner et al., 2007).

Growth conditions

Surface sterilized Arabidopsis thaliana seeds were plated on half strength Murashige and Skoog (MS) media (Duchefa) with 1%(w/v) sucrose and 1% (w/v) agar (pH=5.9). Seeds were stratified for 2 days at 4°C, in the dark. Seedlings were grown vertically in long-day conditions (16h light/8h dark) at 21°C. As light sources light emitting diode production modules (Philips GreenPower) were used in a deep red, far-red, blue combination with a photon density of 140 µmol/m^2/s ± 20%.

Hormonal treatment

Treatments were performed on half strength Murashige and Skoog (MS) media (Duchefa) with 1%(w/v) sucrose and 1% (w/v) agar (pH=5.9). Short term treatments were performed by transfer of plants on media supplemented with 2µM TZ trans-Zeatin (tZ, Sigma) or DMSO for 6 hours.

Confocal Microscopy (microscope specification)

5 days old seedlings were mounted into a microcopy chamber (Nunc™ Lab-Tek™ Chambered Coverglass, Thermo Fisher Scientific). Confocal images were obtained with LSM 800 laser scanning confocal microscopes (Zeiss) equipped with a Plan-Apochromat 40x/1.2 water immersion objective. Fluorescence markers were excited at 488 nm.

Data analysis and Statistics

Data were analyzed using ImagJ software (National Institute of Health, http://rsb.info.nih.gov/ij), GraphPad Prism 8, and Microsoft PowerPoint programs. All data were analysed using Student's t test.

References:

Adamowski, M., & Friml, J. (2015). PIN-Dependent Auxin Transport: Action, Regulation, and Evolution. *The Plant Cell Online*, *27*(1), 20–32. https://doi.org/10.1105/tpc.114.134874

Cavallari, N., Artner, C., & Benkova, E. (2021). Auxin-Regulated Lateral Root Organogenesis. *Cold Spring Harbor Perspectives in Biology*, a039941. https://doi.org/10.1101/cshperspect.a039941

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., Aoyama, T., Costantino, P., & Sabatini, S. (2008). A genetic framework for the control of cell

division and differentiation in the root meristem. *Science*, *322*(5906), 1380–1384. https://doi.org/10.1126/science.1164147

Dhonukshe, P., Aniento, F., Hwang, I., Robinson, D. G., Mravec, J., Stierhof, Y. D., & Friml, J. (2007). Clathrin-Mediated Constitutive Endocytosis of PIN Auxin Efflux Carriers in Arabidopsis. *Current Biology*, *17*(6), 520–527. https://doi.org/10.1016/j.cub.2007.01.052

Feraru, E., Feraru, M. I., Asaoka, R., Paciorek, T., De Rycke, R., Tanaka, H., Nakano, a., & Friml, J. (2012). BEX5/RabA1b Regulates trans-Golgi Network-to-Plasma Membrane Protein Trafficking in Arabidopsis. *The Plant Cell*, *24*(7), 3074–3086. https://doi.org/10.1105/tpc.112.098152

Geldner, N, Friml, J., Stierhof, Y. D., Jürgens, G., & Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature*, *413*(6854), 425–428. https://doi.org/10.1038/35096571

Geldner, Niko, Dénervaud-Tendon, V., Hyman, D. L., Mayer, U., Stierhof, Y.-D., & Chory, J. (2009). Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set. *The Plant Journal*, *59*(1), 169–178. https://doi.org/10.1111/j.1365-313X.2009.03851.x

Geldner, Niko, Friml, J., Stierhof, Y. D., Jürgens, G., & Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature*, *413*(6854), 425–428. https://doi.org/10.1038/35096571

Geldner, Niko, Hyman, D. L., Wang, X., Schumacher, K., & Chory, J. (2007). Endosomal signaling of plant steroid receptor kinase BRI1. *Genes and Development*, *21*(13), 1598–1602. https://doi.org/10.1101/gad.1561307

Jones, B., Ljung, K., Gunnerås, S. A., Petersson, S. V., Tarkowski, P., Graham, N., May, S., Dolezal, K., & Sandberg, G. (2010). Cytokinin regulation of auxin synthesis in Arabidopsis involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction. *Plant Cell*, *22*(9), 2956–2969. https://doi.org/10.1105/tpc.110.074856

Kitakura, S., Vanneste, S., Robert, S., Löfke, C., Teichmann, T., Tanaka, H., & Friml, J. (2011). Clathrin mediates endocytosis and polar distribution of PIN auxin transporters in Arabidopsis. *Plant Cell*, *23*(5), 1920–1931. https://doi.org/10.1105/tpc.111.083030

Kleine-Vehn, J., Leitner, J., Zwiewka, M., Sauer, M., Abas, L., Luschnig, C., & Friml, J. (2008). Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(46), 17812–17817. https://doi.org/10.1073/pnas.0808073105

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., & Benková, E. (2011). Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis. *Developmental Cell*, *21*(4), 796–804. https://doi.org/10.1016/j.devcel.2011.08.014

Marhavý, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J., Schwechheimer, C., Murphy, A., & Benková, E. (2014). Cytokinin Controls Polarity of

PIN1-Dependent Auxin Transport during Lateral Root Organogenesis. *Current Biology*, *24*(9), 1031–1037. https://doi.org/10.1016/j.cub.2014.04.002

Müller, B., & Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature*, *453*(7198), 1094–1097. https://doi.org/10.1038/nature06943

Naramoto, S. (2017). Polar transport in plants mediated by membrane transporters: focus on mechanisms of polar auxin transport. *Current Opinion in Plant Biology*, *40*, 8–14. https://doi.org/10.1016/j.pbi.2017.06.012

Naramoto, S., Kleine-Vehn, J., Robert, S., Fujimoto, M., Dainobu, T., Paciorek, T., Ueda, T., Nakano, A., Van Montagu, M. C. E., Fukuda, H., & Friml, J. (2010). ADP-ribosylation factor machinery mediates endocytosis in plant cells. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(50), 21890–21895. https://doi.org/10.1073/pnas.1016260107

Niko Geldner, 1, Nadine Anders, 1 Hanno Wolters, 1, Jutta Keicher, 1, Wolfgang Kornberger, 1, Philippe Muller, 2, Alain Delbarre, 2, Takashi Ueda, 3, Akihiko Nakano, 3, & and Gerd Jurgens. (2003). The Arabidopsis GNOM ARF-GEF Mediates Endosomal Recycling, Auxin Transport, and Auxin-Dependent Plant Growth Niko. *Cell*, *112*(5), 219–230. https://doi.org/tpc.111.083030 [pii] 10.1105/tpc.111.083030

Ruzicka, K., Simásková, M., Duclercq, J., Petrásek, J., Zazímalová, E., Simon, S., Friml, J., Van Montagu, M. C. E., & Benková, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(11), 4284–4289. https://doi.org/10.1073/pnas.0900060106

Semeradova, H., Montesinos, J. C., & Benkova, E. (2020). All Roads Lead to Auxin: Post-translational Regulation of Auxin Transport by Multiple Hormonal Pathways. *Plant Communications*, *1*(3), 100048. https://doi.org/10.1016/j.xplc.2020.100048

Šimášková, M., O'Brien, J. A., Khan, M., Van Noorden, G., Ötvös, K., Vieten, A., De Clercq, I., Van Haperen, J. M. A., Cuesta, C., Hoyerová, K., Vanneste, S., Marhavý, P., Wabnik, K., Van Breusegem, F., Nowack, M., Murphy, A., Friml, J., Weijers, D., Beeckman, T., & Benková, E. (2015). Cytokinin response factors regulate PIN-FORMED auxin transporters. *Nature Communications*, *6*, 8717. https://doi.org/10.1038/ncomms9717

Tanaka, H., Kitakura, S., De Rycke, R., De Groodt, R., & Friml, J. (2009). Fluorescence Imaging-Based Screen Identifies ARF GEF Component of Early Endosomal Trafficking. *Current Biology*, *19*(5), 391–397. https://doi.org/10.1016/j.cub.2009.01.057

Tanaka, H., Kitakura, S., Rakusová, H., Uemura, T., Feraru, M. I., de Rycke, R., Robert, S., Kakimoto, T., & Friml, J. (2013). Cell Polarity and Patterning by PIN Trafficking through Early Endosomal Compartments in Arabidopsis thaliana. *PLoS Genetics*, *9*(5). https://doi.org/10.1371/journal.pgen.1003540

Teh, O. K., & Moore, I. (2007). An ARF-GEF acting at the Golgi and in selective endocytosis in polarized plant cells. *Nature*, *448*(7152), 493–496. https://doi.org/10.1038/nature06023

Wisniewska, J., Xu, J., Seifartová, D., Brewer, P. B., Růžička, K., Blilou, L., Rouquié, D., Benková, E., Scheres, B., & Friml, J. (2006). Polar PIN localization directs auxin flow in plants. *Science*, *312*(5775), 883. https://doi.org/10.1126/science.1121356

Xu, J., & Scheres, B. (2005). Cell polarity: ROPing the ends together. *Current Opinion in Plant Biology*, *8*(6), 613–618. https://doi.org/10.1016/j.pbi.2005.09.003

Zhao, Z., Andersen, S. U., Ljung, K., Dolezal, K., Miotk, A., Schultheiss, S. J., & Lohmann, J. U. (2010). Hormonal control of the shoot stem-cell niche. *Nature*, *465*(7301), 1089–1092. https://doi.org/10.1038/nature09126

Identification of Cytokinin Binding Proteins using Chemical Proteomics

Identification of Cytokinin Binding Proteins using Chemical Proteomics

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Introduction

Cytokinin receptors

Cytokinins (CKs) are fundamental regulators of plant growth, development, and responses to the environment. They play role in embryogenesis, cell division and differentiation, activity of the root and shoot apical meristems, formation of lateral organs, and others (Osugi and Sakakibara, 2015; Kieber and Schaller, 2018; Wyblouw and De Rybel, 2019; Li et al., 2021).

Cytokinin was first identified as an active cell division and growth altering factor in early 50's of the last century (Skoog and Tsui, 1951). This pioneering work was followed by the isolation of cytokinin along with characterization of its structure and basic biological function (reviewed in Kamínek, 2015). Once the cytokinin was recognized as an integral regulator of plant growth, the search for the cellular machinery that perceives cytokinin began. Despite many cytokinin-binding molecules were identified, in e.g. chinese-cabbage (Berridge et al., 1970), wheat germ (Erion and Fox, 1981) or carrot cell suspension (Kobayashi et al., 1981), it soon became apparent that they do not have the activity of a receptor (Firn, 1987).

The discovery of the actual cytokinin receptors was accelerated by identification of ETR1, the receptor for ethylene. ETR1 is a part of a two-component signaling pathway previously known from bacteria and yeast (Chang et al., 1993). In principle, the two-component signaling pathway is activated upon binding of the ligand to the receptor, followed by the autophosphorylation of the receptor. The phosphoryl group is then transferred to the

phosphotransfer protein that further transfers the phosphoryl group to transcription factors. These transcription factors then ultimately regulate the downstream response. Strikingly, overexpression of CKI1, a homologous kinase to ETR1, allowed CK-independent growth of *Arabidopsis* cultured tissues, suggesting that CK signaling is also transduced by a two-component pathway (Kakimoto, 1996). Moreover, research of gene expression regulation identified that cytokinin-induced transcription factors *ARABIDOPSIS RESPONSE REGULATORS* (ARRs) are similar to two-component response regulators in bacteria (D'Agostino et al., 2000).

Having this knowledge, the cytokinin two-component signaling pathway was almost fully discovered. However, the actual sensor of cytokinin was still missing because CKI1 does not contain a CK-binding domain and does not bind CK in vitro (Yamada et al., 2001). Finally, the first authentic CK receptor *CYTOKININ RESISTANT 1 (CRE1)/ARABIDOPSIS HISTIDINE KINASE 4 (AHK4)* was found using genetic screen for altered cytokinin sensitivity in a collection of *Arabidopsis* mutant plants. An elegant functional confirmation was performed by the rescue of yeast histidine kinase mutant with *CRE1* and supply of cytokinin (Inoue et al., 2001). A complementary study elucidated the whole signaling pathway by its reconstitution in mesophyll protoplasts from Arabidopsis leaves. This study presented confirmation of CRE1 activity in the cytokinin-dependent signaling and introduced two other histidine kinase receptors for cytokinin, AHK2 and AHK3 (Hwang and Sheen, 2001).

In conclusion, the current model of Arabidopsis cytokinin signal transduction pathway proposes cytokinin sensing by histidine kinase receptors, transfer of the signal to ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER proteins (AHPs), translocation of AHPs to the nucleus, subsequent type-B ARR-dependent transcriptional activation, and a negative feedback regulation by the type-A ARRs (reviewed in Kakimoto, 2003; Keshishian and Rashotte, 2015; Kieber and Schaller, 2018; Li et al., 2021).

Binding of the cytokinin to AHKs was further characterized biochemically (Yamada et al., 2001; Romanov et al., 2006; Lomin et al., 2011), structurally (Hothorn et al., 2011), and evolutionarily (Heyl et al., 2007). What remained elusive was the localization of cytokinin receptors within the cell. AHKs are membrane-localized proteins. The initial speculation assumed a plasma membrane localization of receptors sensing the extracellular space, analogically to those in bacteria and yeast (Hwang and Sheen, 2001; Inoue et al., 2001). The

PM localization was also experimentally supported (Kim et al., 2006). However, later on, evidences for the localization rather on the ER have accumulated (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011). Recently, the idea of signaling from the PM was revisited (Zürcher et al., 2016) and extracellular sensing of cytokinin was experimentally demonstrated (Antoniadi et al., 2020; Kubiasová et al., 2020) indicating dual localization of cytokinin receptors both on the ER and the PM.

Cytokinin binding proteins

In parallel to advances in understanding of cytokinin perception by histidine kinases, it has been still speculated, whether cytokinin can conduct part of its activity also more directly, by binding to proteins and influencing their properties (function, stability, localization…). Initially, cytokinin binding proteins were isolated from various plant species *e.g.* from microsomal fraction of carrot suspension cells (Kobayashi et al., 1981) or protonemata of *Funaria hygrometrica* (Gardner et al., 1978). These studies offered mainly information about molecular weight, dissociation constant, and other biochemical characteristics of isolated proteins. Later studies found cytokinin binding factor in wheat germ (*Triticum durum*) CBF-1, a homolog of vicilin like seed storage protein (Erion and Fox, 1981; Brinegar et al., 1985; Brinegar et al., 1988), which suggests a role of cytokinin binding during grain development. Binding of cytokinin to this protein was recently confirmed by modern methods (Simerský et al., 2017). In tobacco (*Nicotiana sylvestris*) leaves, cytokinin binding protein 57 (CBP57) was found. It is a homolog of S-Adenosyl-L-homocystein (SAH) hydrolase, which catalyses hydrolysis of SAH into homocysteine and adenosine. SAH is a competitive inhibitor of methyl transfer reactions, therefore the CBP57 might have a role in methylation of proteins or DNA by regulating the amount of SAH (Mitsui et al., 1996). In tobacco (*Nicotiana tabacum*) callus, cytokinin binding protein 1 and 2 (CBP1 and CBP2) were found. They share a homology with endochitinase and osmotin-like protein (OLP), respectively (Kobayashi et al., 2000). Since OLP is predicted to act in pathogen response and abiotic stress (Viktorova et al., 2012), CBP2 might play a role in cytokinin-governed stress response. Cytokinin-specific binding protein (CSBP), isolated from the soluble fraction of mung bean (*Vigna radiate*), shares partial homology with pathogenesis-related protein family PR-10 (Fujimoto et al., 1998). The crystal structure of CSBP was solved and the binding pocket for cytokinin ligand was verified (Pasternak et al.,

2006). However, later on the specificity of binding to cytokinin was challenged when it was shown that the binding pocket of CSBP can also binds gibberellins (Ruszkowski et al., 2014). Binding of PR-10 related proteins to cytokinin was shown also in *Medicago truncatula* (MtN13) and *Lupinus luteus (LlPR-10.2B)* with suggested role in nodulation or as a cytokinin reservoir, respectively (Fernandes et al., 2008; Fernandes et al., 2009; Ruszkowski et al., 2013). Functional characterization of cytokinin binding protein 70 (CBP70) from maize (*Zea mays*), revealed its nuclear localization, involvement in activation of transcription *in vitro*, and suggested that CBP70 is an elongation factor (Shepelyakovskaya et al., 2002; Brovko et al., 2007; Brovko et al., 2010). Additionally, in *Arabidopsis thaliana* leaves, a nuclear localized 67 KDa cytokinin binding protein participating in translation was found (Selivankina et al., 2004).

Although these studies of cytokinin binding proteins offer a homology-based assumption of a protein function and in-detail characterization of binding sites, an explanation or confirmation of functional consequences of cytokinin binding to these proteins remains to be discovered.

In *Arabidiopsis*, there are cytokinin effects, which might be independent of changes of the gene expression. In particular, cytokinin promotes the degradation of auxin transporter PIN1 even in presence of chemical inhibitors of transcription and translation (Marhavý et al., 2011). Moreover, recent study revealed the effect of cytokinin on microtubule dynamics in animal cells, which lack homologous two-component histidine-kinase receptor pathway that is responsible for cytokinin signaling in plants (Montesinos et al., 2020). In addition, there are examples of alternative signaling pathways of other hormones. Auxin binds Auxin binding protein 1 (ABP1) and by that regulates downstream cellular responses. However, despite multiple development-altering phenotypes of ABP1 gain-of-function lines, the biological significance of auxin binding to ABP1 remains elusive (Gelová et al., 2021). Additionally, recent findings revealed a plasma membrane-originating auxin signaling through TMK1 (Cao et al., 2019) acting in parallel to cytosolic, TIR-dependent canonical pathway (Weijers and Wagner, 2016).

Therefore, it is likely that there might be another, non-canonical mechanism through which cytokinin can influence biological processes in the cell. To tackle this question, we implemented chemical proteomic approach followed by mass-spectrometry analysis. Using recently developed experimental set-up (Simerský et al., 2017), we performed affinity

purification of cytokinin binding proteins from *Arabidopsis thaliana* roots. The mass spectrometry analysis identified 224 putative cytokinin-binding proteins. Twelve candidate proteins were detected with multiple spectra in multiple replicates. One third of these are proteins involved in vesicle formation or sorting. The best-scored candidate with the highest fold change was dynamin-related protein 2A (DRP2A). DRP2A-GFP shows decrease of the signal upon cytokinin treatment and *drp2a-1* mutant exhibits increased cytokinin sensitivity. Altogether, our data suggest possible cytokinin binding to DRP2A and the role of DRP2A in the cytokinin regulated plant growth and development.

Results

Cytokinins modified with a linker maintain hormonal activity

Chemical structure of cytokinins composes of a characteristic adenine-derived purine ring with a substitution group on the N^6 atom (Figure 1A). Majority of naturally occurring cytokinins have isoprenoid-derivate substitution on the N^6 (isopentenyladenine, trans-Zeatin), whereas synthetic cytokinins contain aromatic moiety as a side group (kinetin, benzylaminopurine). We modified either isoprenoid or aromatic types of cytokinin molecules by substitution of C^2 or N^9 hydrogen with a linker, to immobilize them on beads as a bait for cytokinin binding proteins. Compounds VM12, VM2, and VM20 were generated as derivatives of isopentenyladenine (iP), JK11, JK5 and JK10 from benzylaminopurine (BAP), and VM5 was obtained as a derivative of kinetin (Figure 1A).

Typically, enhanced activity of cytokinin leads to severe reduction of the primary root growth and inhibition of lateral root development (Laplaze et al., 2007). Since the linker attached to the cytokinin molecule might interfere with its biological activity, we tested whether effects of modified cytokinins on growth and development of Arabidopsis roots are comparable to these of iP and BAP.

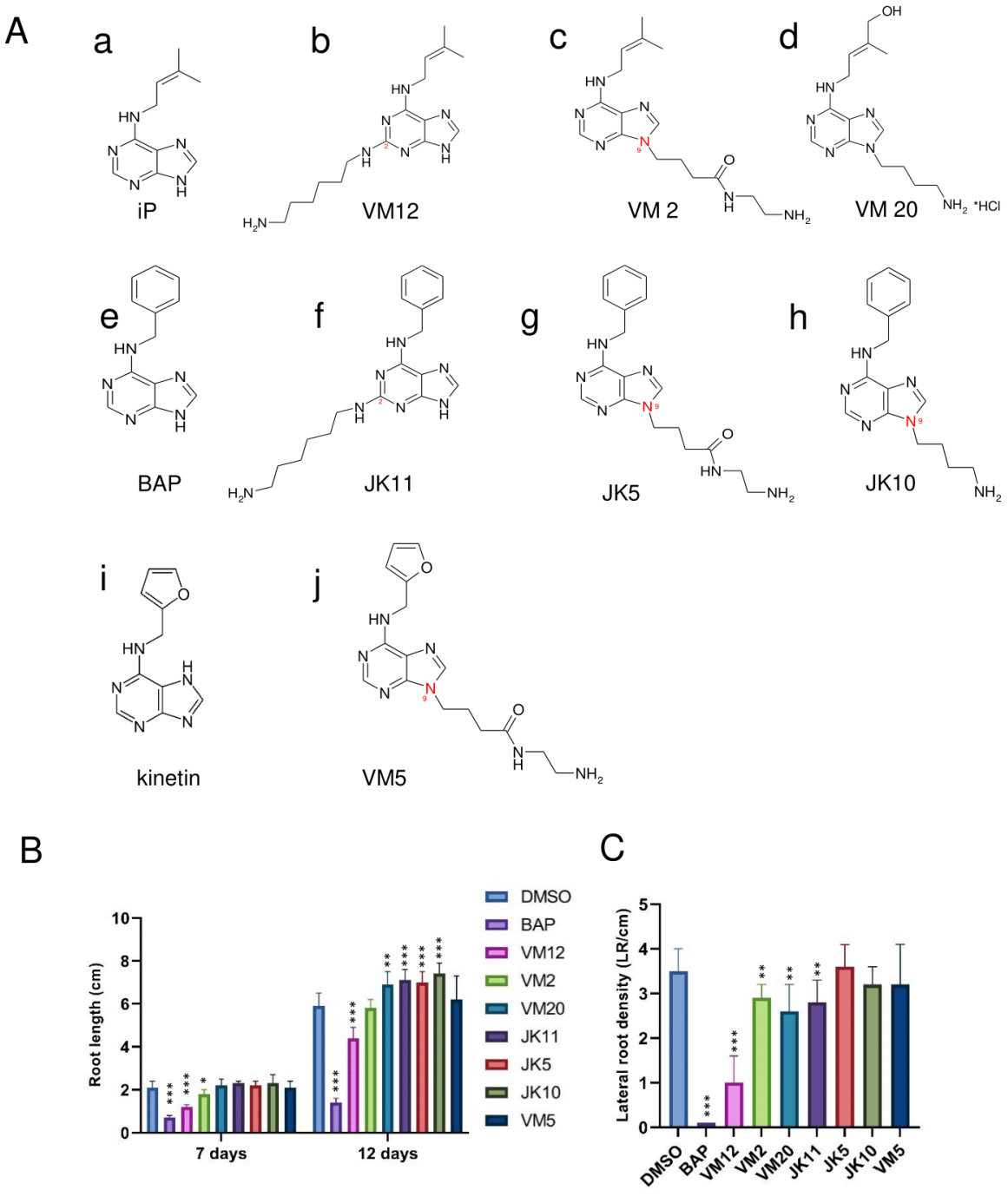


Figure 1: Modified cytokinin compounds and their effect on the plant growth and development *(A) Chemical structures of classical cytokinins (a,e,i) and modified cytokinin compounds (b,c,d,f,g,h,j). (B)Root length of 7- and 12- days old Col-0 plants grown on media supplemented with 1µM, BAP, VM12, VM2, JK11, JK5, VM5, VM20, JK10, and DMSO. (C) Lateral root density of 12- days old Col-0 plants grown on media supplemented with 1µM, BAP, VM12, VM2, JK11, JK5, VM5, VM20, JK10, and DMSO. The bar charts represent mean ± s.d., *** = p < 0.001, ** = p < 0.01, * = p < 0.05 by Student's t-test, n>6.*

First, we assessed the root length and the lateral root density of 7- and 12-days-old plants grown on media supplemented with either BAP or modified cytokinin molecules at 1µM concentration (Figure 1B, C). Treatment with VM12 and VM2 resulted in a significant reduction of the main root length when compared to control treatment. Notably, the inhibitory effects of both molecules were weaker when compared to treatment with BAP, and, the root growth reduction by VM2 was less profound than by VM12. Interestingly, VM20, JK11, JK5, and JK10 displayed mild root growth promoting effects. VM5, a molecule derived from kinetin, did not exhibit any significant effect on the primary root growth (Figure 1B). As for the lateral root density, expectedly, we observed a severe inhibition of emerged lateral roots by BAP (0 LR/cm). From the modified cytokinin molecules, VM12 exhibited the strongest inhibition leading to drop of the lateral root density to 1±0.6 LR/cm when compared to 3.5±0.5 LR/cm in mock treated control roots. Treatment with VM2, VM20 and JK11 resulted in milder, but still significant reduction of the lateral root density (Figure 1C).

Interestingly, VM12 shows stronger inhibitory effects on root system than VM2 and VM20 (Figure 1B, C). While all these compounds are derived from iP, the structure of VM2 and VM20 differ from VM12 in the position of the linker. VM2 and VM20 has a substitution with the linker on N^9, whereas the VM12 molecule has the linker substitution on C^2. Likewise, the inhibitory effect of JK11, a BAP derivate with substitution on C^2, on the LR outgrowth is slightly more profound when compared to JK5 and JK10 that have N^9 positioned linkers (Figure 1C). The difference between biological activity of cytokinin derivatives suggests that the linker position might interfere with the binding of the cytokinin molecule into the pocket of cytokinin receptors and other putative cytokinin binding proteins.

These results, suggesting the importance of the intact N^9, are in accordance with published crystal structure of cytokinin binding proteins (Pasternak et al., 2006; Hothorn et al., 2011; Ruszkowski et al., 2013). Moreover, N^9- glucosyl conjugates of cytokinins are known to be the inactive, storage forms that do not trigger the cytokinin signaling *in vitro* (Spíchal et al., 2004; Kieber and Schaller, 2018). This is consistent with lower biological activity of the VM2, VM20, JK5 and JK10 compounds with N^9 positioned linker when compared to VM12 and JK11 with a linker positioned at C^2.

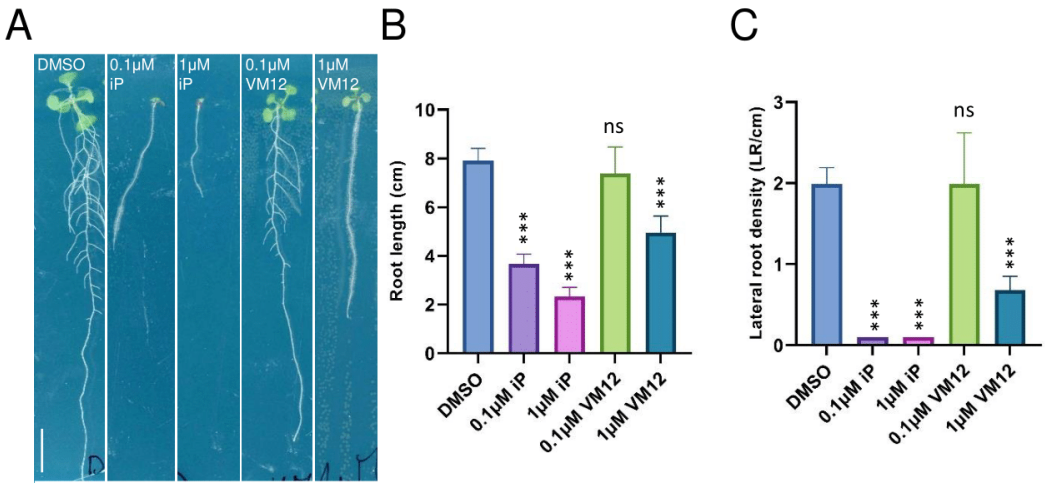


Figure 2: VM12 shows cytokinin-like effects on root growth and lateral root development *(A) 12-days old Col-0 plants grown on media supplemented with DMSO, 0,1µM iP, 1µM iP, 0,1µM VM12 or 1µM VM12. iP and VM12 reduce root length and lateral root density of Col-0. (B) Root length measurements of (A). (C) Lateral root density measurements of (A). The boxplots represent mean ± s.d., *** = p < 0.001, n.s. = p > 0.05 by Student's t-test, n > 4. Scale bare = 1cm.*

Overall, VM12 showed the strongest effects on the root length and the lateral root density among modified cytokinin molecules that we tested (Figure1, Figure S1). To further compare the biological activity of VM12 with iP, various concentrations of these molecules were applied (Figure 2, Figure S1). When compared to iP, which at 100nM and 1µM concentrations reduces the root length to 46±5% and 30±5% of mock treated 12-days-old plants, respectively, the same concentrations of VM12 cause reduction to 93±14% and 63±9%, respectively (Figure 2B). Likewise, while 1µM iP reduces the density of lateral roots to 0% of the mock treated plants, the application of 1µM VM12 decreases LR density to 34±9% of the mock control (Figure 2C). These results indicate that cytokinin activity of VM12 is attenuated when compared to its parent molecule iP, which might be due to the different solubility, permeability or stability of the VM12 molecule compared to iP (personal communication). Altogether, based on the root growth assays, cytokinin activity appears to be best preserved in VM12, the derivative of iP carrying linker at C2 position, out of all the tested compounds.

Modified cytokinin molecules activate canonical cytokinin signaling pathway

To further test VM12 biological activity, we examined activation of the canonical cytokinin and auxin transcriptional pathway by observing the dual *TCS::GFP,DR5::RFP* reporter (Marhavý et al., 2011). *TCS* and *DR5* are hormone-responsive promoters traditionally used as markers for the respective hormone activity (Friml et al., 2003; Müller and Sheen, 2008; Zürcher et al., 2013). 5-days-old plants were treated with either iP or modified cytokinins such as VM12, JK11, JK10, and VM5, which were applied at 2µM concentration for 4 hours. In the meristem of the primary root, we did not observe any apparent changes in *TCS* and *DR5* expression (Figure S3A). Importantly, in the developing lateral root primordia (stages III-V), treatment with 2µM iP as well as 2µM VM12 enhanced expression of the cytokinin sensitive *TCS* reporter when compared to mock treated primordia (Figure 3B, D). Neither iP, nor VM12 activated the TCS expression at the tip of the developing primordia, where *DR5* signal maxima was formed. Concomitantly, treatment with these cytokinin molecules resulted in more diffused DR5 signal expanding from the tip to the base of the primordia. This is in agreement with previous reports, suggesting that auxin maxima formation at tip of the primordia is affected by cytokinin, which attenuate PIN1-dependent transport of auxin (Marhavý et al., 2011; Figure 3C,D). Additionally, brighter *TCS* signal was detected after treatment with iP and VM12 in differentiated tissues of the primary root including, epidermis, cortex, endodermis, and the xylem pole pericycle cells when compared to mock treated roots. Treatment with other modified cytokinin molecules such as JK11, JK10, and VM5 increased the strength of the *TCS::GFP* signal mainly in the central cylinder and partially also in the epidermis of the primary root (Figure S3B).

Taken together, VM12 treatment lead to the strongest activation of the *TCS* cytokinin reporter expression when compared to other compounds such as JK11, JK10, and VM5. These results suggest that similarly to iP, VM12 is able to activate canonical cytokinin signaling and transcriptional response.

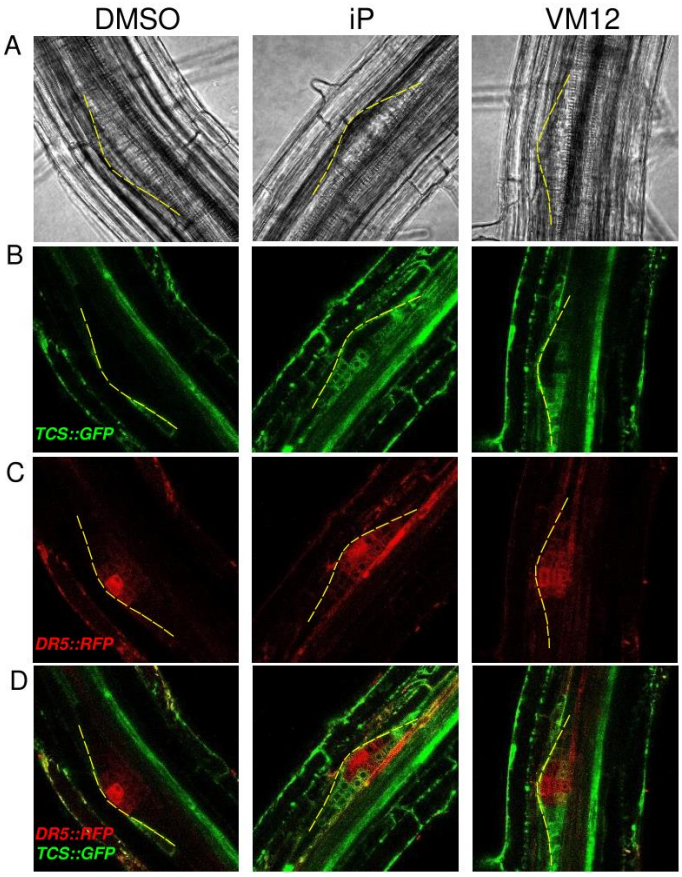


Figure 3: Expression of cytokinin (TCS) and auxin (DR5) reporters upon treatment with iP and VM12 *5-days-old plants were transferred on the media supplemented with DMSO, 2µM iP or 2µM VM12 and treated for 4 hours. iP and VM12 treatment increase the TCS::GFP signal in the developing lateral root primordia and in endodermis, cortex and epidermis tissues of the primary root surrounding the primordia. (A) Bright field images. (B) TCS::GFP expression (green) (C) DR5::RFP expression (red). (D) Overlay of the green and the red channel. Yellow dashed lines represent outline of the developing lateral root primordia.*

Effects of modified cytokinin molecules on PIN1 protein

Important part of the cytokinin-regulated developmental processes involves hormonal cross-talk with auxin. Cytokinin fine-tunes polar auxin transport through modulation of PIN1 expression and trafficking. Upon cytokinin treatment, the abundance of PIN1 at the plasma membrane is significantly reduced (Ruzicka et al., 2009; Marhavý et al., 2011). To test whether modified cytokinin molecules affect PIN1 protein trafficking, 5-days-old seedlings of *PIN1::PIN1-GFP* reporter line were treated for 8 hours with 2µM concentration of iP, VM12, JK11, JK10, and VM5 (Figure 4A). The PIN1-GFP signal at the plasma membrane of pericycle cells in the primary root meristem was measured. In accordance with previous reports, a significant reduction of the PIN1-GFP abundance at plasma membrane after treatment with iP was detected (Figure 4; Marhavý et al., 2011). VM12 showed similar trend in attenuating of the PIN1-GFP membrane signal, but did not reach the significance level (Figure 4B). No other modified cytokinin molecule interfered with the PIN1-GFP signal intensity.

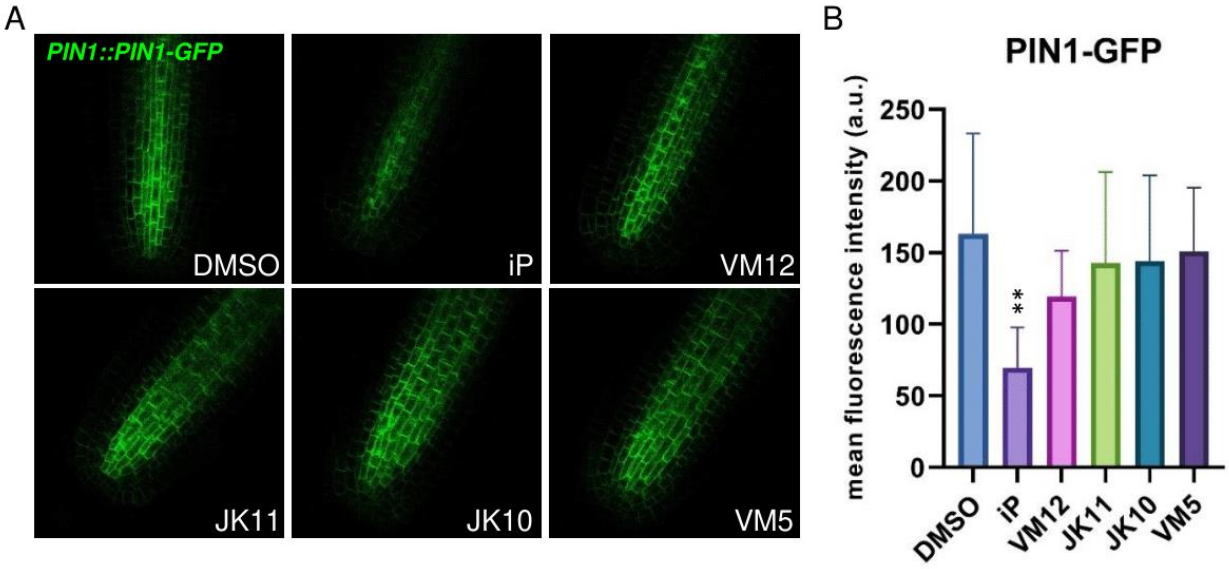


Figure 4: Cytokinin decreases PIN1-GFP membrane signal *(A) Primary root meristem expressing PIN1::PIN1-GFP. Plants were transferred on the media supplemented with 2µM DMSO, iP, VM12, VM5, JK10 and JK11 and treated for 8 hours. (B)Quantification of membrane signal intensity of PIN1-GFP in pericycle cells of the primary root meristem. The boxplots represent mean ± s.d., ** = p < 0.01, by Student's t-test, n > 6.*

In conclusion, the investigation of effects of modified cytokinin molecules on the root morphology, activation of cytokinin signaling pathway and PIN1-GFP signal intensity shows that VM12, iP- derivate with C^2 -attached linker, retains main biological activities innate to natural cytokinins. Therefore, we decided to use the VM12 molecule for immobilization on the beads and affinity purification of cytokinin binding proteins from roots of *Arabidopsis thaliana*.

A chemical proteomic workflow

Given the localization of cytokinin receptors, cytokinin binding occurs in the apoplast or in the lumen of the ER (Antoniadi et al., 2020; Kubiasová et al., 2020). However, the cytokinin binding to other proteins might occur also in other parts of the cell. The pioneering experiments in protonemata of *Funaria hygrometrica* (Gardner et al., 1978) or carrot suspension cells (Kobayashi et al., 1981) identified cytokinin binding proteins in the microsomal fraction and therefore predicted the localization of cytokinin receptors at the plasma membrane or in the ER. However, the isolation of such receptors was not successful. Later reported cytokinin binding proteins were identified in the cytosolic, nuclear or plastid

fraction. (Brinegar et al., 1985; Brinegar et al., 1988; Fujimoto et al., 1998; Shepelyakovskaya et al., 2002; Brovko et al., 2007; Brovko et al., 2010).

In our study, we decided to focus on the microsomal fraction of the cell for multiple reasons. First, previously published literature suggests cytokinin effect on the endomembrane trafficking (Marhavý et al., 2011). Therefore, our interest lies in the group of proteins that are part of or associated with the microsomal fraction of the cell lysate. Second, highly abundant cytosolic proteins might "blind" the mass spectrometry detection since it is biased towards identification of proteins that are present in high amounts. This could limit the detection of low abundant proteins, which might have a key regulatory role. Third, we aimed at the detection of cytokinin receptors, AHKs, which are transmembrane proteins, and their recovery would serve as a validation of our affinity purification approach. We used root tissue of 6-days-old *Arabidopsis thaliana* seedlings to approximate conditions for confocal microscopy and biochemical validation.

The chemical proteomics is a technique that utilizes matrix-immobilized compounds to enrich for interacting proteins from biological samples. Affinity purification and subsequent mass spectrometry allows identification of proteins/protein complexes that bind to immobilized compounds. A major challenge of chemical proteomics is the high abundance of background proteins, which increases the risk of identification of false positives and may lead to misinterpretation of the acquired data. Moreover, iP is a N^6 derivate of adenine, a nucleobase with a purine ring. Adenine molecule and its derivates are very abundant in the cell *e.g.* as building blocks of the DNA/RNA or as the energy-rich ATP molecules. Therefore, many proteins naturally recognizing and binding to adenine structure might also bind to iP in an unspecific manner in our assay.

In order to increase the stringency of the affinity purification, we followed protocol by Simerský et al. (2017) and included several variants of pull-down controls (Figure 5, 3). All control pull-down variants were performed in parallel to the regular pull down, with the same protein lysate as input material. In the regular pull-down we detect proteins that bind to cytokinin, but also proteins with broader binding specificity and some background proteins with unspecific binding

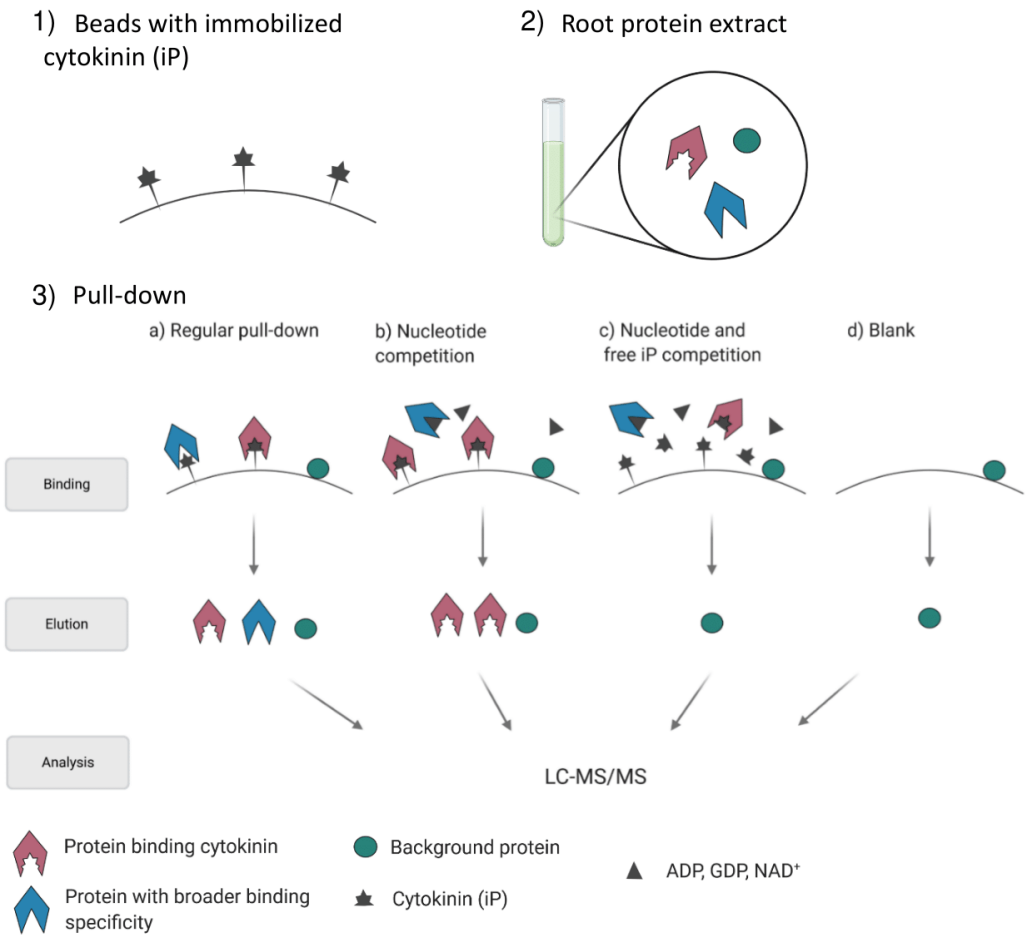


Figure 5: Experimental design of the cytokinin binding protein pull-down *(1)Schematic depicting of cytokinin molecule attached to a bead with a linker. (2)Schematic depicting of a protein lysate. (3)Proteins binding to cytokinin and proteins with broader specificity are identified in regular pull-down (a). During competition with nucleotides (b), proteins with broader specificity will get outcompeted. Specifically bound proteins are enriched. In competition with nucleotides and free iP (c), specifically bound proteins and proteins with broader specificity are outcompeted. Only non-specific, background proteins remain. Blank (d) is a negative control with beads without attached ligand. Only the non-specific proteins will be identified.*

(Figure 5, 3a). In the first control pull-down, we added free nucleotides (ADP, GDP and NAD^+), which outcompete proteins with broader binding specificity (Figure 5, 3b). Therefore, we expect increase in the concentration of proteins with high affinity to cytokinin compared to the regular pull-down, because outcompeted proteins with broader specificity do not occupy binding sites for true cytokinin binding proteins. In the second control variant, we add free iP and nucleotides (Figure 5, 3c) with aim to outcompete proteins with broader specificity as well as true cytokinin binding proteins. The last control pull-down is blank, in which proteins

binding only non-specifically *e.g.* to the surface of the affinity beads are detected (Figure 5, 3d).

The final comparison of the mass spectrometry analysis of individual pull-down experiments will yield proteins binding specifically to cytokinin. This is achieved by filtering the data for peptides that are present in the nucleotide competition pull-down (Figure 5, 3b) in higher amounts than in the regular pull-down (Figure 5, 3a), and at the same time, they are not detected in the iP competitive pull-down (Figure 5, 3c) or blank (Figure 5, 3d).

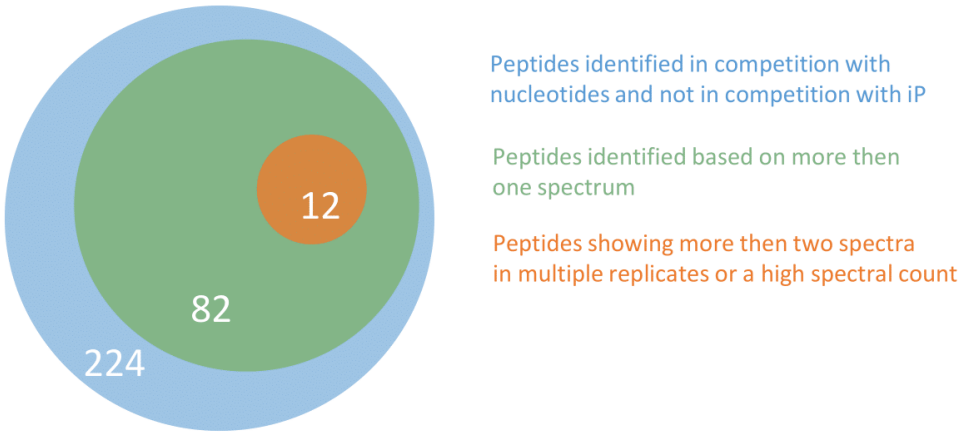
Functional annotation of potential cytokinin binding proteins

We identified 969 proteins in the regular pull-down, 1087 proteins in the nucleotide competition pull down, 1055 proteins in the iP competition pull down and ~150 proteins in blank. We were unable to identify AHKs in any replicate of our experiment. This might be due to the low abundance of AHK proteins, difficulties with the isolation since they are transmembrane proteins or their inability to bind cytokinin that is immobilized on the surface of beads.

Here, we introduce group of proteins identified in higher amounts in the nucleotide competition pull-down than in the regular pull-down and concomitantly absent in the iP competition pull-down. In four replicates we identified in total 224 proteins. 142 of them were identified based only on 1 spectrum in 1 replicate, therefore we don't consider them highly relevant. Out of the remaining 82 proteins, 19 were found in more than one replicate or had high spectral count. Twelve peptides out of these 19 were identified based on more than two spectra, hence seemed to be meaningful for further analysis (Figure 6A). These twelve proteins listed in Figure 6B are considered promising candidates for further investigation.

We searched for orthologues of previously identified cytokinin binding proteins among the 224 identified proteins using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Some of the proteins previously reported to bind to cytokinin are involved in transcription or translation. For example, the CBP70 from maize and CBP67 from barley and Arabidopsis were found to participate in transcription (Kulaeva et al.,

A



B

	Prey - Acc. No.	Prey Gene	Prey - protein	Spectra in Nuc. comp. pull-down	Spectra in Regular pull-down	Saint score	Fold change
1	Q9SE83	DRP2A	Dynamin-2A	8|6|4|4	0|0|0	0.99	55
2	Q9C5N2	TMN9	Transmembrane 9 superfamily member 9	10|0|0|0	0|0|0	0.33	25
3	Q8LF20	At2g25430	Putative clathrin assembly protein (ATECA4)	0|0|4|4	0|0|0	0.66	20
4	Q8LF21	DRP1C	Dynamin-related protein 1C	5|1|0|2	0|0|0	0.55	20
5	O81796	IDH3	Isocitrate dehydrogenase [NAD] regulatory subunit 3	0|0|3|2	0|0|0	0.52	12.5
6	O82209	At2g19680	Copia-like retroelement pol polyprotein	1|1|1|0	0|0|0	0	7.5
7	Q9LHE7	FYPP3	Phytochrome-associated serine/threonine-protein phosphatase 3	1|0|2|1	0|0|1	0.06	3
8	A0A2H1ZEG9	Hsp70-15	Heat shock protein 70	0|2|2|3	1|0|1	0.08	2.62
9	A8MS03	RPS6	Ribosomal protein S6	2|1|0|0	0|1|0	0.06	2.25
10	A0A1P8AZ64	GAD4	Glutamate decarboxylase	1|0|1|1	1|0|0	0	2.25
11	O22715	AP1M2	AP-1 complex subunit mu-2	1|0|1|1	0|1|0	0	2.25
12	Q9FHM7	HIR4	Hypersensitive-induced response protein 4	1|1|1|0	0|1|0	0	2.25

Figure 6: Venn diagram and list of identified putative cytokinin binding proteins *(A) Venn diagram of proteins identified in the nucleotide competitive assay (B) List of candidates appearing in multiple spectra in multiple replicates Prey - Acc. No.: Accession number of identified gene, Prey gene: Shortcut of the name of the gene, Prey-protein: Name of the gene, Spectra in Nuc. Comp. pull-down: Amount of spectra detected for the respective protein in each of the four replicates of nucleotide competition pull-down, Spectra in Regular pull-down: Spectra identified in three replicates of the regular pull-down without competition, Saint score: Probabilistic score for affinity purification, Fold change: Amount of enrichment in the nucleotide-competitive pull-down compared to iP-competitive pull down.*

2000; Selivankina et al., 2004; Brovko et al., 2010). However, their sequences are not publicly available, therefore we were not able to look for orthologues in Arabidopsis. Although we found proteins involved in transcription (MED37A, MRG7.19, At1g02080), they were among

the 142 proteins identification of which was based only on 1 spectra. Therefore, suggesting functional cytokinin binding would be a pure speculation. The involvement of cytokinin in translation was suggested because cytokinin was found to bind chinese-cabbage ribosomes (Berridge et al., 1970). We found Ribosomal protein S6 (RPS6) among the twelve candidate proteins. However, the relevance of RPS6 can't be verified in the literature because the study suggesting the chinese-cabbage ribosomal binding to cytokinin does not specify which molecules are responsible for cytokinin binding. Cytokinin binding factor CBF-1 isolated from wheat, associates with ribosomes (Brinegar et al., 1985), but CBF-1 does not have an orthologue in Arabidopsis.

In tobacco, S-adenosyl-L-cysteine hydrolase (SAH), CBP1, and CBP2 were reported to bind cytokinin (Mitsui et al., 1996; Kobayashi et al., 2000). Despite there are orthologues of these proteins in Arabidopsis, we did not detect them in our analysis. There are no clear Arabidopsis orthologues for the Mung bean CSBP or the *Mediacgo truncatula* nodulin MtN13. We did identified a NODULIN-LIKE21 among the subset of proteins, which were identified based on one spectra but it shares no significant sequence similarity with MtN13.

Altogether, we did not find any orthologues of previously published proteins binding cytokinin among 224 proteins present in the nucleotide competition pull down and absent in the iP competition pull down. This is not surprising, since most of the studies were performed on other species and on a different tissue/subcellular fraction than in our project. Therefore, our dataset offers new and unique insights into the cytokinin action and potential cytokinin binding proteins in the microsomal fraction of the protein lysate from *Arabidopsis thaliana* roots.

Candidate proteins have a role in vesicular trafficking

Out of the twelve cytokinin binding protein candidates (Figure 6B), four are involved in the process of vesicular trafficking. Namely, we identified Putative clathrin assembly protein (ATECA4), AP-1 complex subunit mu-2 (AP1M2), Dynamin-related protein 2A (DRP2A), and Dynamin-related protein 1C (DRP1C). These proteins participate in various steps of the vesicle trafficking. The vesicle formation starts with clustering of the coating proteins at the parent membrane, continues with cargo recruitment and membrane curvature, followed by constriction and fission of the vesicle (Kaksonen and Roux, 2018; Narasimhan et al., 2020).

The ATECA4 might play a role in the vesical formation (Gadeyne et al., 2014), the DRP2A and DRP2B in the fission of the vesicle (Huang et al., 2015) and the AP1M2 in cargo sorting to the vesicle on the level of TGN (Wang et al., 2013; Shimada et al., 2018).

DRP2A - potential cytokinin binding protein identified by chemical proteomics

The highest scored candidate in our dataset is the Dynamin-related protein 2A (DRP2A), which was detected in multiple spectra of all the replicates in the nucleotide competition pull-down and it was not found in any replicate of the iP competition or the regular pull-down.

Plant dynamin-related proteins (DRPs), similarly to their animal homologs, are involved in scission of the nascent vesicle from the parental membrane (Hong et al., 2003; Fujimoto and Tsutsumi, 2014). They play a major role in the process of clathrin mediated endocytosis (CME), cell and organelle division, and polarity establishment (Fujimoto et al., 2008; Konopka and Bednarek, 2008; Backues et al., 2010; Fujimoto et al., 2010; Taylor, 2011; Huang et al., 2015). The DRP2 group, which shares the highest similarity to animal dynamins, has two members, DRP2A and DRP2B. DRP2A and DRP2B have over 90% of amino acid similarity and are thought to act redundantly in the plant growth and development (Taylor, 2011; Huang et al., 2015). Therefore, it is striking that we identified only DRP2A as a protein selectively binding to cytokinin in our study. Importantly, this is not a biased detection due to the prevalent presence of only one of the paralogs in the analyzed tissue. Whereas we identified both DRP2A and DRP2B in the nucleotide competition pull-down, only DRP2A was absent after the competition with free iP, suggesting that it was selectively outcompeted (Figure S6). Altogether, our data suggest that DRP2A might specifically bind to cytokinin.

***drp2* mutants show mild oversensitivity to cytokinin**

To examine whether cytokinin-regulated developmental processes involve DRP2s, we tested sensitivity of *drp2a-1* and *drp2b-2* mutants to the hormone. The double mutant *drp2a drp2b* is gametophyte lethal and previous observations of single mutants have not revealed any obvious phenotype defects (Backues et al., 2010). Nonetheless, recent study shows altered sensitivity of

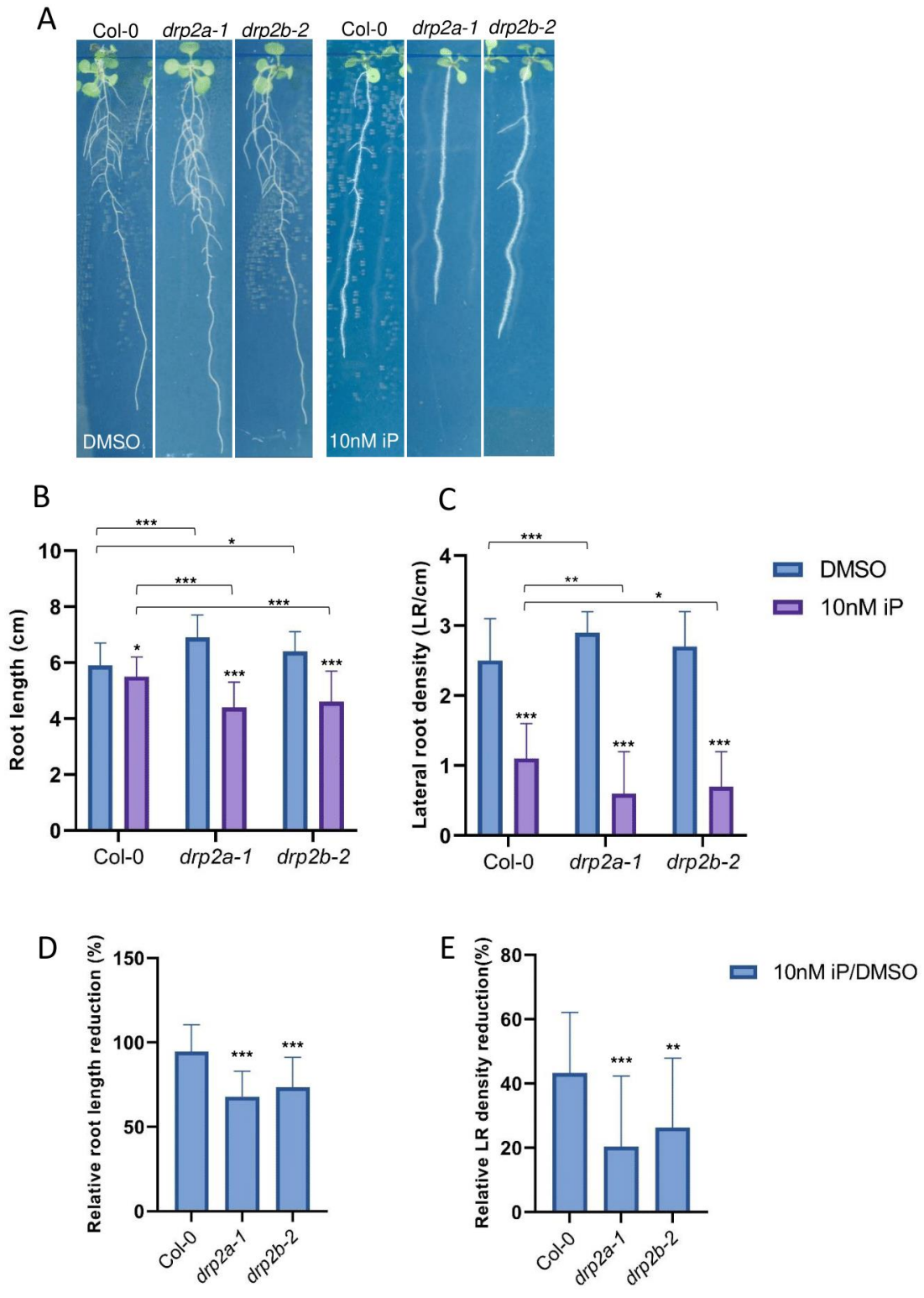


Figure 7: drp2 mutants show enhanced sensitivity to cytokinin *(description on the next page)*

Figure 7: drp2 mutants show enhanced sensitivity to cytokinin *(A) 12 days old plants (Col-0, drp2a-1, drp2b-2) grown on media supplemented with DMSO or 10nM iP. (B) Quantification of the root length of (A). (C) Quantification of the lateral root density of (A). (D) % of root length of plants grown on 10 nM iP relative to DMSO. (E) % of lateral root density of plants grown on 10 nM iP relative to DMSO*

drp2b but not *drp2a* to flg22 peptide (the active peptide derivative of bacterial flagellin, Smith et al., 2014) suggesting a potential functional diversification of the two homologues in Arabidopsis. To test their cytokinin sensitivity, *drp2a* and *drp2b* mutants were grown for 12 days on mock or media supplemented with 10nM iP. In control mock conditions, *drp2a-1* mutant has a longer primary root and higher lateral root density when compared to Col-0, whereas *drp2b-2* mutant showed longer primary root but the same lateral root density as Col-0 (Figure 7A,B,C). On cytokinin, both *drp2a-1* and *drp2b-2* mutants exhibited shorter root and lower lateral root density than Col-0, which suggests oversensitivity to the hormone. Whereas the Col-0 root length is reduced to 94±16% of the mock treated control when grown on cytokinin, the *drp2a-1* is reduced to 64±15% and the *drp2b-2* to 73±18% of the root length of the respective mutant grown on mock medium (Figure 7D). The lateral root density of Col-0 is reduced to 44±19% on cytokinin, and the *drp2a-1* and the *drp2b-2* decreases to 20±22% and 26±22%, respectively, compared to the lateral root density of respective genotype on DMSO (Figure 7E). Increased sensitivity of *drp2* mutants to cytokinin hints at a role of *DRP2* genes in the cytokinin mediated regulation of the root growth and the lateral root development.

Monitoring of the DRP2A and DRP2B signal

To explore whether/how cytokinin might interfere with the DRP2A activity, we monitored subcellular localization of DRP2A-GFP and DRP2B-GFP in epidermal cells of the primary root meristem and in lateral root primordia under control conditions and after cytokinin application (Figure 8). To examine the subcellular localization of DRP2s in detail, we compared two optical sections planes, through the middle and through the surface of the cell (Figure 8D). A strong DRP2A-GFP and DRP2B-GFP signal in the newly forming cell plate in the dividing cells of the primary root meristem (Figure 8E, pink rectangle) and lateral root primordia (Figure 8B) was detected. Additionally, punctuate pattern of DRP2A-GFP as well as DRP2B-GFP signal in the cytoplasm and in close proximity of the plasma membrane was observed

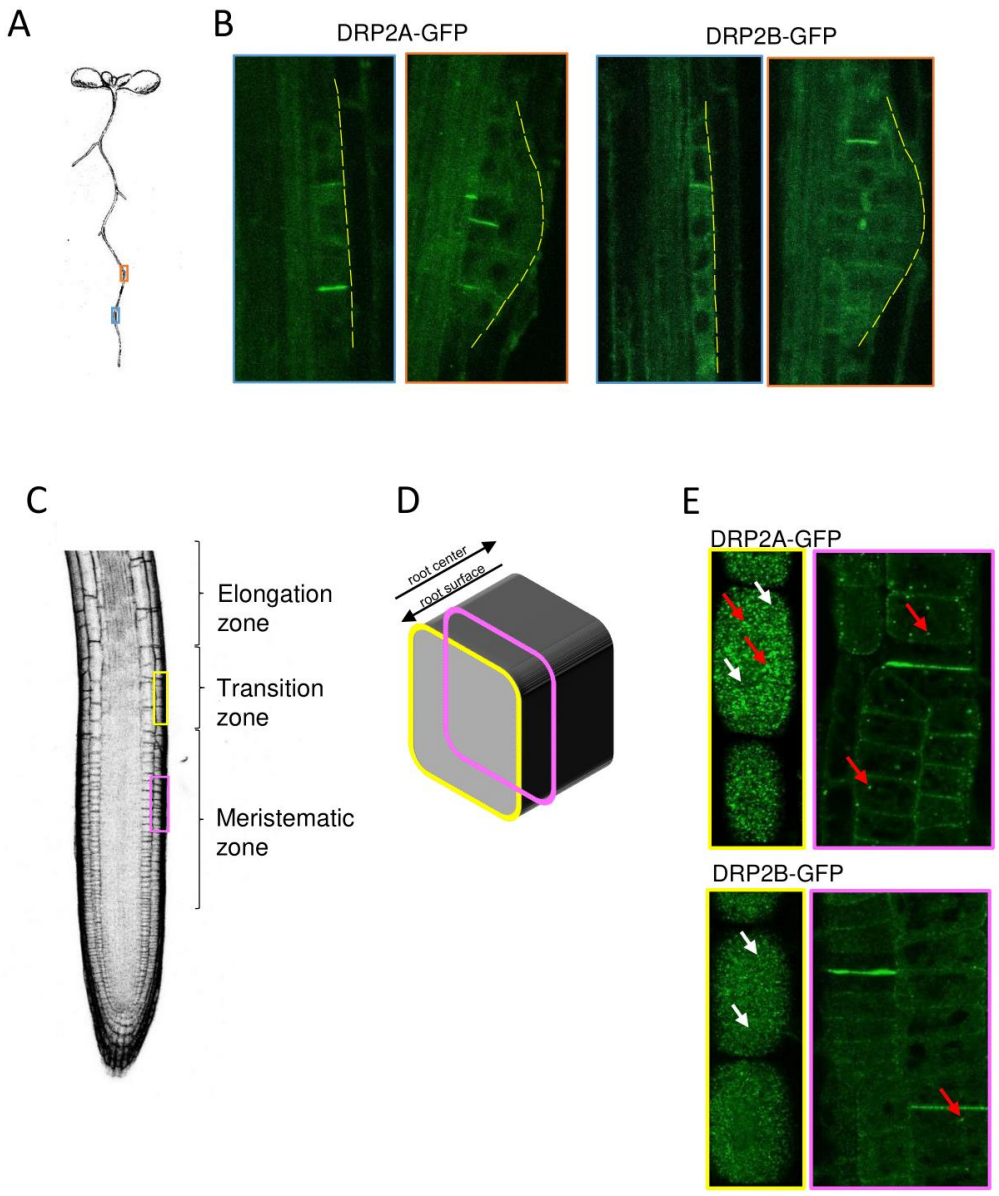


Figure 8. Expression pattern of DRP2A::DRP2A-GFP and DRP2B:DRP2B-GFP in the lateral root primordia and primary root meristem *(description on the next page)*

(Figure 8E, yellow rectangle). Intriguingly, we noted a difference in the subcellular pattern between DRP2A and DRP2B. Although both of the DRP2s localize to small puncta on (or in close proximity to) the plasma membrane (Figure 8E, white arrows), some of the DRP2A-GFP-positive signal aggregates were larger than the DRP2B and localized in the cytoplasm (Figure 8E, red arrows). The subcellular pattern of DRP2A-GFP and DRP2B-GFP is in accordance to the published DRP2A and DRP2B lines in terms of strong localization to the forming cell plate in epidermal cells of the primary root meristem (Huang et al., 2015). In this study DRP2A and DRB2B co-localized with markers of post-Golgi organelles and with each other (Huang et al.,

Figure 8. Expression pattern of DRP2A::DRP2A-GFP and DRP2B:DRP2B-GFP in the lateral root primordia and primary root meristem *(A) Schematic depiction of a seedling with highlighted regions of imaging by blue and orange rectangles. (B) CLSM images of DRP2A-GFP and DRP2B-GFP expression in developing lateral root primordia. The signal is mainly decorating newly formed cell plate in dividing cells. (C) Primary root meristem with highlighted regions of imaging by yellow and pink rectangles. (D) Schematic depicting of optical sections by CLSM. Yellow rectangle represents an optical section on the surface of the cell, pink rectangle represents an optical section in the middle of the cell. (E) CLSM images of DRP2A-GFP and DRP2B-GFP expression in meristematic zone (pink rectangle) and Z-stack image of transition zone (yellow rectangle) of the primary root. The signal is decorating forming cell plates. Many small dots are visible in both DRP2A-GFP and DRP2B-GFP expressing plants (white arrows) mainly in the proximity of or on the plasma membrane. There are also larger dots, rather deeper in the cell, which are much more abundant in case of DRP2A-GFP than DRP2B-GFP (red arrows). Yellow dashed lines represent outline of the developing lateral root primordia.*

2015). However, it does not report differences in the size and the distribution of the dot-shaped structures of DRP2A and DRP2B as much as it was observed in lines used in our study. Whereas there is a possibility, that different signal intensity and distribution between DRP2A and DRP2B that we observed might reflect a different expression levels, it might also be the native localization of DRP2A and DRP2B. It was reported that while DRP2B associates with the plasma membrane (Fujimoto et al., 2010), DRP2A also plays a role in the trans-Golgi trafficking (Jin et al., 2001). Therefore, the larger structures observed in the DRP2A-GFP lines (Figure 8E, red arrows) might be DRP2A residing on the TGN participating in vesicle formation from this organelle as previously suggested (Jin et al., 2001).

DRP2A-GFP signal intensity decreases upon cytokinin treatment

While the biological relevance of the cytokinin binding to DRP2A is completely unknown, we hypothesized that cytokinin binding might affect the DRP2A protein abundance and/or its subcellular localization. To avoid potential bias and variability caused by division of cells in the root meristematic zone we focused on the root epidermal cells at the transition zone prior entering to elongation phase (Figure 8C, yellow rectangle). Importantly, transition of root meristematic cells from proliferation to elongation phase is tightly controlled by cytokinins, thus epidermal cell at the transition zone provide a very suitable model to monitor cytokinin - DRP2 interaction (Dello Ioio et al., 2008; Di Mambro et al., 2017).

First, we investigated time and concentration dependence of the cytokinin effects on DRP2A-GFP. 5-days-old *DRP2A::DRP2A-GFP* transgenic seedlings were treated with 2µM iP for 2, 4 and 6 hours. The signal intensity was measured and a significant decrease of the DRP2A-GFP signal within 4 hours was detected, further dropping at 6 hours compared to the DMSO treated control (Figure 9A, B). Therefore, we decided to use 4-hour treatment as a standard time frame for further experiments.

Since the 2µM iP concentration is much higher than the endogenous physiological levels of cytokinins, we tested DRP2A-GFP sensitivity to lower cytokinins concentrations. 5-days-old seedlings were exposed to 2µM, 200nM, and 20nM iP for 4 hours (Figure 9C, D). In addition, 2µM adenine was included to validate the specificity of the cytokinin effect. Importantly, treatment with adenine did not significantly altered intensity of the DRP2A-GFP signal. This suggests that the reduction of the DRP2A-GFP signal intensity is dependent on the cytokinin activity of the iP molecule and it is not triggered by other molecule with similar structure. When compared to the mock treatment, a significant decrease of the DRP2A-GFP signal in response to all tested concentrations of iP was detected. No significant differences in reduction of DRP2A-GFP signal intensity triggered by 2µM, 200nM, and 20nM iP could be detected. Therefore, we decided to use the lowest iP concentration (20nM) for further experiments, since it is the closest to the endogenous levels out of all concentrations that we tested.

Typically, root cells after exiting the meristematic zone, proceed through the transition zone, where they lose their proliferation capacity before undergoing a rapid expansion and differentiation in the elongation zone (Figure 9E). As cytokinin plays a key function in regulation of cell transition from proliferative to differentiation phase (Dello Ioio et al., 2008; Di Mambro et al., 2017) we investigated whether the cytokinin effect on DRP2A-GFP signal intensity is dependent on differentiation status of the cells. Noteworthy, in mock treated roots, the DRP2A-GFP signal in the epidermal cells decreases as they progress to differentiation phase. The DRP2A-GFP signal intensity is significantly higher in the transition

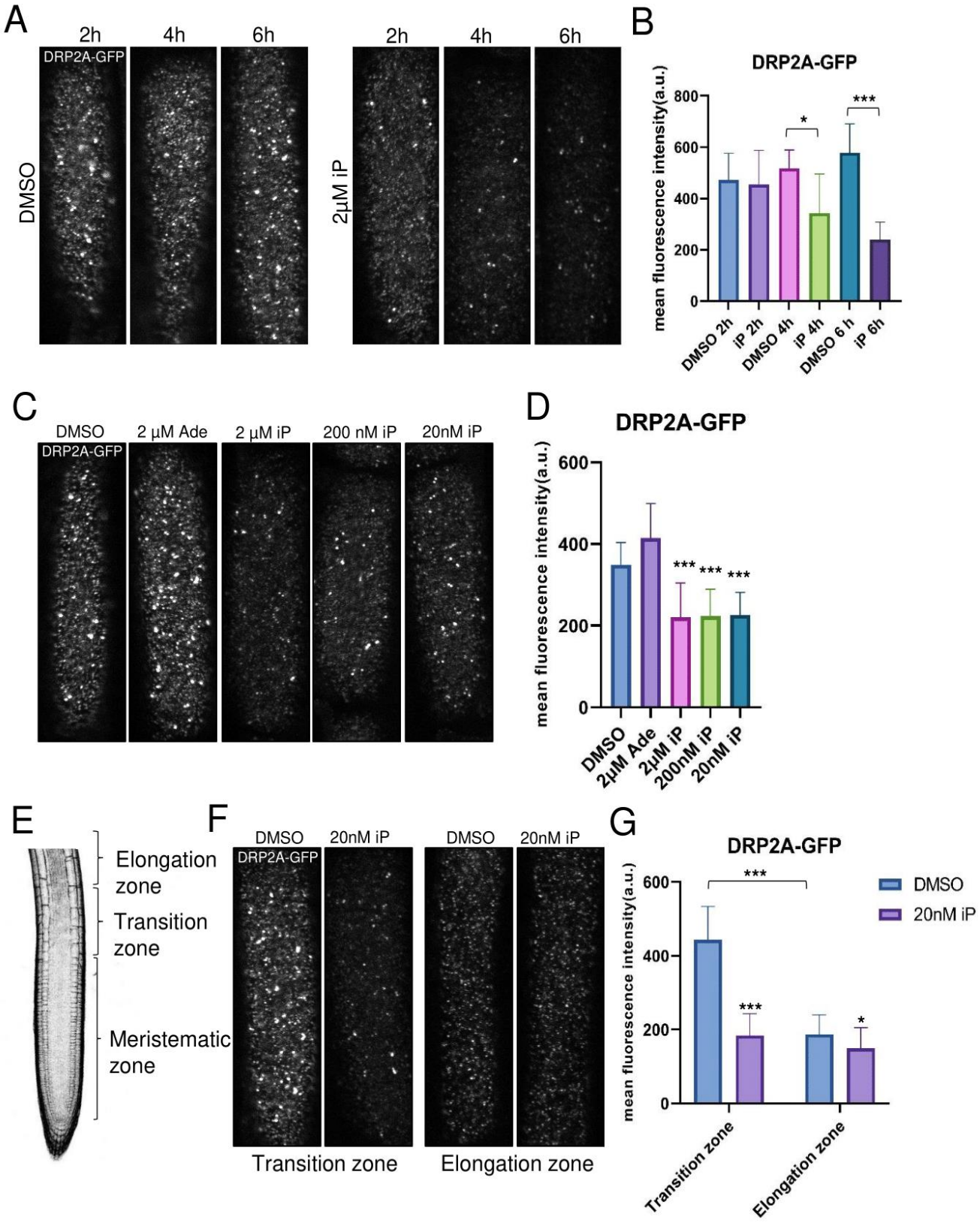


Figure 9: DRP2A-GFP signal upon cytokinin treatment *(description on the next page)*

Figure 9: DRP2A-GFP signal upon cytokinin treatment *(A) Z-stack CLSM image of root transition zone of DRP2A::DRP2A-GFP transferred on the media supplemented with DMSO or 2µM iP and treated for 2, 4 and 6 hours. (B) Quantification of the mean intensity of (A) signal in representative region of interest in the cell. (C) Z-stack CLSM image of transition zone of DRP2A::DRP2A-GFP transferred on the media supplemented with DMSO, 2µM iP, 2µM adenine, 200nM iP, and 20nM iP and treated for 4 hours. (D) Quantification of the mean intensity of (C) signal in representative region of interest in the cell. (E) Representation of different developmental zones in the root meristem of the primary root. (F) Z-stack CLSM image of transition and elongation zone of DRP2A::DRP2A-GFP transferred on the media supplemented with DMSO or 20nM iP and treated for 4 hours. (G) Quantification of the mean intensity of (F) signal in representative region of interest in the cell. The boxplots represent mean ± s.d., *** = p < 0.001, * = p < 0.05, n.s. = p > 0.05 by Student's t-test, n > 6. Experiments were performed in 3 independent replicates.*

zone than in the elongation zone of the root meristem (Figure 9F, G). Furthermore, we noticed a change in the subcellular pattern of the DRP2A-GFP, such as smaller DRP2A-GFP positive aggregations in cells at the elongation compared to the transition zone (Figure 9F, G). 20nM cytokinin treatment for 4 hours resulted in significant decrease of the DRP2A-GFP signal in epidermal cells at both, the transition and the elongation zone when compared to mock treated cells in respective root zones. However, the absolute reduction of the DRP2A-GFP signal in the elongation zone was lower when compared to this detected in the transition zone (Figure 9F, G).

In conclusion, cytokinin decreases the signal intensity of the DRP2A-GFP in epidermal cells of the root meristem in time-dependent and concentration-independent manner. The decrease of the DRP2A-GFP signal intensity is also dependent on the developmental phase of the cells with much greater cytokinin effect in the transition zone compared to the elongation zone.

The effect of cytokinin on other proteins involved in endocytosis

To further characterize the cytokinin effect on the DRP2A-GFP, we examined whether the decrease of the signal intensity is specific to the DRP2A-GFP or it might interfere with the activity of other proteins of the endocytic machinery. Our pull-down assay recovered DRP2A, but not DRP2B as a cytokinin binding protein (Figure S6). Hence, we tested whether DRP2B differs in the sensitivity to cytokinin when compared to DRP2A. 5-days-old seedlings were treated with 20nM iP for 4 hours and the intensity of the DRP2B-GFP or DRP2A-GFP signal in the epidermal cells at the transition and elongation zones was measured (Figure 10). We found that in the transition zone, upon treatment with cytokinin the DRP2A-GFP signal

decreases to 57±4% of the mock-treated control whereas the DRP2B-GFP signal intensity decreases to 72±11% of the DMSO-treated control (Figure 10A, B, C). In the elongation zone, there was no significant difference between mock and cytokinin treatment in either DRP2A-GFP or DRP2B-GFP (Figure 10A, C, D).

Next, we examined whether cytokinin affects the endocytosis marker clathrin light chain (CLC)-GFP, which might suggest general effect on endocytosis. Upon treatment with 20nM iP for 4 hours, CLC-GFP signal intensity showed a modest but significant decrease (81±5% of the mock-treated control) in the transition zone but not the in elongation zone (Figure 10E, B, D).

Altogether, we observed cytokinin-induced decrease of the DRP2A-GFP signal in the epidermal cells at the primary root transition zone. The signal intensity decrease of DRP2B-GFP or CLC-GFP upon cytokinin treatment was much weaker than the decrease of the signal intensity of DRP2A-GFP. This suggests protein-specific effect of cytokinin on the DRP2A-GFP preferentially in the transition zone of the root meristem.

Discussion

Cytokinin is a key regulator of plant growth and development. Apart from the classical histidine-kinase signaling pathway (Kieber and Schaller, 2018) it might also act in parallel, by directly binding to proteins and influencing their function and/or stability.

We used affinity purification followed by mass spectrometry to search for cytokinin binding proteins. To select a physiologically relevant bait for affinity purification, we tested biological activity of multiple modified cytokinin molecules with attached linker. We identified molecule VM12, an iP derivate with linker attached on the C^2 of the purine ring. VM12 is the most similar to cytokinin in terms of effects on the root growth, the lateral root density, and on activation of the canonical transcriptional response.

Interestingly, VM12 does not reach the level of biological activity of iP in any of the experiments. This might be due to slightly different chemical properties e.g. increased hydrophobicity

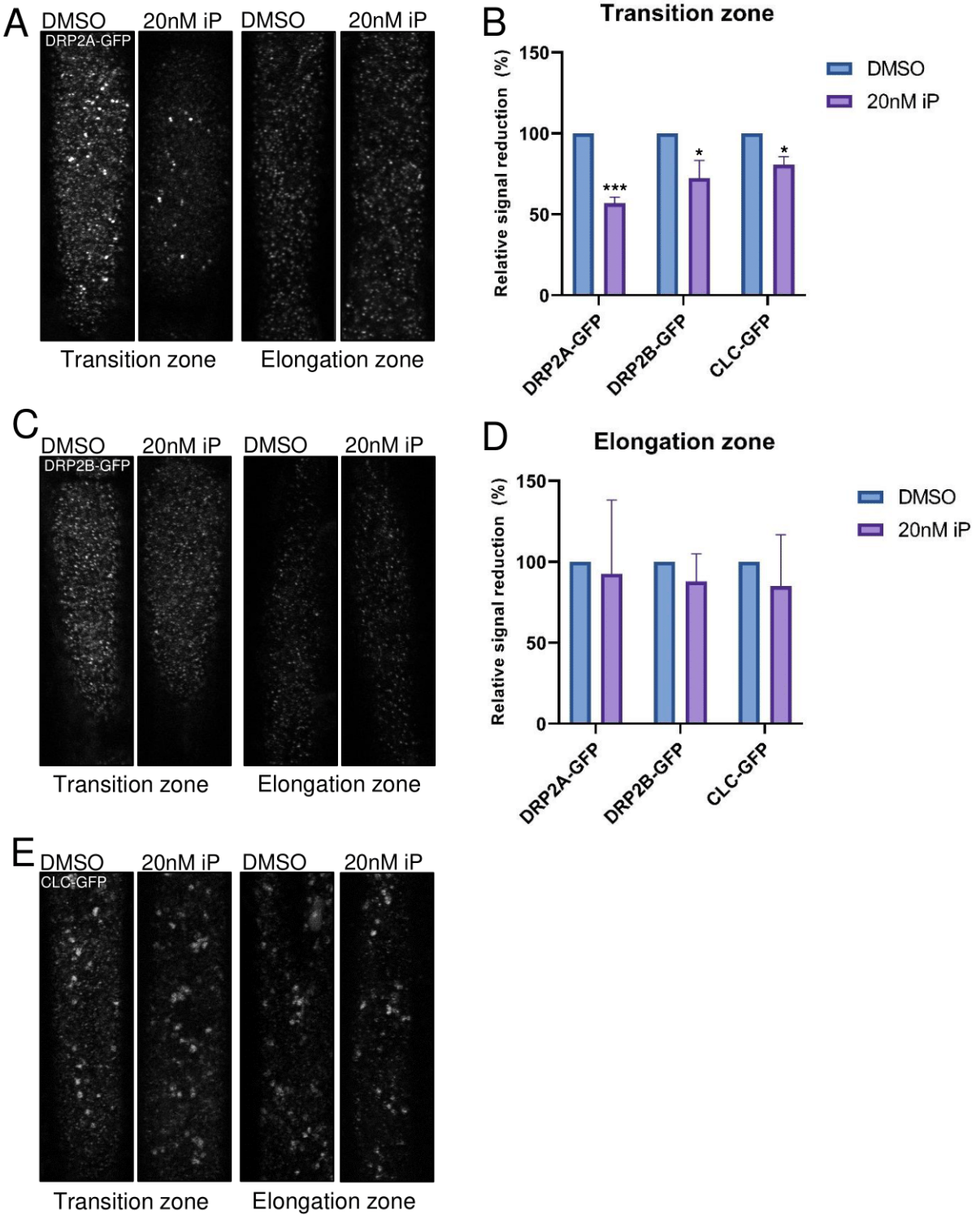


Figure 10: DRP2A-GFP, DRP2B-GFP and CLC-GFP signal upon cytokinin treatment
(description on the next page)

(personal communication). Theoretically, there is a possibility that the VM12 molecule decays and the fraction of the free iP molecules trigger the mild response of the plant. We tested the

Figure 10: DRP2A-GFP, DRP2B-GFP and CLC-GFP signal upon cytokinin treatment *(A) Z-stack CLSM image of transition and elongation zone of DRP2A::DRP2A-GFP transferred on the media supplemented with DMSO or 20nM iP and treated for 4 hours. (B) Quantification of the mean intensity of (A, C, E) in transition zone signal in representative region of interest in the cell. (C) Z-stack CLSM image of transition and elongation zone of DRP2B::DRP2B-GFP transferred on the media supplemented with DMSO or 20nM iP and treated for 4 hours. (D) Quantification of the mean intensity of (A, C, E) in elongation zone signal in representative region of interest in the cell. (E) Z-stack CLSM image of transition and elongation zone of CLC::CLC-GFP transferred on the media supplemented with DMSO or 20nM iP and treated for 4 hours. The boxplots represent mean ± s.d., *** = p < 0.001, * = p < 0.05, n.s. = p > 0.05 by Student's t-test, n > 6. Experiments were performed in 3 independent replicates.*

stability of VM12 powder and it is intact more than a year from the synthesis (data not shown). Although we did not analyze the stability of VM12 in the media on which the plants were grown/treated, we assume it is unlikely that the effect of VM12 would be due to a free cytokinin from VM12 molecules metabolized by the plant. The linker is attached to the C^2 position of the iP, which is not the target of the typical cytokinin catabolism enzymes such as cytokinin oxidases (Schmülling et al., 2003).

Other molecules like VM2, VM20, JK11, JK5, and JK10 showed a mild effect on the root growth or the lateral root density (Figure 1B, C). Moreover, VM5, JK10, and JK11 increased the TCS reporter signal in the central cylinder and differentiated root epidermal cells (Figure S3). Therefore, these molecules are potential candidates to be a bait in future affinity-purification experiments. Especially, JK11, which is derived from BAP, might be a suitable alternative to VM12 derived from iP. Using BAP-derived or tZ-derived molecules for affinity purification might broaden the spectrum of cytokinin binding proteins since different cytokinins can have different biochemical properties and binding abilities (Spíchal et al., 2004; Kieber and Schaller, 2018).

Our search revealed twelve candidate proteins that were identified based on multiple spectra in multiple replicates, none of which shares homology with previously identified cytokinin binding proteins. Among these candidates, we detected four proteins that are involved in endomembrane trafficking, in particular in vesicular formation (ATECA4, DRP2A, and DRP1C) and sorting (AP1M2). This suggests that cytokinin might influence the process of vesicular formation at TGN and/or at the plasma membrane and thereby it might regulate the vesicular trafficking of proteins in the cell. For example, the PIN1 targeting to lytic vacuoles for

degradation as DRP2A was indeed found to play a role in the trafficking from the TGN to the vacuole (Jin et al., 2001). However, the fact that we identified multiple proteins that participate in the vesicular trafficking does not mean that all off them must necessarily interact with cytokinin. Some of them might be pulled-down due to the interaction with the actual cytokinin binding protein *e.g.* Putative clathrin assembly protein (ATECA4) might interact with DRP2A and DRP1C through TPLATE COMPLEX MUNISCIN-LIKE (TML) (Gadeyne et al., 2014).

The most promising candidate from our list is the DRP2A. Interestingly, despite the large amino acid similarity between DRP2A and its closest homologue DRP2B, proteotypic peptides of DRP2B were not outcompeted in the iP competition pull-down (Figure S6). This hints at DRP2A affinity to cytokinin and possibly also a functional diversification between DRP2A and DRP2B. The putative difference between DRP2A and DRP2B is further supported by the confocal live imaging, which shows that DRP2A and DRP2B differ in the sensitivity to cytokinin. Reduction of DRP2A-GFP signal in response to cytokinin is much more profound when compared to DRP2B-GFP (Figure 10). Altogether, although DRP2A and DRP2B are largely redundant in their activity (Backues et al., 2010), the difference in the subcellular localization pattern and cytokinin sensitivity suggests that they might participate selectively in hormone regulated trafficking processes in the cell. This hints at the fine functional difference between DRP2A and DRP2B and at specificity of cytokinin action by selectively binding to only one of these homologues.

Despite it is assumed that DRP2A and DRP2B are fully redundant (Backues et al., 2010), their specific functions in regulation of plant pathogen resistance has been recently reported. DRP2B was found to have a role in the response to the flg22 (the peptide derivate of bacterial flagellin) and to the flagellated bacteria *Pseudomonas syringae pv. tomato* DC3000. The flg22-triggered internalization of the FLS2 receptor that mediates the host defense responses was partially dependent on DRP2B but not DRP2A protein (Smith et al., 2014). Increased susceptibility to pathogen infection of *drp2b-2* mutant might be caused by absence of the *DRP2B* gene, but also by higher abundance of DRP2A since that *drp2b-2* has increased expression of the DRP2A (Huang et al., 2015). Cytokinin was previously shown to affect plant immunity by promoting resistance to *Pseudomonas syringae pv. tomato DC3000*, which is the

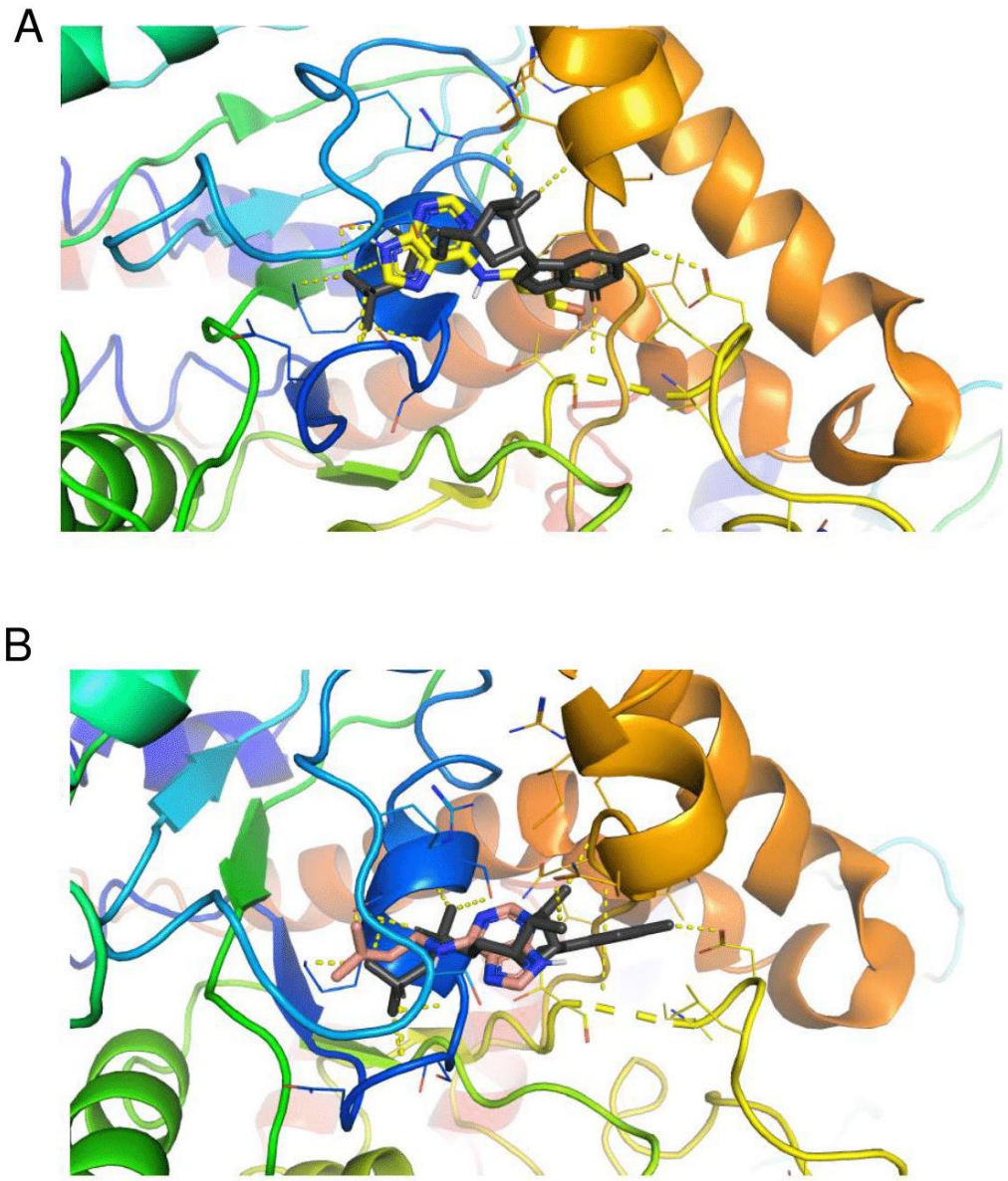


Figure 11: Docking of the molecule of trans-Zeatin into the GDP-binding pocket of DRP1A (Crystal structure) *(A) Trans-Zeatin in the active site of DRP1A (B) iP in the active site of DRP1A*

same strain as used in the *drp2b-2* pathogen response assays (Choi et al., 2010; Großkinsky et al., 2016). This suggests that DRP2s are the point of interaction between plant and pathogens and cytokinin might play a role of the signaling molecule in this process.

It seems counter intuitive, that cytokinin caused decrease of the DRP2s-GFP signal, which should make the plant more susceptible to the pathogen (*drp2b-2* shows immune defects), whereas Choi et al.(2010) and Großkinsky et al.(2016) show that cytokinin promotes the resistance to the pathogen. However, it is important to notice that while our microscopy

observations were performed in roots, most of the pathogen-sensitivity experiments are conducted in leaves. Interestingly, our preliminary biochemical analysis shows, that whereas there is a decrease of the DRP2A-GFP in roots upon cytokinin treatment, this is not the case in the shoot (data not shown).

Another remarkable characteristic of the cytokinin effect on DRP2A-GFP is that it is much more pronounced in cells at the transition zone, compared to the cells at the elongation zone of the primary root meristem (Figure 9F, G). Cytokinin plays a key role in determining the size of the meristem by regulating the differentiation of the cells (Dello Ioio et al., 2008; Di Mambro et al., 2017). Therefore, in roots, cytokinin might affect the size of the root meristem by influencing the DRP2A-GFP in the transition zone.

It is tempting to speculate that DRP2A and DRP1C were found in the screen because they are GTPases, therefore they are prone to bind molecules with a purine ring structure like cytokinin. We do not see general enrichment for GTPases in our dataset. Moreover, unspecific ATPase/GTPase binding was filtered out by using the nucleotide competitive pull-down. However, possibility that cytokinin binds DRP2A and DRP1C in the GTP-binding pocket cannot be excluded.

Although DRP1A was not found in our dataset, its crystal structure is available (Chen et al., 2012). The DRP2s can be modeled based on the 31% homology with the DRP1A. Preliminary computer modeling of docking of cytokinin molecules (tZ, iP) found that cytokinin can bind to the GTP-binding cavity of DRP1A. In case of tZ, hydrogen bonding to the amino acid ASN242 can be observed, on the other hand in case of iP a hydrogen bond with SER44 is observed. The binding energy of both cytokinin ligands is in the range of -5.5 kcal / mol to -6.7 kcal / mol, suggesting high affinity of the ligand to the protein (Figure 11). Nevertheless, it is important to model also the binding of the modified cytokinin molecule VM12 in further modeling experiments, to exclude the possibility that the presence of the linker interferes with the binding.

High affinity of cytokinin to the GTP-binding cavity promotes speculation that cytokinin effect on the DRP2A is caused by competitive binding with the GTP. Although this hypothesis seems plausible, results from treatment of DRP2A-GFP plants with adenine, which shares the purine ring structure with cytokinin and ATP/GTP and therefore it can probably also bind to the GTP

pocket did not result in DRP2A-GFP signal decrease. This suggest more specific cytokinin effect then just competitive binding to the GTP pocket of DRP2A.

Conclusion and perspectives

The chemical genomics approach offers a great potential for exploration of previously undescribed biological activity of chemical compounds. In our study, we used immobilized cytokinin (iP) to pull-down potential protein binding partners from the root tissue lysate of *Arabidopsis thaliana*. Among the 12 top candidates, we identified four proteins involved in endomembrane trafficking. The best scored cytokinin-binding protein candidate is DRP2A.

Further validation of the cytokinin binding to candidate proteins needs to be performed. First of all, cytokinin-DRP2A interaction should be verified by an independent method such as Surface plasmon resonance (SPR, Jain et al., 2016). We are currently working on the purification of the functional DRP2A protein necessary for this experiment. Drug Affinity Responsive Target Stability assay (DARTS, Pai et al., 2015) is another independent method suitable for testing of DRP2A-cytokinin binding, which is currently ongoing, using plants expressing *DRP2A::DRP2A-GFP*.

Since we observed that cytokinin treatment decreases the signal intensity of DRP2A-GFP in the epidermal cells, we are also performing further experiments including RT-QPCR of *DRP2A* (and *DRP2B*), to exclude the effect of cytokinin on DRP2A protein synthesis.

In addition, as a complementary approach, we are implementing bioinformatics tools to model iP docking to putative binding sites on a DRP2A protein. In case we identify a specific binding site, we will perform the site-directed mutagenesis and investigate the role of this mutation in plant growth and development as well as in the response to cytokinin.

Modeling of the docking of cytokinin to DRP2A might provide mechanistic and temporal insights into consequences of cytokinin binding. DRP2s have two main conformation states, the pre-fission and the post-fission, depending on the phase of the vesicle formation (Yan et al., 2018). Whether cytokinin might bind into the pre-fission DRP2A, interfering with the process of the fission or into the post-fission, *e.g.* preventing the recruitment of the DRP2A to the forming vesicle, remains to be discovered. We also plan to compare the model of iP

docking to DRP2A with DRP2B, and by that attempt to explain the specific identification of DRP2A.

These experiments together with in-detail investigation of the role of cytokinin-binding in endocytosis and other trafficking processes by recently published tools (Johnson et al., 2020) should shed more light on the physiological relevance of cytokinin binding proteins identified in our study.

Supplementary figures

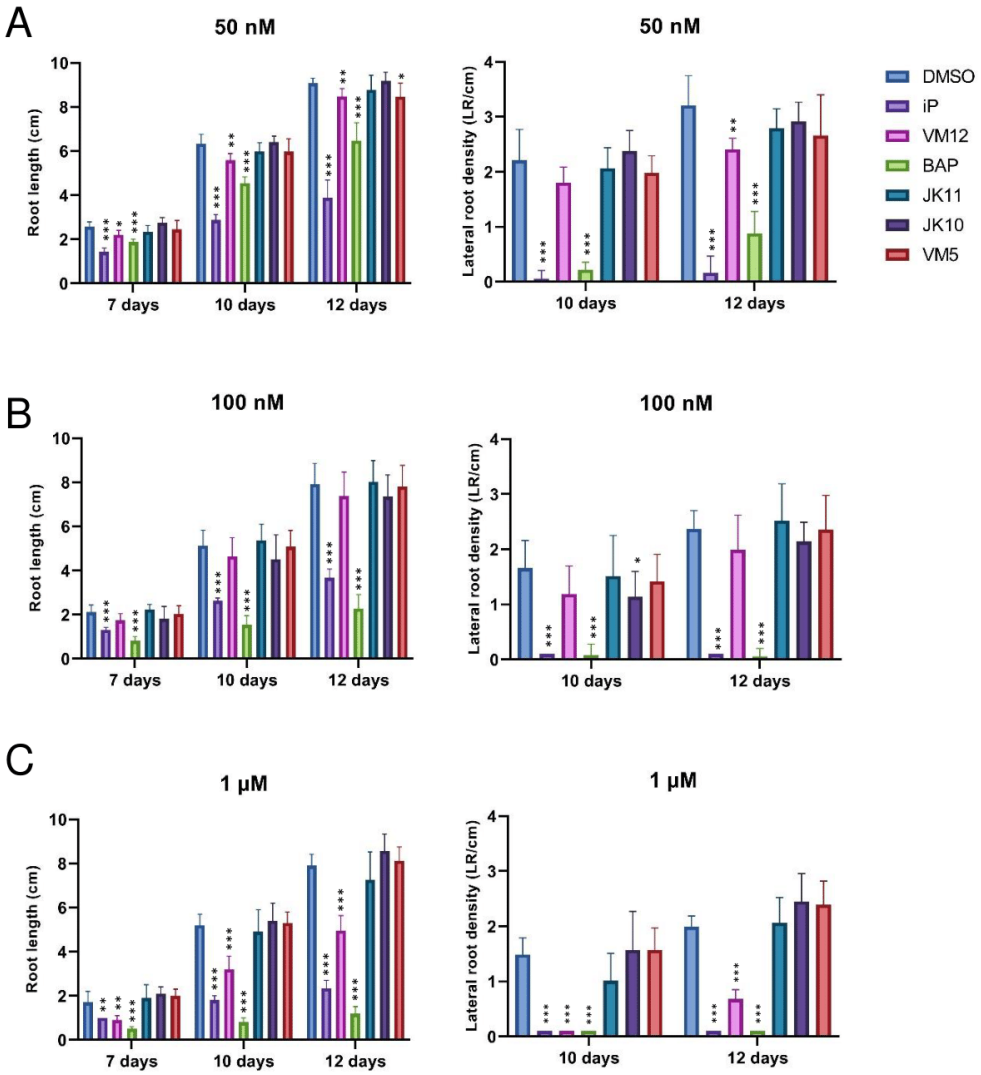


Figure S1: Effect of the modified cytokinin compounds on plant growth and development *(A)Root length and lateral root density measurements of plants grown on media supplemented with 50nM DMSO, BAP, iP, VM5, VM12, JK10, and JK5. (B) Root length and lateral root density measurements of plants grown on media supplemented with 100nM DMSO, BAP, iP, VM5, VM12, JK10, and JK5. (C) Root length and lateral root density measurements of plants grown on media supplemented with 1µ DMSO, BAP, iP, VM5, VM12, JK10, and JK5. The bar charts represent mean ± s.d., *** = p < 0.001, ** = p < 0.01, * = p < 0.05 by Student's t-test, n>5.*

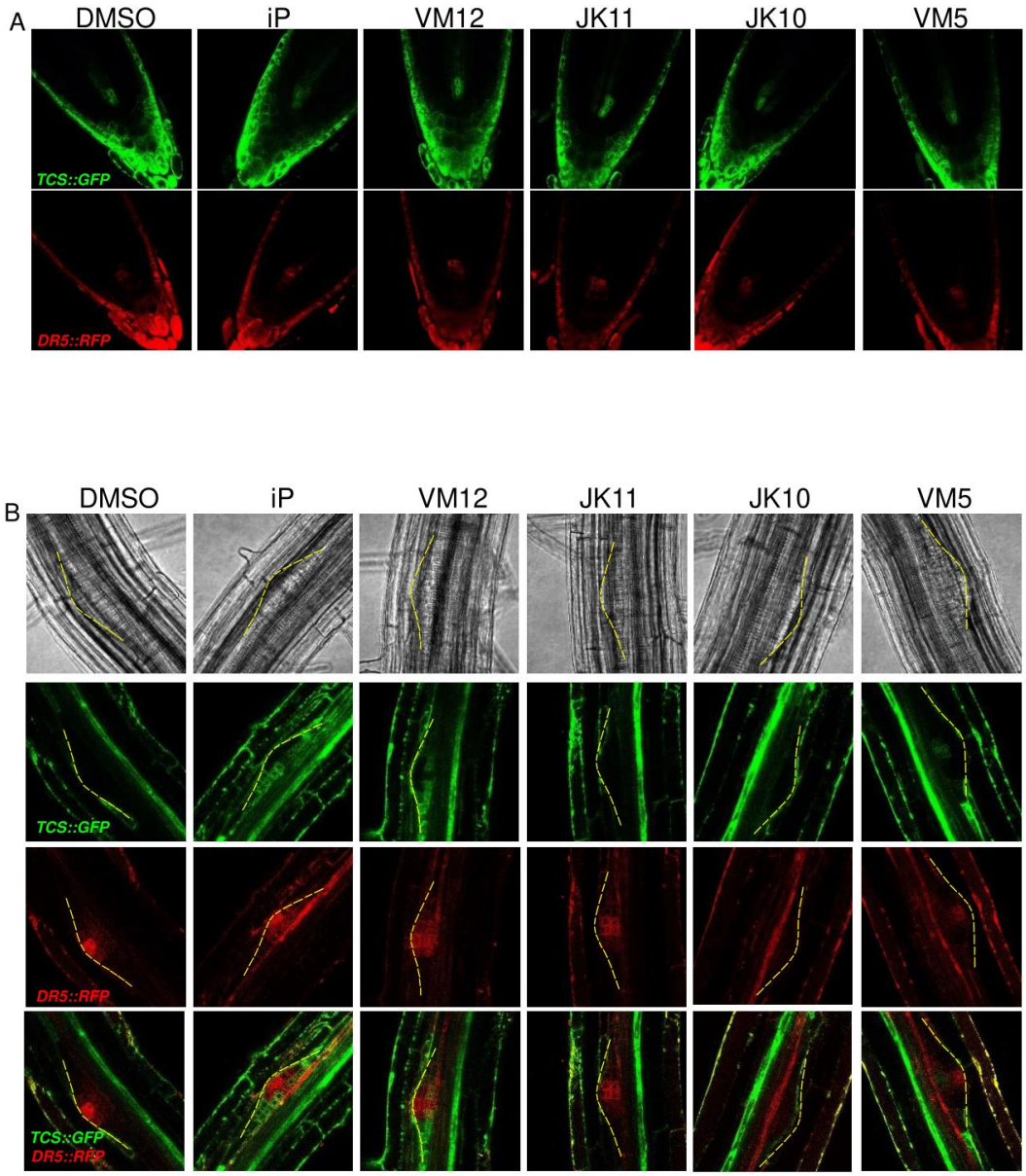


Figure S3: Expression of cytokinin (TCS) and auxin (DR5) reporters upon treatment with iP and modified cytokinins *5-days-old plants were transferred on the media supplemented with DMSO, 2µM iP, 2µM VM12, 2µM VM5, 2µM JK10, 2µM JK11 and treated for 4 hours. (A) TCS::GFP (green), DR5::RFP (red) expression in primary root meristem. We did not observe DR5 or TCS upregulation upon treatment with cytokinin or modified cytokinin molecules. (B) iP and VM12 treatment increases the TCS::GFP signal in the developing lateral root primordia and in endodermis, cortex and epidermis tissues of the surrounding primary root. VM5, JK10, and JK11 increase the TCS::GFP expression in the xylem pole pericycle cells and other tissues of the primary root. Yellow dashed lines represent outline of the developing lateral root primordia*

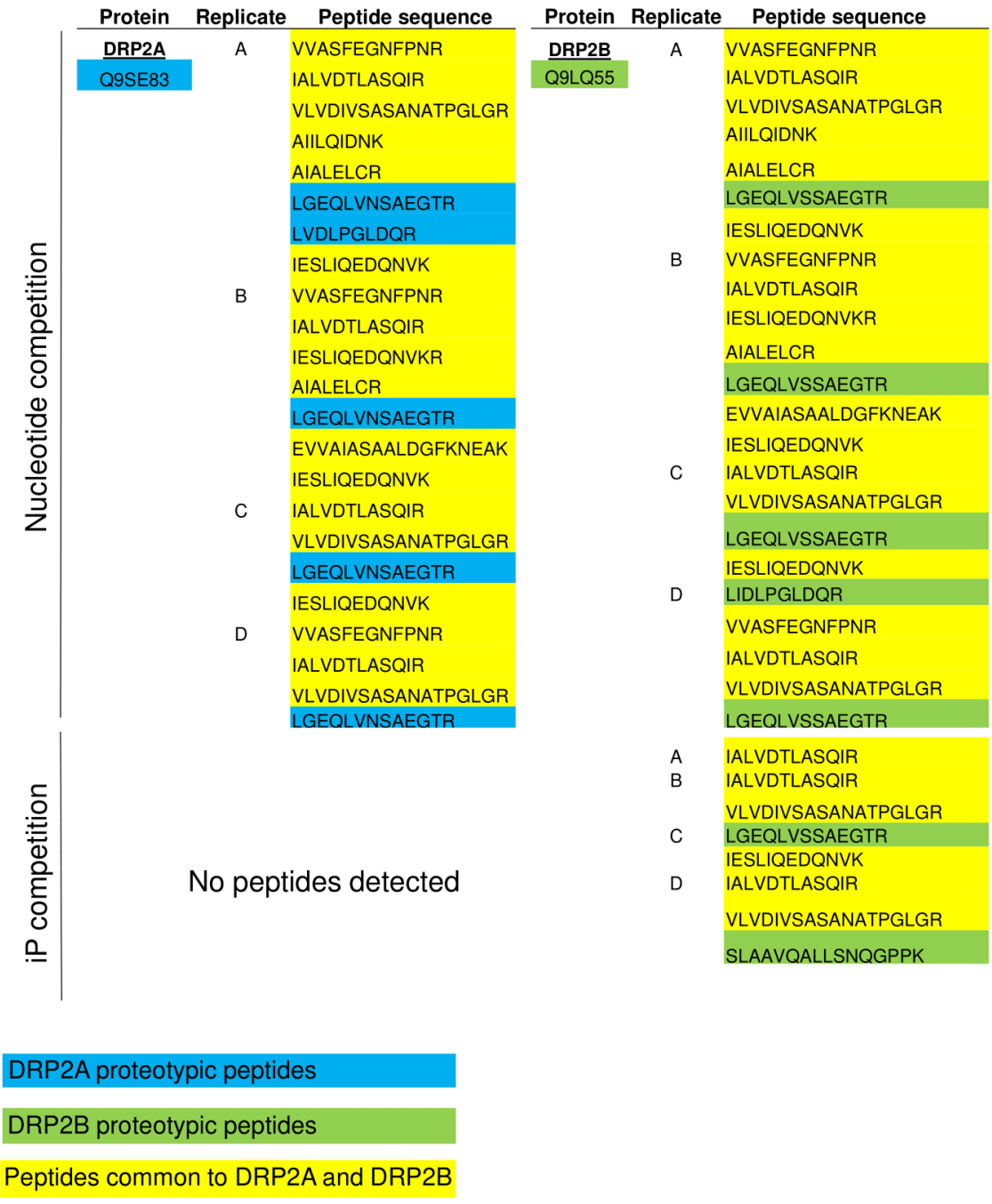


Figure S6: DRP2A (not DRP2B) identified as cytokinin binding protein *List of individual peptides identified by mass spectrometry. DRP2A peptides do not appear in the mass spectrometry results form iP competitive pull-down, suggesting their out competition by free iP. (A,B,C,D) represent replicate1-4*

Methods

Synthesis of cytokinin derivates

The chemicals used were purchased from Sigma-Aldrich (MO, USA) and were of analytical purity grade at least. Cytokinin ligands with 6-aminohexyl linker attached at C^2 or at N^9 position were synthesized at Department of Chemical Biology and Genetics, Faculty of Science, Palacký University, Czech Republic, following the procedure described earlier (Antoniadi et al., 2020; Simerský et al., 2017). The structure was confirmed by ^{1}H NMR spectra measured on an Avance AV 300 (Bruker Daltonics, Germany) NMR spectrometer at a temperature of 300 K and a frequency of 300.13 MHz.

Cytokinin affinity matrix preparation

NHS-activated Sepharose™ 4 Fast Flow beads (GE Healthcare, United Kingdom) were washed with a threefold volume of DMSO and mixed with equal volume of 20 mM solution of C2-AHA-iP ligand in DMSO. After the incubation performed at room temperature under continuous stirring overnight, the beads were washed in three successive steps with DMSO, 50% (v/v) DMSO and water. Non-reacted NHS- groups were inactivated with 1 M ethanolamine according to the manufacturer's instructions. In the last step, the affinity matrix was washed with water and 20% (v/v) ethanol and stored in the latter solution at 4 °C until required.To determine the efficiency of the ligand immobilization, an aliquot of the affinity beads was resuspended in 50% (v/v) glycerol and its absorbance was determined by UV–Vis measurement at 290 nm. The concentration of immobilized C2-AHA-iP was subsequently calculated following Lambert–Beer's law. Blank beads blocked with ethanolamine were prepared following the reaction scheme described above, omitting the ligand immobilization step.

Plant material

Plant material used in this study: *Arabidopsis thaliana* plants, ecotype Col-0. Mutants *drp2a-1 (*SALK_071036) and *drp2b-2(*SALK_134887) were obtained from The Salk Institue. TCS::GFP DR5::RFP (Marhavý et al., 2011), DRP2A::DRP2A-GFP, DRP2B::DRP2B-GFP(unpublished, generated by Nataliia Gnyliukh, Friml lab), CLC::CLC-GFP(Konopka et al., 2008), PIN1::PIN1-GFP (Benková et al., 2003).

Growth conditions and material

Surface sterilized seeds were plated on half strength Murashige and Skoog (MS) media (Duchefa) with 1%(w/v) sucrose and 1% (w/v) agar (pH=5.9). Seeds were stratified for 2 days at 4°C, in the dark. Seedlings were grown vertically in long-day conditions (16h light/8h dark) at 21°C. As light sources light emitting diode production modules (Philips GreenPower) were used in a deep red, far-red, blue combination with a photon density of 140 µmol/m^2/s ± 20%.

Hormonal treatment

All treatments were performed on a half strength Murashige and Skoog (MS) media (Duchefa) with 1%(w/v) sucrose and 1% (w/v) agar (pH=5.9). For long term experiments the plants were

grown on MS media supplemented with indicated concentrations of **N^6-(2-Isopentenyl) adenine** (iP, Sigma), modified cytokinin molecules or DMSO. Short term treatments were performed by transfer of plants on media supplemented with indicated concentrations of iP, Adenine or DMSO.

Protein extraction

Roots of 7-day-old seedlings of were harvested, flesh frozen in liquid nitrogen and ground in a grinder to a fine powder. Protein extraction was performed on ice for 30 minutes. The plant material mass to extraction buffer volume ratio was 1:3, the composition of the buffer was as follows: 50 mM Tris/HCl of pH 8.0, 100 mM NaCl, 2.5 mM $MgCl_2$, 2 mM $CaCl_2$, 5% (v/v) glycerol, 0.2% (v/v) Igepal Ca-630, a protease inhibitor cocktail cOmplete (Roche, Switzerland), 1 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4. Subsequently, two centrifugations at 4 °C (at 10,000g for 10 min and at 25,000g for 20 min,respectively) were performed to clear the supernatant. Protein concentration was determined using 2-D Quant Kit (GE Healthcare, IL, USA).

Affinity enrichment procedure and eluate processing

Complete affinity experiment comprised four pull-down variants. In a regular pull-down the extracted proteins were simply incubated with C2-AHA-iP affinity beads. A nucleotide-competitive pull-down involved the incubation of C2-AHA-iP affinity beads with protein extract in the presence of natural nucleotides (2.5 mM ADP, 2.5 mM GDP and 2.5 mM NAD^+). In an iP-competitive pull-down the affinity beads were incubated with protein extract in the presence of natural nucleotides and free iP (2.5 mM ADP, 2.5 mM GDP and 2.5 mM NAD^+ and 0.15 mM iP). A blank pull-down represented a control in which the extracted proteins were incubated with blank beads blocked with ethanolamine (see Figure 5). The affinity experiments were performed in triplicates.

An aliquot of the protein extract corresponding to a protein content of 300 µg was supplemented with extraction buffer to a final volume of 300 µl. Either affinity beads, or blank beads (100 µl) were washed with 3 ml of the extraction buffer and subjected to in vitro incubation with the diluted protein extract at 4 °C under continuous stirring for 2 h. Afterwards, the mixture was transferred to a Micro Bio Spin Column (Bio-Rad Laboratories, CA, USA), gravity drained and successively washed with the extraction buffer and its variants containing 500 mM and 100 mM NaCl, respectively. The detained proteins were eluted with 250 µl of 100 mM formic acid into a glass vial containing 62.5 µl of 1M triethylammonium bicarbonate buffer pH 8.5, flesh frozen in liquid nitrogen and stored at -80 °C until required.

Proteolytic digestion of isolated proteins

The isolated proteins were digested in-solution in the glass vial. The sample was reduced with 6 µl of 500 mM DTT (60 minutes, 56 °C) and alkylated with 17.5 µl of 1 M IAA. Trypsin digestion was performed overnight at 37 °C. The digest was acidified with 10 µl of formic acid and desalted on C-18 Micro SpinColumns (Harvard Apparatus, MA, USA) according to the manufacturer's instructions. Purified sample was evaporated in a vacuum centrifuge (Concentrator plus, Eppendorf, Germany) and stored at -80 °C until processed further.

Peptide LC–MS/MS analysis

The peptide analysis was performed on a nanoflow capillary liquid chromatography RSLCnano 3000 (Dionex, Thermo Fisher Scientific, CA, USA) coupled to tandem mass spectrometry (UHR-QTOF maXis; Bruker Daltonik, Germany) via nanoESI source (Bruker Daltonik, Germany). The chromatography system comprised a precolumn 75 µm × 3 cm, IntegraFrit (New Objective, CA, USA) and an analytical capillary column 50 µm × 20 cm, SilicaTip (New Objective, CA, USA), both filled with a reverse phase Reprosil GOLD C18, 3 µm (Dr. Maisch GmbH, Germany). Peptides retained on the precolumn were isocratically washed with 20 µl 2% FA for 10 minutes. After washing, the peptides were separated on the analytical column by a 80 minute multi-step gradient at a constant flow rate of 200 nl/min (Buffer A: 0,1% FA, buffer B: 90 % ACN in H_2O, 0,1% FA. 0 min, 5 % B; 13 min, 5 % B; 53 min, 25 % B; 60 min, 35 % B; 62 min, 55 % B; 64 min, 85 % B; 66 min, 85 % B; 69 min, 5 % B; 80 min, 5 % B).

The MS analysis was carried out in a data-dependent acquisition and CID method for peptide identification. The settings of the MS analyzer were as follows: Source (Captive Spray 1080 V; Dry gas: 8 l/min; Dry temperature: 150 °C); Tune Page (Ion funnel RF 400 Vpp; Multipole RF 400 Vpp; Quadrupole ion energy 3.5 eV; collision energy 6 eV; Collision RF 1250 Vpp; transfer time 90 µs; pre- puls storage 12 µs); MS/MS mode (auto MS/MS, switch threshold from MS to MSMS mode 500 cts, active exclusion after 1 spectra for 20 s); Mass range 100–1800 m/z with MS time acquisition 500 ms and MS/MS time acquisition 60–250 ms according to precursor intensity.

Mass spectrometry data analysis

Raw data were processed by DataAnalysis 4.3x64 (Bruker Daltonics, Germany) and extracted MS and MSMS spectra were saved as mgf files (mascot generic file, Matrix Science, England). Protein identification was successively performed using SearchGUI v.3.2.20 software (Vaudel et al., 2011) which applies three independent search algorithms for peptide and protein identification (X! Tandem, Craig & Beavis, 2004; MS Amanda, (Dorfer et al., 2014); MS-GF+, (Kim & Pevzner, 2014). The reference Arabidopsis thaliana protein databases (xxxxxx sequences, downloaded on 20xx-xx-xx) was downloaded from UNIPROT repository (www.unipro.org). Identification parameters were set as following: trypsin proteolytic enzyme, carbamidomethylation of cysteine as a fixed modification and methionin oxidation, acetylation of N-protein termini, deamination for asparagine and glutamine as variable modifications.

Data from all three identification algorithms were evaluated and filtered by PeptideShaker v1.16.15 (Vaudel et al., 2015). Text files containing comprehensive protein and peptide identifications including also quantitative data in the form of evaluated spectral counts were exported for each affinity purification experiment. These text files were further processed by APOSTL software (Kuenzi et al., 2016) designed to statistically evaluate proteomic data from affinity experiments in order to identify putative interactors.

Genotyping

For genotyping we used the primers: drp2a-1_LP: ACACCTCATCAACACAAAGGC, drp2a-1_RP: AAACAACTGACATTCCATGGG, drp2b-2_LP: ATAGCCTAATTGGGCATCCAG, drp2b-2_RP: TATAGCATCGTTGTGCTGTGC.

Microscopy

Confocal images were obtained with LSM 800 laser scanning confocal microscopes (Zeiss) equipped with a Plan-Apochromat 40x/1.2 water or Plan-Apochromat 100x /1.4 Oil immersion objective. Fluorescence markers were excited at 488 nm (GFP).

Microscopy data analysis

Data were analyzed using ImagJ software (National Institute of Health, http://rsb.info.nih.gov/ij), GraphPad Prism 8, and Microsoft PowerPoint programs.

Statistical analysis

All data were analysed using one-way ANOVA with a Tukey multiple comparisons test or two-tailed Student's *t* test.

Supplemental References

Antoniadi I, Novak O, Gelova Z, Johnson A, Plihal O, Simersky R, Mik V, Vain T, Mateo-Bonmati E, Karady M, Pernisova M, Plackova L, Opassathian K, Hejatko J, Robert S, Friml J, Dolezal K, Ljung K, Turnbull C (2020) Cell-surface receptors enable perception of extracellular cytokinins. Nat Commun 11:4284.

Beck S, Michalski A, Raether O, Lubeck M, Kaspar S, Goedecke N, Baessmann C, Hornburg D, Meier F, Paron I, Kulak NA, Cox J, Mann M (2015) The Impact II, a very high-resolution quadrupole time-of-flight instrument (QTOF) for deep shotgun proteomics. Mol Cell Proteomics 14:2014–2029.

Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M (2011) Andromeda: A peptide search engine integrated into the MaxQuant environment. J Proteome Res 10:1794–1805.

Craig R, Beavis RC (2004) TANDEM: matching proteins with mass spectra. Bioinformatics 20:1466-7.

Dorfer V, Pichler P, Stranzl T, Stadlmann J, Taus T, Winkler S, Mechtler K (2014) MS Amanda, a Universal Identification Algorithm Optimized for High Accuracy Tandem Mass Spectra. J. Proteome Res 13:3679-3684.

Holub J, Hanuš J, Hanke DE, Strnad M (1998) Biological activity of hydroxybenzyladenines. Plant Growth Regul 26:109-115.

Kim S, Pevzner PA (2014) MS-GF+ makes progress towards a universal database search tool for proteomics. Nat Commun 5:5277.

Kuenzi BM, Borne AL, Li J, Haura EB, Eschrich SA, Koomen JM, Rix U, Stewart PA (2016) APOSTL: An Interactive Galaxy Pipeline for Reproducible Analysis of Affinity Proteomics Data. J Proteome Res 15:4747-4754.

Simerský R, Chamrád I, Kania J, Strnad M, Šebela M, Lenobel R (2017) Chemical Proteomic Analysis of 6-Benzylaminopurine Molecular Partners in Wheat Grains. Plant Cell Rep 36:1561–1570.

Vaudel M, Barsnes H, Berven FS, Sickmann A, Martens L. (2011) SearchGUI: An open-source graphical user interface for simultaneous OMSSA and X!Tandem searches. Proteomics 11(5):996-9. doi: 10.1002/pmic.201000595.

Vaudel M, Burkhart JM, Zahedi RP, Oveland E, Berven FS, Sickmann A, Martens L, Barsnes H (2015) PeptideShaker enables reanalysis of MS-derived proteomics data sets Nature Biotechnol 33:22–24.

References

Antoniadi, I., Novák, O., Gelová, Z., Johnson, A., Plíhal, O., Simerský, R., Mik, V., Vain, T., Mateo-Bonmatí, E., Karady, M., et al. (2020). Cell-surface receptors enable perception of extracellular cytokinins. *Nat. Commun.* 11.

Backues, S. K., Korasick, D. A., Heese, A., and Bednareka, S. Y. (2010). The arabidopsis dynamin-related protein2 family is essential for gametophyte development. *Plant Cell* 22:3218–3231.

Berridge, M. V, Ralph, R. K., and Letham, D. S. (1970). The binding of kinetin to plant ribosomes. *Biochem. J.* 119:75–84.

Brinegar, A. C., Stevens, A., and Fox, J. E. (1985). Biosynthesis and Degradation of a Wheat Embryo Cytokinin-Binding Protein during Embryogenesis and Germination. *Plant Physiol.* 79:706–710.

Brinegar, A. C., Cooper, G., Stevens, A., Hauer, C. R., Shabanowitz, J., Hunt, D. F., and Fox, J. E. (1988). Characterization of a benzyladenine binding-site peptide isolated from a wheat cytokinin-binding protein : Sequence analysis and identification of a single affinity-labeled histidine residue by mass spectrometry. *Proc. Natl. Acad. Sci. USA* 85:5927–5931.

Brovko, F. A., Vasil'eva, V. S., Shepelyakovskaya, A. O., Selivankina, S. Y., Kudoyarova, G. R., Nosov, A. V., Moshkov, D. A., Laman, A. G., Boziev, K. M., Kusnetsov, V. V., et al. (2007). Cytokinin-binding protein (70 kDa): Localization in tissues and cells of etiolated maize seedlings and its putative function. *J. Exp. Bot.* 58:2479–2490.

Brovko, F. A., Vasil'Eva, V. S., Lushnikova, A. L., Selivankina, S. Y., Karavaiko, N. N., Boziev, K. M., Shepelyakovskaya, A. O., Moshkov, D. A., Pavlik, L. L., Kusnetsov, V. V., et al. (2010). Cytokinin-binding protein (70 kDa) from etioplasts and amyloplasts of etiolated maize seedlings and chloroplasts of green plants and its putative function. *J. Exp. Bot.*

61:3461–3474.

Caesar, K., Thamm, A. M. K., Witth??ft, J., Elgass, K., Huppenberger, P., Grefen, C., Horak, J., and Harter, K. (2011). Evidence for the localization of the Arabidopsis cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. *J. Exp. Bot.* 62:5571–5580.

Cao, M., Chen, R., Li, P., Yu, Y., Zheng, R., Ge, D., Zheng, W., Wang, X., Gu, Y., Gelová, Z., et al. (2019). TMK1-mediated auxin signalling regulates differential growth of the apical hook. *Nature* 568:240–243.

Chang, C., Kwok, S. F., Bleecker, A. B., and Meyerowitz, E. M. (1993). Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. *Science (80-.).* 262:539 LP – 544.

Chen, X., Xu, X., Sun, Y., Zhou, J., Ma, Y., Yan, L., and Lou, Z. (2012). Purification, crystallization and preliminary X-ray crystallographic analysis of {\it Arabidopsis thaliana} dynamin-related protein 1A GTPase-GED fusion protein. *Acta Crystallogr. Sect. F* 68:69–72.

Choi, J., Huh, S. U., Kojima, M., Sakakibara, H., Paek, K. H., and Hwang, I. (2010). The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in arabidopsis. *Dev. Cell* 19:284–295.

D'Agostino, I. B., Deruere, J., and Kieber, J. J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiol* 124:1706–1717.

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., Aoyama, T., Costantino, P., and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. *Science (80-.).* 322:1380–1384.

Di Mambro, R., De Ruvo, M., Pacifici, E., Salvi, E., Sozzani, R., Benfey, P. N., Busch, W., Novak, O., Ljung, K., Di Paola, L., et al. (2017). Auxin minimum triggers the developmental switch from cell division to cell differentiation in the Arabidopsis root. *Proc. Natl. Acad. Sci. U. S. A.* 114:E7641–E7649.

Erion, J. L., and Fox, J. E. (1981). Purification and Properties of a Protein Which Binds Cytokinin-active 6-Substituted Purines 1. *Plant Physiol.* 67:156–162.

Fernandes, H., Pasternak, O., Bujacz, G., Bujacz, A., Sikorski, M. M., and Jaskolski, M. (2008). Lupinus luteus Pathogenesis-Related Protein as a Reservoir for Cytokinin. *J. Mol. Biol.* 378:1040–1051.

Fernandes, H., Bujacz, A., Bujacz, G., Jelen, F., Jasinski, M., Kachlicki, P., Otlewski, J., Sikorski, M. M., and Jaskolski, M. (2009). Cytokinin-induced structural adaptability of a Lupinus luteus PR-10 protein 276:1596–1609.

Firn, R. D. (1987). Too Many Binding Proteins, not Enough Receptors? In *Plant Hormone Receptors* (ed. Klämbt, D.), pp. 1–11. Berlin, Heidelberg: Springer Berlin Heidelberg.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. *Nature* 426:147–153.

Fujimoto, M., and Tsutsumi, N. (2014). Dynamin-related proteins in plant post-Golgi traffic. *Front. Plant Sci.* 5:1–8.

Fujimoto, Y., Nagata, R., Fukasawa, H., Yano, K., Azuma, M., Iida, A., and Sugimoto, S. (1998). Purification and cDNA cloning of cytokinin-specific binding protein from mung bean (Vigna radiata). *Eur. J. Biochem.* 802:794–802.

Fujimoto, M., Arimura, S. I., Nakazono, M., and Tsutsumi, N. (2008). Arabidopsis dynamin-related protein DRP2B is co-localized with DRP1A on the leading edge of the forming cell plate. *Plant Cell Rep.* 27:1581–1586.

Fujimoto, M., Arimura, S. I., Ueda, T., Takanashi, H., Hayashi, Y., Nakano, A., and Tsutsumi, N. (2010). Arabidopsis dynamin-related proteins DRP2B and DRP1A participate together in clathrin-coated vesicle formation during endocytosis. *Proc. Natl. Acad. Sci. U. S. A.* 107:6094–6099.

Gadeyne, A., Sánchez-Rodríguez, C., Vanneste, S., Di Rubbo, S., Zauber, H., Vanneste, K., Van Leene, J., De Winne, N., Eeckhout, D., Persiau, G., et al. (2014). The TPLATE adaptor complex drives clathrin-mediated endocytosis in plants. *Cell* 156:691–704.

Gardner, G., Sussman, M. R., and Kende, H. (1978). In vitro cytokinin binding to a particulate cell fraction from protonemata of Funaria hygrometrica. *Planta* 143:67–73.

Gelová, Z., Gallei, M., Pernisová, M., Brunoud, G., Zhang, X., Glanc, M., Li, L., Michalko, J., Pavlovičová, Z., Verstraeten, I., et al. (2021). Developmental roles of Auxin Binding Protein 1 in Arabidopsis thaliana. *Plant Sci.* 303.

Großkinsky, D. K., Tafner, R., Moreno, M. V., Stenglein, S. A., De Salamone, I. E. G., Nelson, L. M., Novák, O., Strnad, M., Van Der Graaff, E., and Roitsch, T. (2016). Cytokinin production by Pseudomonas fluorescens G20-18 determines biocontrol activity against Pseudomonas syringae in Arabidopsis. *Sci. Rep.* 6:1–11.

Heyl, A., Wulfetange, K., Pils, B., Nielsen, N., Romanov, G. A., and Schmülling, T. (2007). Evolutionary proteomics identifies amino acids essential for ligand-binding of the cytokinin receptor CHASE domain. *BMC Evol. Biol.* 7:1–8.

Hong, Z., Bednarek, S. Y., Blumwald, E., Hwang, I., Jurgens, G., Menzel, D., Osteryoung, K. W., Raikhel, N. V., Shinozaki, K., Tsutsumi, N., et al. (2003). A unified nomenclature for Arabidopsis dynamin-related large GTPases based on homology and possible functions. *Plant Mol. Biol.* 53:261–265.

Hothorn, M., Dabi, T., and Chory, J. (2011). Structural basis for cytokinin recognition by Arabidopsis thaliana histidine kinase 4. *Nat. Chem. Biol.* 7:766–768.

Huang, J., Fujimoto, M., Fujiwara, M., Fukao, Y., Arimura, S. I., and Tsutsumi, N. (2015). Arabidopsis dynamin-related proteins, DRP2A and DRP2B, function coordinately in post-Golgi trafficking. *Biochem. Biophys. Res. Commun.* 456:238–244.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. *Nature* 413:383–9.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki,

K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. *Nature* 409:1060–1063.

Jain, P., Arora, D., and Bhatla, S. C. (2016). Surface Plasmon Resonance Based Recent Advances in Understanding Plant Development and Related Processes. *Biochem. Anal. Biochem.* 05.

Jin, J. B., Kim, Y. A., Kim, S. J., Lee, S. H., Kim, D. H., Cheong, G. W., and Hwang, I. (2001). A new dynamin-like protein, ADL6, is involved in trafficking from the trans-Golgi network to the central vacuole in Arabidopsis. *Plant Cell* 13:1511–1525.

Johnson, A., Gnyliukh, N., Kaufmann, W. A., Narasimhan, M., Vert, G., Bednarek, S. Y., and Friml, J. (2020). *Experimental toolbox for quantitative evaluation of clathrin-mediated endocytosis in the plant model Arabidopsis*.

Kakimoto, T. (1996). CKI1, a Histidine Kinase Homolog Implicated in Cytokinin Signal Transduction. *Science (80-.).* 274:982 LP – 985.

Kakimoto, T. (2003). PERCEPTION AND SIGNAL TRANSDUCTION OF CYTOKININS. *Annu. Rev. Plant Biol.* 54:605–627.

Kaksonen, M., and Roux, A. (2018). Mechanisms of clathrin-mediated endocytosis. *Nat. Publ. Gr.* 19:313–326.

Kamínek, M. (2015). Tracking the Story of Cytokinin Research. *J. Plant Growth Regul.* 34:723–739.

Keshishian, E. A., and Rashotte, A. M. (2015). Plant cytokinin signalling. *Essays Biochem.* 58:13–27.

Kieber, J. J., and Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development* 145:1–7.

Kim, H. J., Ryu, H., Hong, S. H., Woo, H. R., Lim, P. O., Lee, I. C., Sheen, J., Nam, H. G., and Hwang, I. (2006). Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 103:814–819.

Kobayashi, K., Zbell, B., and Reinert, J. (1981). A high affinity binding site for cytokinin to a particulate fraction in carrot suspension cells. *Protoplasma* 106:145–155.

Kobayashi, K., Fukuda, M., Igarashi, D., and Sunaoshi, M. (2000). Cytokinin-binding proteins from tobacco callus share homology with osmotin-like protein and an endochitinase. *Plant Cell Physiol.* 41:148–157.

Konopka, C. A., and Bednarek, S. Y. (2008). Comparison of the dynamics and functional redundancy of the Arabidopsis dynamin-related isoforms DRP1A and DRP1C during plant development. *Plant Physiol.* 147:1590–1602.

Kubiasová, K., Montesinos, J. C., Šamajová, O., Nisler, J., Mik, V., Semerádová, H., Plíhalová, L., Novák, O., Marhavý, P., Cavallari, N., et al. (2020). Cytokinin fluoroprobe reveals multiple sites of cytokinin perception at plasma membrane and endoplasmic reticulum. *Nat. Commun.* 11:1–11.

Kulaeva, O. N., Karavaiko, N. N., Selivankina, S. Y., Kusnetsov, V. V., Zemlyachenko, Y. V., Cherepneva, G. N., Maslova, G. G., Lukevich, T. V., Smith, A. R., and Hall, M. A. (2000). Nuclear and chloroplast cytokinin-binding proteins from barley leaves participating in transcription regulation. *Plant Growth Regul.* 32:329–335.

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M. B., et al. (2007). Cytokinins Act Directly on Lateral Root Founder Cells to Inhibit Root Initiation. *Plant Cell Online* 19:3889–3900.

Li, S. M., Zheng, H. X., Zhang, X. S., and Sui, N. (2021). Cytokinins as central regulators during plant growth and stress response. *Plant Cell Rep.* 40:271–282.

Lomin, S. N., Yonekura-Sakakibara, K., Romanov, G. A., and Sakakibara, H. (2011). Ligand-binding properties and subcellular localization of maize cytokinin receptors. *J. Exp. Bot.* 62:5149–5159.

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., et al. (2011). Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis. *Dev. Cell* 21:796–804.

Mitsui, S., Wakasugi, T., and Sugiura, M. (1996). A cytokinin-binding protein complex from tobacco leaves. *Plant Growth Regul.* 18:39–43.

Montesinos, J. C., Abuzeineh, A., Kopf, A., Juanes-Garcia, A., Ötvös, K., Petrášek, J., Sixt, M., and Benková, E. (2020). Phytohormone cytokinin guides microtubule dynamics during cell progression from proliferative to differentiated stage. *EMBO J.* 39:1–22.

Müller, B., and Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453:1094–1097.

Narasimhan, M., Johnson, A., Prizak, R., Kaufmann, W. A., Tan, S., and Casillas-pe, B. (2020). Evolutionarily unique mechanistic framework of clathrin-mediated endocytosis in plants Advance Access published 2020.

Osugi, A., and Sakakibara, H. (2015). Q&A: How do plants respond to cytokinins and what is their importance? *BMC Biol.* 13:102.

Pai, M. Y., Lomenick, B., Hwang, H., Schiestl, R., McBride, W., Loo, J. A., and Huang, J. (2015). Drug Affinity Responsive Target Stability (DARTS) for Small Molecule Target Identification. *Physiol. Behav.* 176:139–148.

Pasternak, O., Bujacz, G. D., Fujimoto, Y., Hashimoto, Y., Jelen, F., Otlewski, J., Sikorski, M. M., and Jaskolski, M. (2006). Crystal structure of Vigna radiata cytokinin-specific binding protein in complex with zeatin. *Plant Cell* 18:2622–2634.

Romanov, G. A., Lomin, S. N., and Schmülling, T. (2006). Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. *J. Exp. Bot.* 57:4051–4058.

Ruszkowski, M., Szpotkowski, K., Sikorski, M., and Jaskolski, M. (2013). The landscape of cytokinin binding by a plant nodulin. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 69:2365–

2380.

Ruszkowski, M., Sliwiak, J., Ciesielska, A., Barciszewski, J., Sikorski, M., and Jaskolski, M. (2014). Specific binding of gibberellic acid by Cytokinin-Specific Binding Proteins: A new aspect of plant hormone-binding proteins with the PR-10 fold. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 70:2032–2041.

Ruzicka, K., Simásková, M., Duclercq, J., Petrásek, J., Zazímalová, E., Simon, S., Friml, J., Van Montagu, M. C. E., and Benková, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. *Proc. Natl. Acad. Sci. U. S. A.* 106:4284–4289.

Schmülling, T., Riefler, M., Krupková, E., and Bartrina, I. (2003). Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. *J Plant Res* Advance Access published 2003.

Selivankina, S. Y., Karavaiko, N. N., Maslova, G. G., Zubkova, N. K., Prokoptseva, O. S., Smith, A. R., Hall, M. A., and Kulaeva, O. N. (2004). Cytokinin-binding protein from Arabidopsis thaliana leaves participating in transcription regulation. *Plant Growth Regul.* 43:15–26.

Shepelyakovskaya, A. O., Brovko, F. A., and Kulaeva, O. N. (2002). A Cytokinin-Binding 70-kD Protein Is Localized Predominantly in the Root Meristem. *Russ. J. Plant Physiol.* 49:113–120.

Shimada, T., Kunieda, T., Sumi, S., Koumoto, Y., Tamura, K., Hatano, K., Ueda, H., and Hara-nishimura, I. (2018). The AP-1 Complex is Required for Proper Mucilage Formation in Arabidopsis Seeds. *Plant Cell Physiol.* 59:2331–2338.

Simerský, R., Chamrád, I., Kania, J., Strnad, M., Šebela, M., and Lenobel, R. (2017). Chemical proteomic analysis of 6-benzylaminopurine molecular partners in wheat grains. *Plant Cell Rep.* Advance Access published 2017, doi:10.1007/s00299-017-2174-4.

Skoog, F., and Tsui, C. (1951). Growth substances and the formation of buds in plant tissues. *Skoog F Plant Growth Subst. Univ. Wisconsin Press. Madison* Advance Access published 1951.

Smith, J. M., Leslie, M. E., Robinson, S. J., Korasick, D. A., Zhang, T., Backues, S. K., Cornish, P. V., Koo, A. J., Bednarek, S. Y., and Heese, A. (2014). Loss of Arabidopsis thaliana Dynamin-Related Protein 2B Reveals Separation of Innate Immune Signaling Pathways. *PLoS Pathog.* 10.

Spíchal, L., Rakova, N. Y., Riefler, M., Mizuno, T., Romanov, G. A., Strnad, M., and Schmülling, T. (2004). Two cytokinin receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. *Plant Cell Physiol.* 45:1299–1305.

Taylor, N. G. (2011). A role for Arabidopsis dynamin related proteins DRP2A/B in endocytosis; DRP2 function is essential for plant growth. *Plant Mol. Biol.* 76:117–129.

Viktorova, J., Krasny, L., Kamlar, M., Novakova, M., Mackova, M., and Macek, T. (2012). Osmotin, a pathogenesis-related protein. *Curr. Protein Pept. Sci.* 13:672–681.

Wang, J. G., Li, S., Zhao, X. Y., Zhou, L. Z., Huang, G. Q., Feng, C., and Zhang, Y. (2013).

HAPLESS13, the Arabidopsis μ1 adaptin, is essential for protein sorting at the trans-Golgi network/early endosome. *Plant Physiol.* 162:1897–1910.

Weijers, D., and Wagner, D. (2016). Transcriptional Responses to the Auxin Hormone. *Annu. Rev. Plant Biol.* 67:539–574.

Wulfetange, K., Lomin, S. N., Romanov, G. a, Stolz, A., Heyl, A., and Schmülling, T. (2011). The cytokinin receptors of Arabidopsis are located mainly to the endoplasmic reticulum. *Plant Physiol.* 156:1808–1818.

Wyblouw, B., and De Rybel, B. (2019). Cytokinin – A Developing Story. *Trends Plant Sci.* 24:177–185.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T., and Mizuno, T. (2001). The arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant Cell Physiol.* 42:1017–1023.

Yan, L., Yuanyuan Ma, Sun, Y., Gao, J., Chen, X., Liu, J., Wang, C., Rao, Z., Lou, Z., and 1Laboratory (2018). Structural basis for mechanochemical role of Arabidopsis thaliana dynamin-related protein in membrane fissio. *J. Mol. Cell Biol.* 3:378–381.

Zürcher, E., Tavor-Deslex, D., Lituiev, D., Enkerli, K., Tarr, P. T., and Müller, B. (2013). A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signaling network in planta. *Plant Physiol.* 161:1066–75.

Zürcher, E., Liu, J., Di Donato, M., Geisler, M., and Müller, B. (2016). Plant development regulated by cytokinin sinks. *Science (80-.).* 353:1027–1030.

Monitoring of Proteomic and Phosphoproteomic Changes in Arabidopsis thaliana Roots upon Treatment with Cytokinin

Monitoring of Proteomic and Phosphoproteomic Changes in Arabidopsis thaliana Roots upon Treatment with Cytokinin

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Introduction

Cytokinin is fundamental regulator of plant growth, development, and response to the environment (Kieber & Schaller, 2018). The majority of the cytokinin research in the past has been focused on effects dependent on the canonical signaling pathway, involving regulation of the gene expression (Gupta & Rashotte, 2012; Kieber & Schaller, 2018; Wyblouw & De Rybel, 2019). Although gene expression profiling brings comprehensive view on the response to the hormone (Bhargava et al., 2013), it does not provide an exhaustive explanation of cytokinin biological activity. In parallel to the gene expression, the regulation of molecular processes on post-transcriptional and post-translational level dramatically contributes to the final output of the transcriptional changes. Therefore, a complementary approach of proteome profiling offers valuable insights into cytokinin effect on abundance and posttranslational modifications of proteins (PTMs).

PMTs (*e.g.* phosphorylation, acetylation or methylation) are amino acid side chain modifications, influencing protein properties such as stability, activity, localization or interactions with binding partners. They greatly expand the complexity of the proteome and enable fast, dynamic regulation of proteins. The most common and extensively studied modification among PTMs is phosphorylation (Adam & Hunter, 2018). The phosphorylation status of a protein is regulated by kinases, which phosphorylate the substrate protein; and phosphatases, which catalyze the de-phosphorylation.

Many biological processes governed by plant hormones include regulation of phosphorylation (Camoni et al., 2018; Jagodzik et al., 2018; Mao & Li, 2020; Máthé et al., 2021; Soma et al., 2021; Tan et al., 2021). In fact, the canonical cytokinin signaling consists of the

phosphorylation cascade leading to transcriptional changes (Kieber & Schaller, 2018; Li et al., 2021). However, the amount of the data about the impact of cytokinin on phosphorylation of other proteins is rather limited (Dautel et al., 2016).

Experiments with chemical suppressors revealed that the cytokinin-triggered degradation of PIN1 is independent of transcription and translation (Marhavý et al., 2011). Therefore, to search for molecular components of this novel cytokinin action, we investigated the putative regulation on the posttranslational level.

Here, the label-free quantitative mass spectrometry workflow is used to analyze changes in abundance and phosphorylation of proteins triggered by cytokinin. We generated a unique dataset of early changes in the global proteome and phosphoproteome of *Arabidopsis thaliana* roots upon cytokinin treatment.

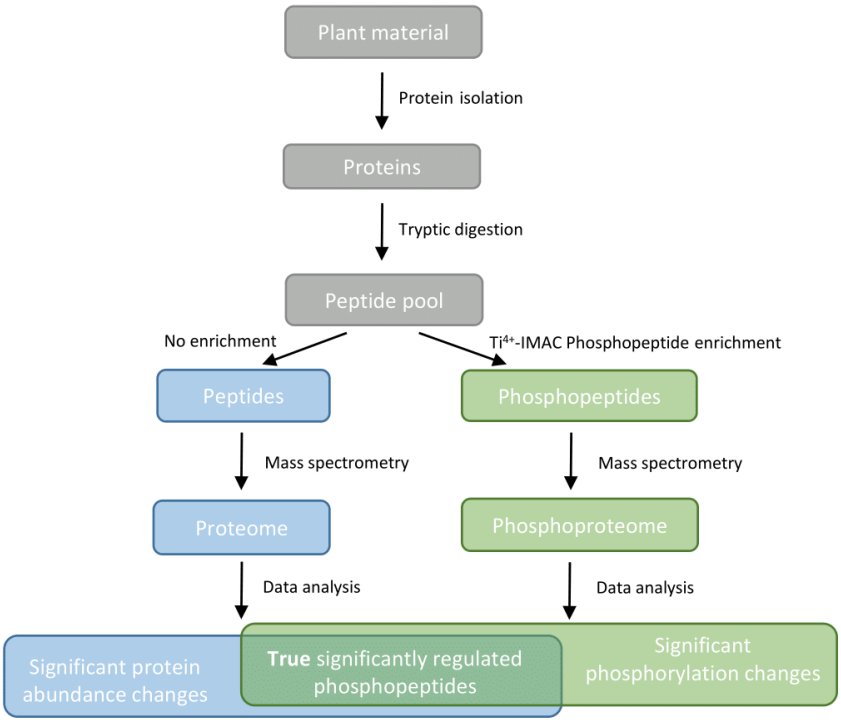

Figure 1: The design of the key steps of the (phospho)proteomic analysis

Results and Discussion

Sample preparation workflow

To identify new components involved in plant's response to cytokinin on the post-translational level, we performed mass spectrometry analysis of proteins extracted from *Arabidopsis thaliana* roots that were treated with mock or cytokinin. The experiment was designed according to recently published phosphoproteomic workflow (Figure1, Vu et al., 2016). *Arabidopsis thaliana* wild type (Col-0) seedlings were grown on the solid AM+ media for 7 days. They were sprayed with mock (DMSO) or cytokinin (2µM, Trans-Zeatin, tZ). Roots were collected after 15, 30, and 60 minutes of the treatment and flash frozen in liquid nitrogen (Figure 2A). The spraying treatment and collection of the roots was performed always during the same time of the day in four biological replicates (in four consecutive days). Samples were stored in -80°C until subsequent analyses.

Proteins were isolated from the roots and subjected to tryptic digestion (protocol according to Vu et al., 2016). All samples underwent the proteomic and phosphoproteomic analysis in parallel. Therefore, the phosphoproteome can be theoretically normalized to the proteome; hence, the true changes in the phosphorylation (and not changes in the protein abundance) can be obtained (Figure1). The key additional step in the phosphoproteomic analysis was that the protein extracts were loaded on titanium immobilized metal-ion affinity chromatography (Ti^{4+}IMAC) column. This column binds phosphorylated peptides and separates them from the non-phosphorylated peptide pool. The increased phosphorylation of a particular peptide is reflected in the enrichment of this peptide on the column. Therefore, in subsequent MS/MS analysis of the eluted phosphopeptides, the abundance of a given peptide is proportional to the level of its phosphorylation (Figure 1).

Q-PCR analysis of cytokinin response

To confirm the efficiency of the cytokinin treatment and to exclude contamination with the hormone in the mock conditions, all collected samples were subjected to the real time Q-PCR. Using Q-PCR, the expression levels of two early responsive cytokinin-induced reporter genes,

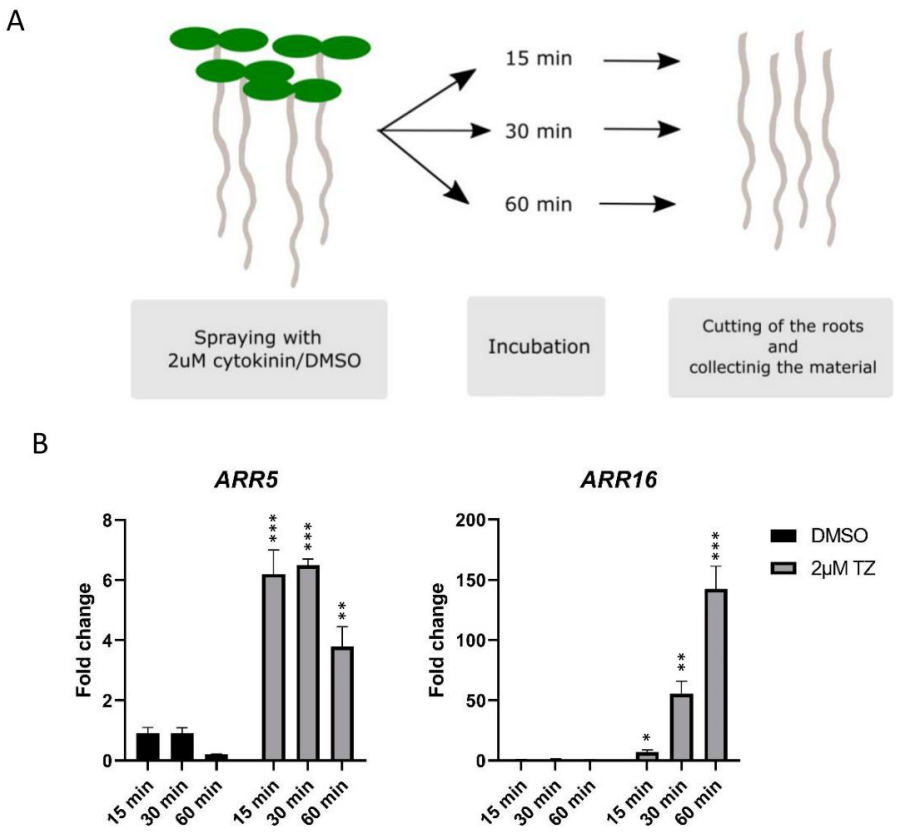

Figure 2. Sample preparation and treatment verification *(A) Process of the sample preparation for the (phospho)proteomic analysis. (B) Real-Time quantitative PCR of two cytokinin responsive genes ARR5 and ARR16 performed on samples harvested for (phospho)proteomic analysis. The bars represent mean ± SE., *** = p < 0.001, ** = p < 0.01, * = p < 0.05 by Student's t-test, n = 4.*

ARR5 and *ARR16* (D'Agostino et al., 2000), were monitored. *ARR5* expression showed 6.2 and 6.5 fold increase after 15 and 30 minutes of the cytokinin treatment compared to mock, respectively. After 60 minutes of the cytokinin treatment, the *ARR5* expression was lower than in previous time points but sill significantly (3.5 fold) higher than mock. *ARR16* expression upon cytokinin treatment was 7, 55, 142 fold higher than in mock at 15, 30 and 60 minutes, respectively. Altogether, although cytokinin reporters *ARR5* and *ARR16* exhibited different kinetics of the response from each other, they were both clearly upregulated after the cytokinin treatment but not in the mock conditions (Figure 2B). This verifies the correctness of the experimental procedure and confirms that samples are suitable for further analysis.

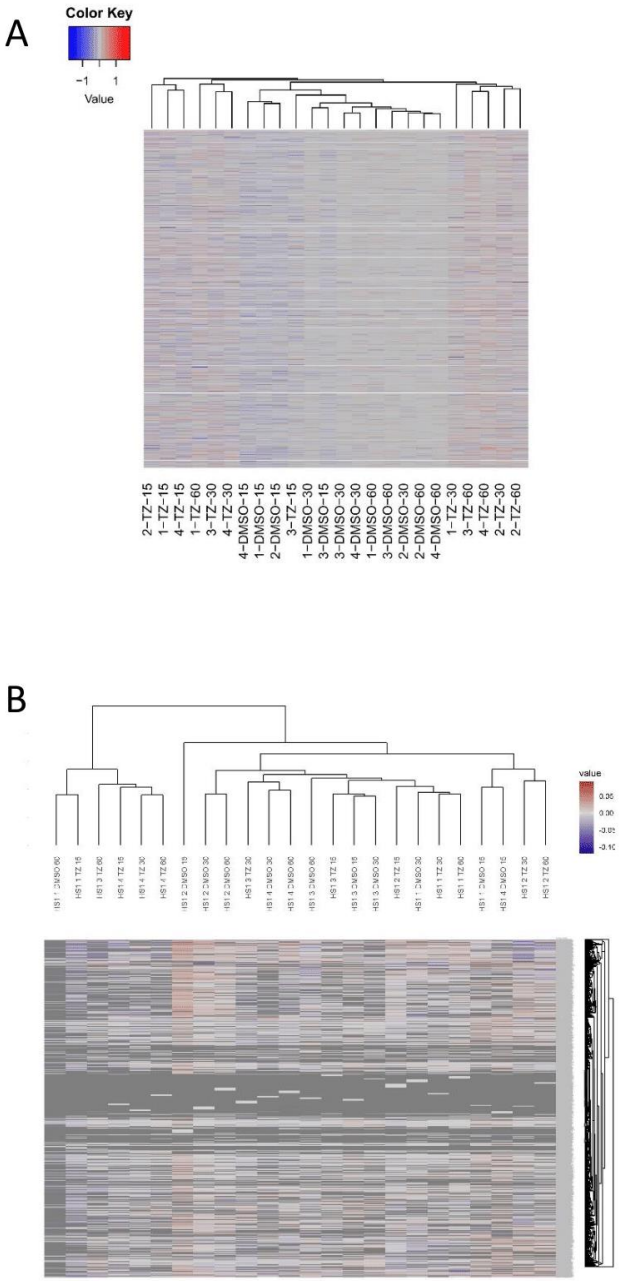

Figure 3: Heat map clustering of log_2 intensity values of peptides *(A) Proteome. (B) Phosphoproteome. The blue color shows lower and the red color shows higher amount of expression (proteome) or phosphorylation (phosphoproteome). TZ = trans Zeatin, DMSO= mock, 15, 30, 60 = minutes of the treatment, 1,2,3,4 = number of the replicate*

Analysis of the Arabidopsis phosphoproteome upon cytokinin treatment

Altogether, we recovered 4342 peptides in the analysis of the proteome and 7051 peptides with at least one phospho-group in the phosphoproteomic analysis. The overlap between peptides identified in the phosphoproteomics and the proteomics was very low. Therefore, we did not opt for normalizing phosphopeptide ratios to parent protein groups since it would result in very high amount of lost values.

The $\log_2$ intensity values of peptides are depicted in the heat maps (Figure 3). Whereas the heat map of the proteome shows clustering of the samples according to the treatment and the time point (Figure 3A), the heat map of the phosphoproteome does not exhibit such clear clustering (Figure 3B).

The profile of the global proteome shows mainly positive $\log_2$(tZ/DMSO) values after 15 minutes of the treatment, which suggests increased abundance of proteins in tZ condition or decrease of the protein abundance in the DMSO condition (Figure 3A, Figure S4A). After 30 and 60 minutes of the treatment, the $\log_2$(tZ/DMSO) global profiles do not show general prevalence for positive or negative values (Figure S4B, C). In the proteome, after 15 minutes of the treatment, 387 peptides showed significantly different ($p<0.05$) abundance between mock and tZ at the false discovery rate (FDR) 10%. There were no peptides with significantly different abundance between the mock and the cytokinin treatment in the 30 and 60 minutes time points at FDR 10%. When we allow 30% FDR, 17 peptides are found significantly different between the cytokinin and the mock after 30 minutes of the treatment and 9 peptides after 60 minutes of the treatment. The top-10 peptides that showed the most significant difference in abundance between the cytokinin and the mock treatment in each time point are listed in Figure 5. The significantly regulated peptides of the proteomic dataset play roles in *e.g.* oxidative stress response (MSD1, Karan & Subudhi, 2014), defense against pathogens (DOX1,Vicente et al., 2012; FAAH, Kim et al., 2009), translation (RPL32A) or general metabolism (GLN2, CHS, BGLU23, FBA8), however, none of them suggests clear, specific connection to cytokinin governed processes. Interestingly, the upregulation of PIP1, a water transporter (Boursiac et al., 2005), after 60 minutes of cytokinin treatment might reflect the role of cytokinin in osmotic stress (Tran et al., 2007).

Since we are primarily interested in the changes of protein phosphorylation, we focus further on the analysis of the phosphoproteomic dataset. The phosphoproteome profile shows

mainly negative log_2(tZ/DMSO) values after 15 minutes of the treatment, which suggests a global decrease in phosphorylation of peptides in samples incubated with cytokinin compared to mock (Figure S4D). After 30 minutes of the treatment, the phosphoproteome profile does not show clear shift in global phosphorylation between mock and cytokinin treatment (Figure S4E). After 60 minutes, the log_2(tZ/DMSO) generally shifts to positive values, which suggests global increase in phosphorylation after the cytokinin treatment compared to mock (Figure S4F). In the phosphoproteome, after 15 minutes of the treatment, 866 peptides were significantly differently ($p<0.05$) phosphorylated between mock and tZ at FDR 10%. At FDR 10%, there were almost no significantly different peptides between the mock and the cytokinin treatment in the 30 and 60 minutes time point. When we allow 30% FDR, 5 peptides are found significantly different after 30 minutes of the treatment and 153 peptides after 60 minutes of the treatment between mock and cytokinin. The top-10 peptides that showed the most significant phosphorylation difference between cytokinin and mock treatment in each time point are listed in Figure 6. Among the top-10 candidates with cytokinin-regulated phosphorylation, there are proteins involved in *e.g.* calcium transport (ACA8, Costa et al., 2017), transcription and translation (At1g29350, At1g76810, At4g31880, RPP3A) without clear and specific connection to cytokinin-governed processes.

Interestingly, AtEH1, that is downregulated after 15 and 30 minutes, is a component of the TPLATE complex, with role in endocytosis and autophagy (Wang et al., 2019). Also, RGTA1, the geranylgeranyl transferase responsible for posttranslational lipid modification of Rab proteins, which is key for their attachment to the membrane (Shi et al., 2016), is upregulated after 15 minutes of cytokinin treatment. Significant change in phosphorylation of these proteins might hint at cytokinin regulation of endomembrane trafficking.

A

15 minutes		
Gene	Name	Regulation in tZ compared to DMSO
JAL33	Jacalin-related lectin 33	up, FDR = 10%
MSD1	Superoxide dismutase [Mn] 1	up, FDR = 10%
RPL32A	60S ribosomal protein L32-1	up, FDR = 10%
DOX1	Alpha-dioxygenase 1	up, FDR = 10%
NUDT1	Nudix hydrolase 1	down, FDR = 10%
FAAH	Fatty acid amide hydrolase	up, FDR = 10%
ACT2	Actin-2;Actin 2	up, FDR = 10%
GRH1	GRR1-like protein 1	up, FDR = 10%
TUBA6	Tubulin alpha-6 chain;Tubulin alpha chain	up, FDR = 10%
RANBP1A	Ran-binding protein 1 homolog a	up, FDR = 10%

B

30 minutes		
Gene	Name	Regulation in tZ compared to DMSO
VCR	Varicose-related protein	up, FDR = 30%
GER2	Putative GDP-L-fucose synthase 2	down, FDR = 30%
RPL36A	60S ribosomal protein L36-1	down, FDR = 30%
GLN2	Glutamine synthetase	down, FDR = 30%
CHS	Chalcone synthase	up, FDR = 30%
At5g19440	At5g19440	up, FDR = 30%
TSN1	Ribonuclease TUDOR 1, Isoform 2	down, FDR = 30%
At2g28790	Pathogenesis-related thaumatin superfamily protein	down, FDR = 30%
DAP	LL-diaminopimelate aminotransferase	up, FDR = 30%
At2g32240	Early endosome antigen	up, FDR = 30%

C

60 minutes		
Gene	Name	Regulation in tZ compared to DMSO
At4g16260	Glycosyl hydrolase superfamily protein;	down, FDR = 30%
BGLU23	Beta-glucosidase 23;	up, FDR = 30%
PIP1-1	Aquaporin PIP1-1;	up, FDR = 30%
RPP0C	60S acidic ribosomal protein P0-3;	up, FDR = 30%
MPT3	Mitochondrial phosphate carrier protein 3	up, FDR = 30%
GPX6	Probable phospholipid hydroperoxide glutathione peroxidase 6	up, FDR = 30%
FBA8	Fructose-bisphosphate aldolase 8	up, FDR = 30%
CSY2	Citrate synthase 2	up, FDR = 30%
PBC1	Proteasome subunit beta type-3-A	up, FDR = 30%
At1g22410	Phospho-2-dehydro-3-deoxyheptonate aldolase	

Figure 5: List of 10 most significantly differently peptides in terms of abundance in each time point (Proteomic dataset) *(A) 15 minutes, (B) 30 minutes, (C) 60 minutes, FDR= False Discovery Rate*

A

15 minutes		
Gene	*Phosphorylation site and name*	*Regulation in tZ compared to DMSO*
ACA8	pS29___Calcium-transporting ATPase 8, plasma membrane-type	up, FDR = 10%
At1g29350	pS452___RNA polymerase II degradation factor-like protein	down, FDR = 10%
At1g76810	pS131___Eukaryotic translation initiation factor 2 (eIF-2) family protein	down, FDR = 10%
AtEH1	pS961-pS970___Calcium-binding EF hand family protein	down, FDR = 10%
RGTA1	pS606___Geranylgeranyl transferase type-2 subunit alpha 1	up, FDR = 10%
TOM2AH3	pS84___Tetraspanin-19, Isoform 3	down, FDR = 10%
IQD32	pS180___Protein IQ-DOMAIN 32	down, FDR = 10%
SNL5	pS717___SIN3-like 5	down, FDR = 10%
At1g16270	pS671___F3O9.7 protein	up, FDR = 10%
At4g24840	pS8___Oligomeric Golgi complex subunit-like protein	down, FDR = 10%

B

30 minutes		
Gene	*Phosphorylation site and name*	*Regulation in tZ compared to DMSO*
ACA8	pS29___Calcium-transporting ATPase 8, plasma membrane-type	up, FDR = 30%
At1g78750	pS308-pS309___F-box/FBD/LRR-repeat protein At1g78750	up, FDR = 30%
At4g31880	pS557___Transcriptional regulator	up, FDR = 30%
DEGP9	pS39___Protease Do-like 9	up, FDR = 30%
AtEH1	pS788___Calcium-binding EF hand family protein	down, FDR = 30%
PP2AB1	pS466-pS469___Serine/threonine protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform, Isoform 2	
CPK8	pS36___Calcium-dependent protein kinase 8	
At3g12020	pS501___Kinesin-like protein KIN-7K, chloroplastic	
KAC2	pT784___Kinesin like protein for actin based chloroplast movement 2	
F19C24.19	pS472-pS478___At1g51580	

C

60 minutes		
Gene	*Phosphorylation site and name*	Regulation in tZ compared to DMSO
PCKA	pS62-pT65___Phosphoenolpyruvate carboxykinase (ATP)	up, FDR = 30%
MUA22.12	pT295-pS301___Major facilitator superfamily protein	down, FDR = 30%
RPP3A	pS106___60S acidic ribosomal protein P3-1	down, FDR = 30%
FTSHI4	pS403___Probable inactive ATP-dependent zinc metalloprotease FTSHI 4	down, FDR = 30%
PCKA	pS62-pT65___Phosphoenolpyruvate carboxykinase (ATP)	down, FDR = 30%
RPP1A	pS102___60S acidic ribosomal protein P1-1	up, FDR = 30%
PATL2	pS77___Patellin-2	down, FDR = 30%
PUX13	pS254___Plant UBX domain-containing protein 13	down, FDR = 30%
At5g11970	pS56___ABC family ABC transporter, putative (DUF3511)	down, FDR = 30%
At3g09890	pY30___Ankyrin repeat family protein	down, FDR = 30%

Figure 6: List of 10 most significantly differently phosphorylated peptides in each time point (Phosphoproteomic dataset) *(A)15 minutes, (B) 30 minutes, (C) 60 minutes FDR= False Discovery Rate*

Conclusion

The role of cytokinin in plant growth and development has been studied mainly on the level of transcriptional changes (Kieber & Schaller, 2018). Interestingly, several studies suggest also transcription-independent effects of cytokinin on vesicular trafficking or cytoskeleton dynamics (Marhavý et al., 2011; Montesinos et al., 2020). Therefore, we performed analysis of the global proteomic and phosphoproteomic changes upon cytokinin treatment.

In recent years, analysis of phosphorylation changes upon treatment with hormones, nitrate or abiotic stress are emerging (Dautel et al., 2016; Kamal et al., 2020; Vega et al., 2020). Observations of changes on the posttranslational level offers advantage over the transcriptomic analysis because it captures the actual abundance and PTMs of the proteins in the cell.

We identified changes in abundance/phosphorylation of many peptides upon cytokinin treatment. The significantly regulated proteins are involved in various biological processes e.g. transcription, translation, calcium transport or general metabolism. However, since we are interested the most in the cytokinin role in the endomembrane trafficking, we focus our follow-up investigation of the candidates that are involved in this process.

Supplemental figures

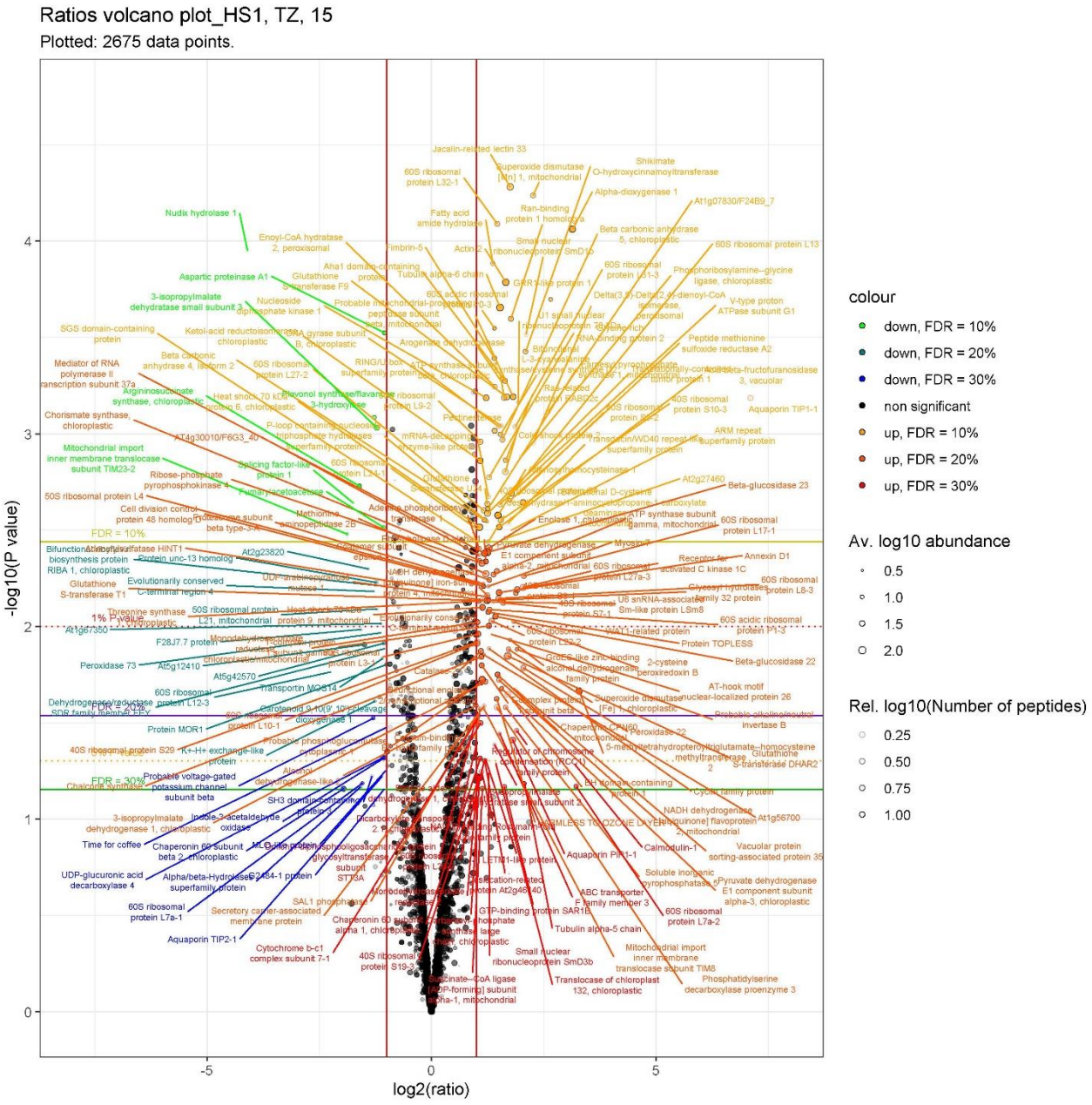

***Figure S4A: Volcano plots of global proteome (15 minutes of the treatment)**, x axis= log_2 (tZ /DMSO) ratio, y axis = -log_{10}(P value), P value was calculated according by t test. Red vertical lines demark 5% of the most extreme values.*

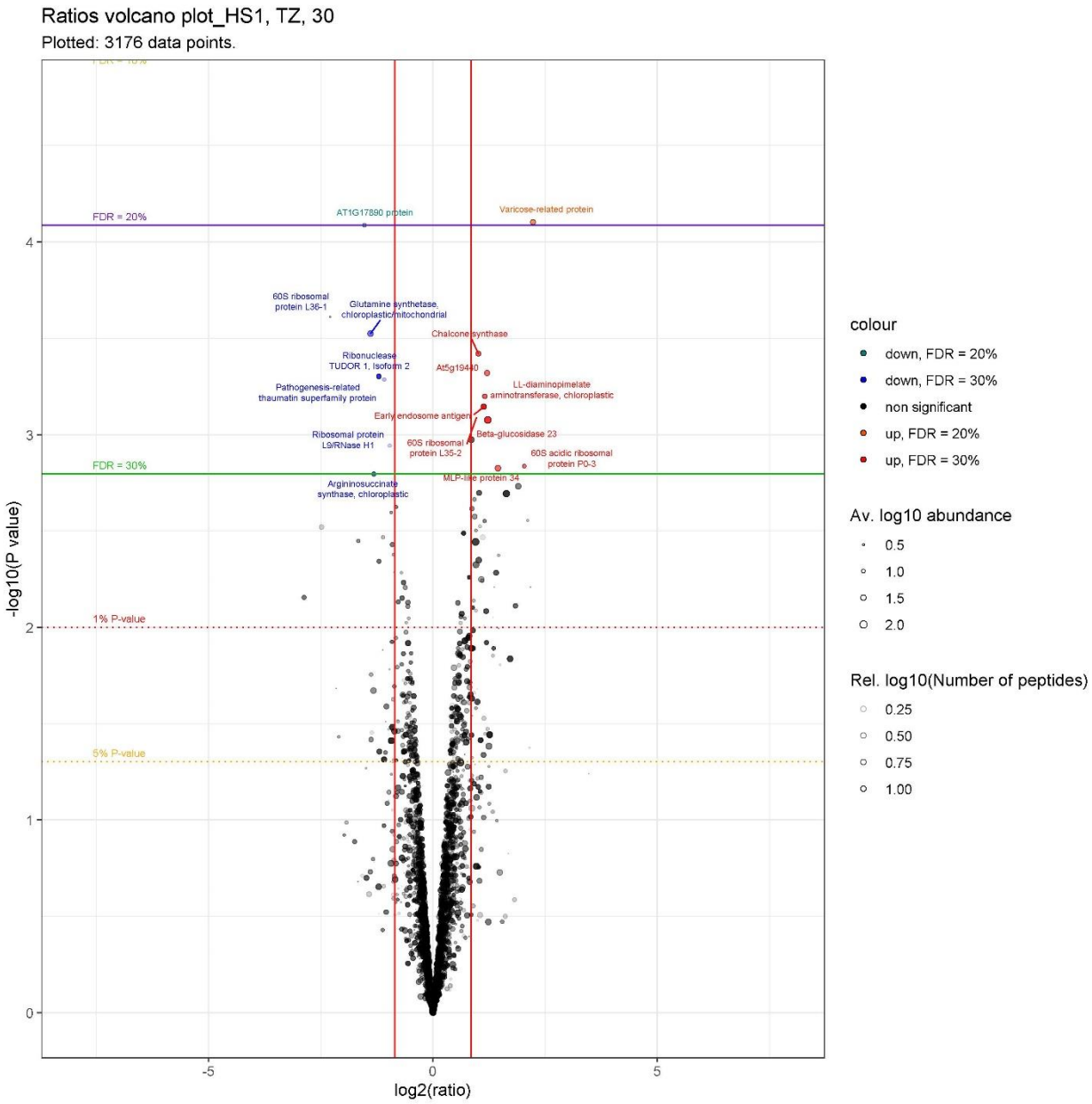

***Figure S4B: Volcano plots of global proteome (30 minutes of the treatment)**, x axis= log_2 (tZ /DMSO) ratio, y axis = -log_{10}(P value), P value was calculated according by t test. Red vertical lines demark 5% of the most extreme values.*

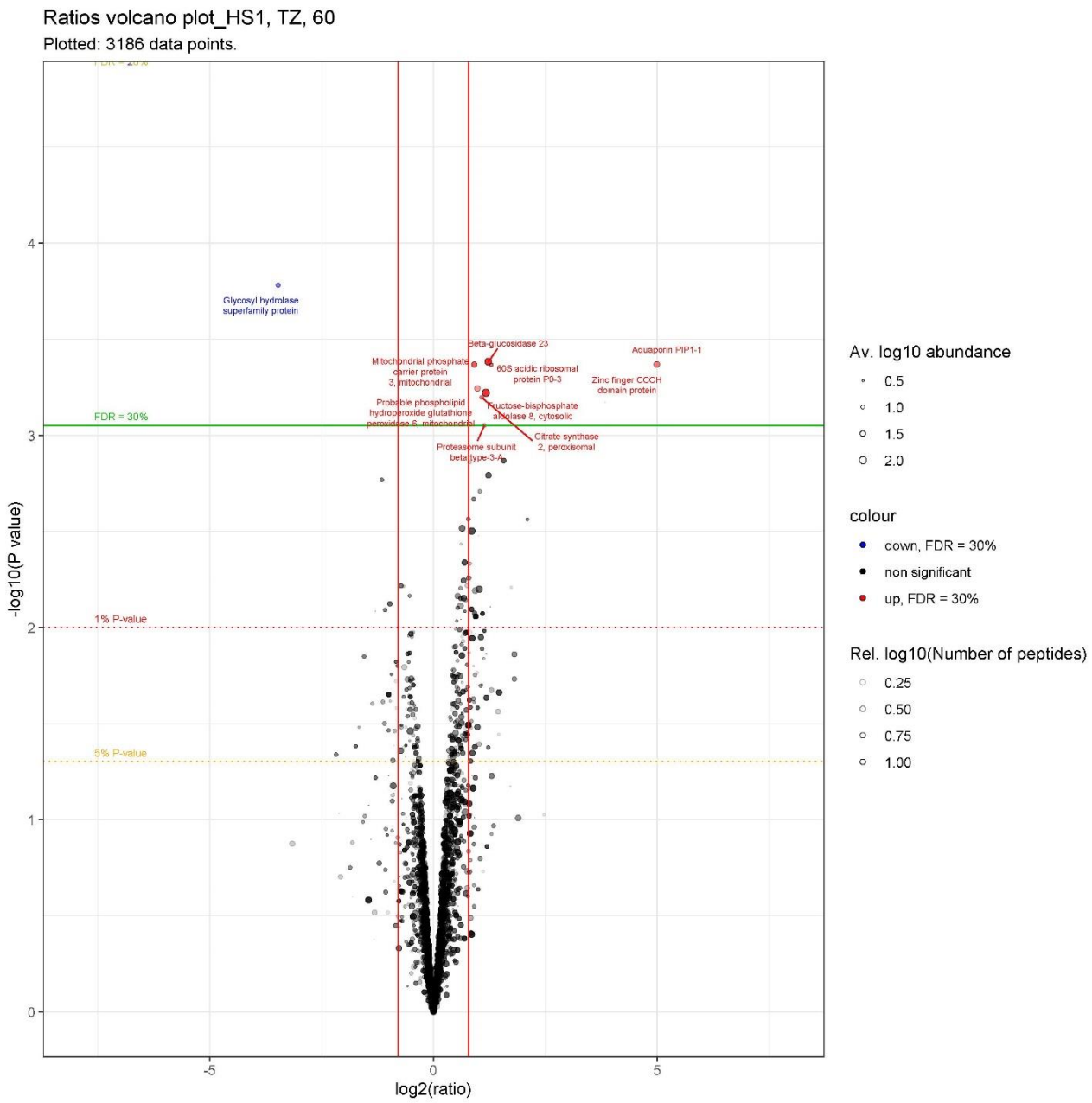

***Figure S4C: Volcano plots of global proteome (60 minutes of the treatment)**, x axis= log_2 (tZ /DMSO) ratio, y axis = -log_{10}(P value), P value was calculated according by t test. Red vertical lines demark 5% of the most extreme values.*

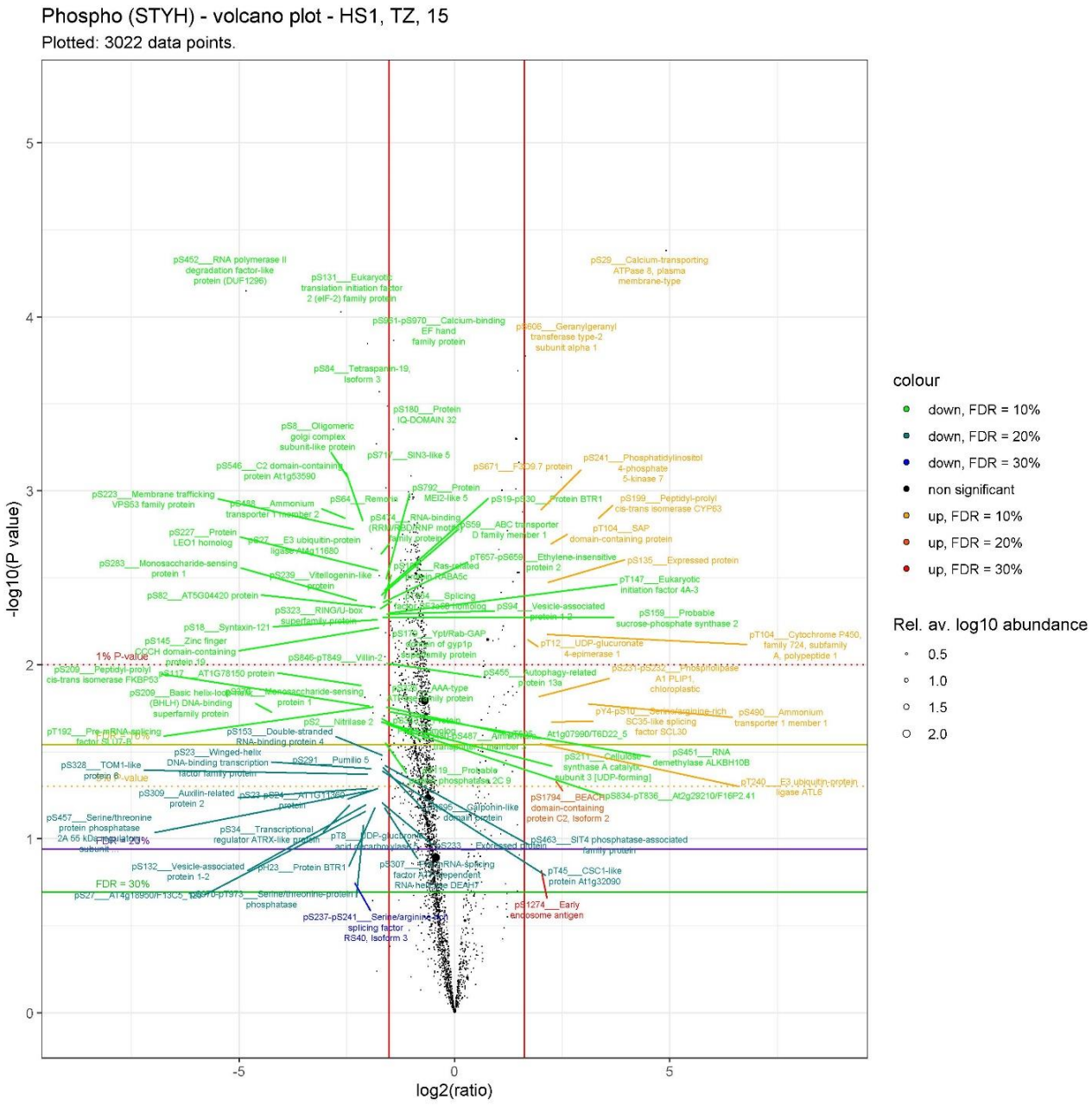

***Figure S4D: Volcano plots of global phosphoproteome (15 minutes of the treatment)**, x axis= log_2 (tZ /DMSO) ratio, y axis = -log_{10}(P value), P value was calculated according by t test. Red vertical lines demark 5% of the most extreme values*

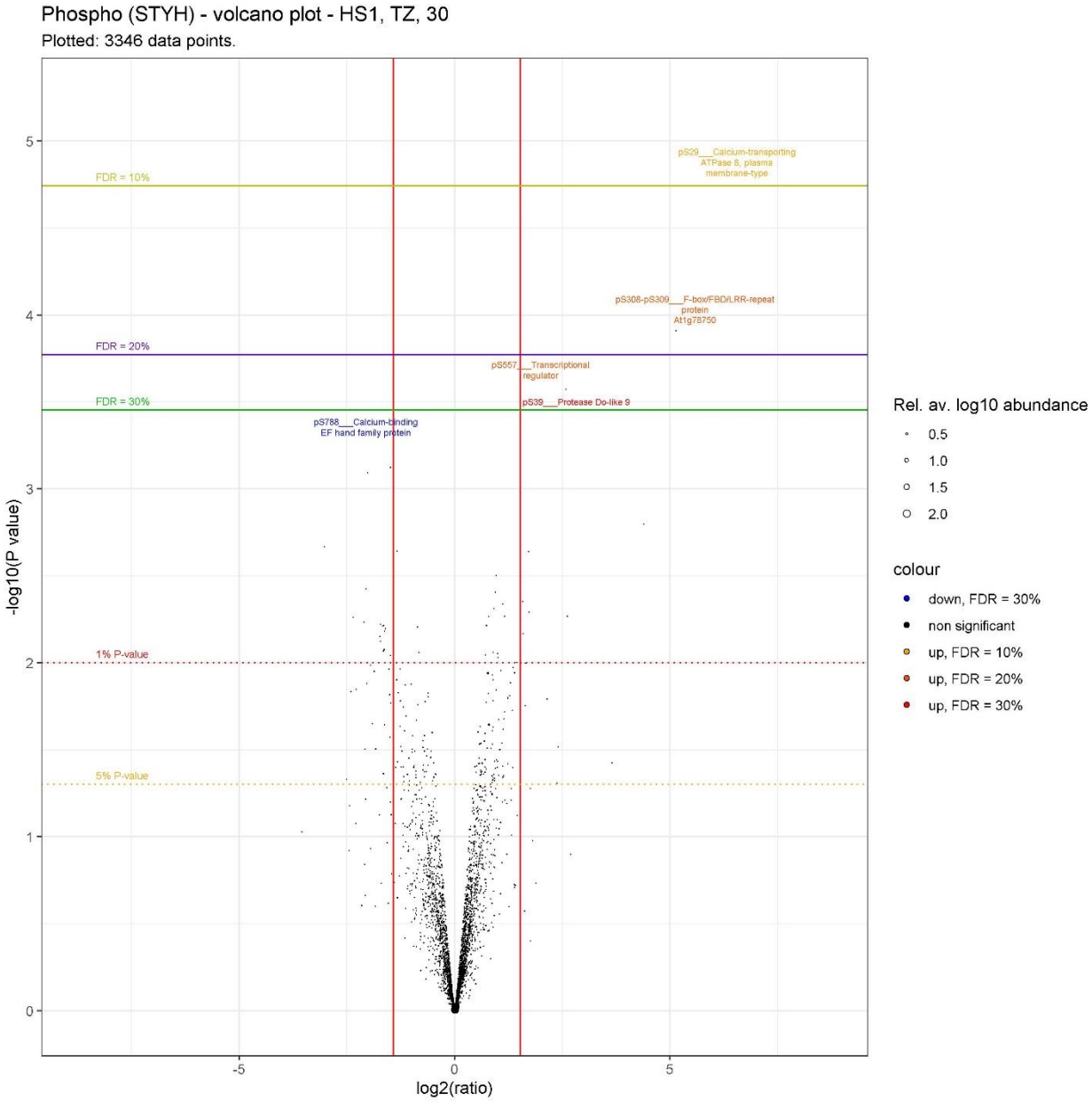

***Figure S4E: Volcano plots of global phosphoproteome (30 minutes of the treatment)**, x axis= log_2 (tZ /DMSO) ratio, y axis = -log_{10}(P value), P value was calculated according by t test. Red vertical lines demark 5% of the most extreme values*

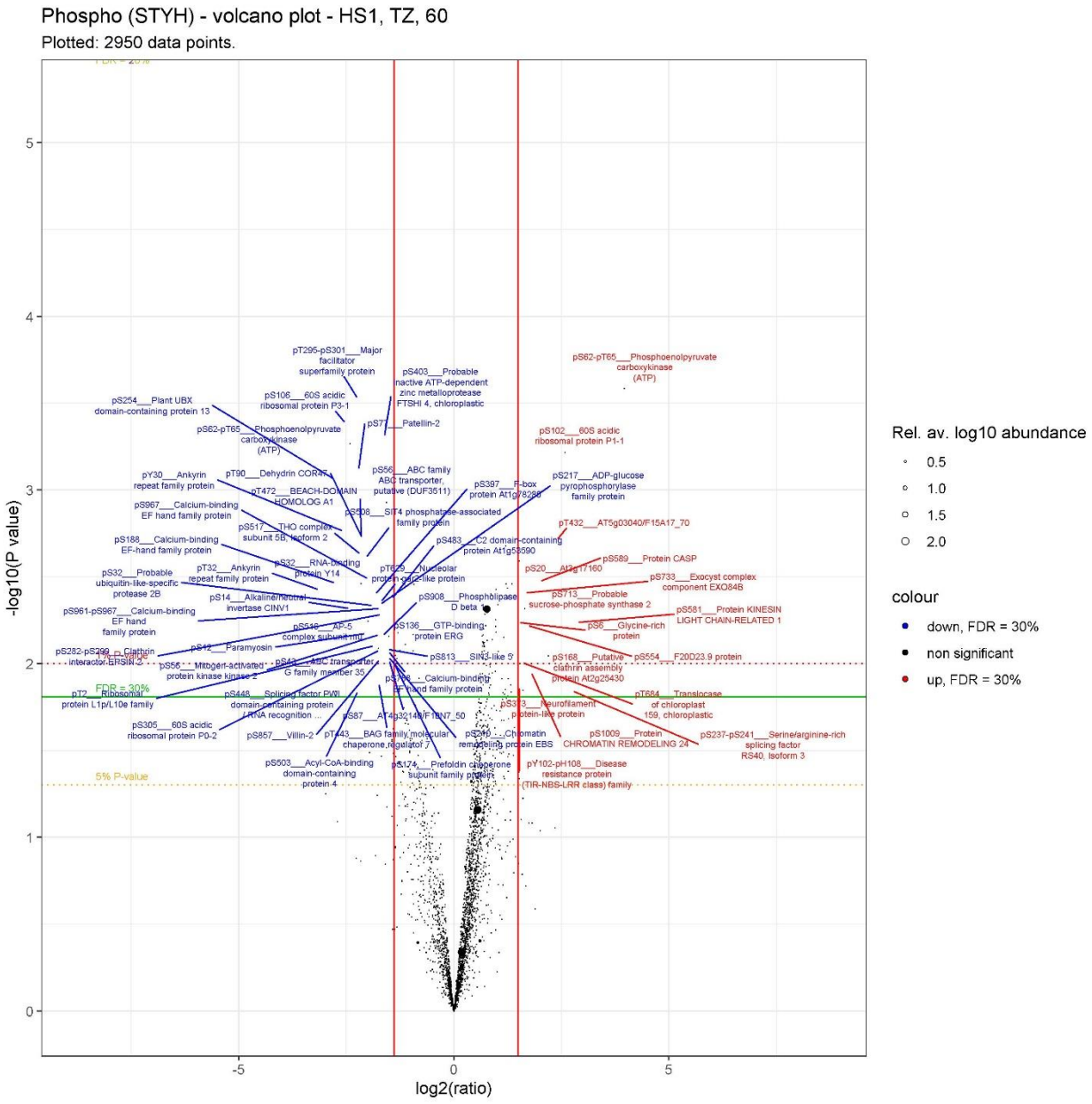

Figure S4F: Volcano plots of global phosphoproteome (60 minutes of the treatment)*, x axis= log_2 (tZ /DMSO) ratio, y axis = -log_{10}(P value), P value was calculated according by t test. Red vertical lines demark 5% of the most extreme values*

Methods

Plant growth and treatment

Seedlings of Arabidopsis thaliana (ecotype Columbia) were grown on vertically held plates with half-strength Murashige and Skoog (MS) medium (pH=5.9) solidified with 0.8% agar at 22 °C in continuous light. Seven days postgermination (dpg), the plants were sprayed with 500µl half strength MS liquid media supplemented with 5µM trans-Zeatin (Sigma) or DMSO (Sigma). Plates were incubated horizontally for (15, 30 and 60 min), roots were cut off and flash-frozen in liquid nitrogen.

QPCR

RNA was extracted from flash-frozen roots with TRIzol reagent (Thermo Fisher Scientific) and cDNA was synthetized with an iScript cDNA kit (Bio-Rad). PCR was performed with gene-specific primers for *ARR5* (ARR5_F: AGTTCGGTTGGATTTGAGGATCTG, ARR5_R: TCCAGTCATCCCAGGCATAGAG) and *ARR16* (ARR16_F: CGTAAACTCGTTGAGAGGTTGCTC, ARR16_R: GCATTCTCTGCTGTTGTCACTTTG). The housekeeping gene *TUB2* was used as the normalization control.

Data analysis

Raw files were searched in MaxQuant (1.6.10.43) against the standard *Arabidopsis thaliana* proteome from UniProtKB. "Carbamidomethyl (C)" was set as fixed modification. Variable modifications were set to "Oxidation (M)", "Acetyl (Protein N-term)", "Deamidation (NQ)", "Gln->pyro-Glu" and "Phospho (STYH)" - the latter to allow potential discoveries of phosphorylated histidines, a modification which has been reported in plants (Nongpiur et al., 2012). Second peptide search and match-between-runs were activated. All false discovery rates (FDRs) were set to 1%. Phospho-enriched and corresponding total proteome samples were defined as separate experiment. MaxQuant output was then post-processed in R from the **evidence.txt** table (which contains peptide-spectrum-matches + matches between runs). Potential contaminants and reverse database hits were excluded, leaving 682 063 individual peptide observations. Evidence intensities were normalized to the ratio between the median of the sample type (phospho or total). The "long" evidences table was aggregated into a "wide" table of 34 830 peptidoforms (modification-specific peptides), 7051 of which with at least one phospho-group (Figure3A). Peptidoform intensities were calculated as the sum of individual evidence intensities and normalized in several steps: first, intensity values were normalized to the ratio between the median intensity over the whole experiment vs sample median; then variance stabilization normalization was applied; the Levenberg-Marquardt procedure was applied to identify normalization factors minimizing the squared sum of pairwise sample-to-sample differences; finally, since a preliminary PCA analysis had revealed an observable replicate-specific batch effect, the data was batch-corrected using ComBat from the sva package. Peptide TZ to DMSO ratios were calculated within replicates for each time point. Protein groups were inferred from observed peptides (the minimum proteins IDs required to explain observed peptides are in the "**Leading protein IDs**" column), requiring at least two distinct peptidoforms for protein group discovery. Protein group-level label-free expression values were calculated as the average of the cross-samples intensities profile of individual peptides weighted by the inverse of peptide posterior-error-probability of discovery, excluding phospho-peptides and their unmodified counterpart. Average values and

ratios over replicate were computed, and ratios were tested using the limma package, performing for each time point a Moderated t-test. The Benjamini-Hochberg procedure was applied to calculate P-value significance thresholds for 10, 20 or 30% accepted FDR. In addition, only average ratios greater than the 95% less extreme ratios between individual DMSO replicates were considered. For phospho-peptides analysis, a similar testing strategy was applied but on phospho-peptide ratios. We identified 4342 peptides in the analysis of the proteome (Figure 3B). However, it was decided to not normalize phospho-peptide ratios to parent protein groups because this would have resulted in too many values being lost. In terms of residues, 83.4% of observed phosphorylations were on serine, 14.4% on threonine, 1.2% on histidine and 1.1% on tyrosine, validating the decision to include histidine as a potential site in addition to the 3 classical phosphosites.
QPCR plots were generated using GraphPad Prism 8, and Microsoft PowerPoint programs.
QPCR data were analysed using Student's t test.

References

Adam, K., & Hunter, T. (2018). Histidine kinases and the missing phosphoproteome from prokaryotes to eukaryotes. *Laboratory Investigation*, *98*(2), 233–247. https://doi.org/10.1038/labinvest.2017.118

Boursiac, Y., Chen, S., Luu, D. T., Sorieul, M., Van Den Dries, N., & Maurel, C. (2005). Early effects of salinity on water transport in Arabidopsis roots. Molecular and cellular features of aquaporin expression. *Plant Physiology*, *139*(2), 790–805. https://doi.org/10.1104/pp.105.065029

Bhargava, A., Clabaugh, I., To, J. P., Maxwell, B. B., Chiang, Y. H., Schaller, G. E., ... & Kieber, J. J. (2013). Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-Seq in Arabidopsis. *Plant Physiology*, *162*(1), 272-294.

Camoni, L., Visconti, S., Aducci, P., & Marra, M. (2018). 14-3-3 proteins in plant hormone signaling: Doing several things at once. *Frontiers in Plant Science*, *9*(March), 1–8. https://doi.org/10.3389/fpls.2018.00297

Costa, A., Luoni, L., Marrano, C. A., Hashimoto, K., Köster, P., Giacometti, S., De Michelis, M. I., Kudla, J., & Bonza, M. C. (2017). Ca2+-dependent phosphoregulation of the plasma membrane Ca2+-ATPase ACA8 modulates stimulus-induced calcium signatures. *Journal of Experimental Botany*, *68*(12), 3215–3230. https://doi.org/10.1093/jxb/erx162

D'Agostino, I. B., Deruere, J., & Kieber, J. J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiol*, *124*(December), 1706–1717. https://doi.org/10.1104/pp.124.4.1706

Dautel, R., Wu, X. N., Heunemann, M., Schulze, W. X., & Harter, K. (2016). The Sensor Histidine Kinases AHK2 and AHK3 Proceed into Multiple Serine/Threonine/Tyrosine Phosphorylation Pathways in Arabidopsis thaliana. *Molecular Plant*, *9*(1), 182–186. https://doi.org/10.1016/j.molp.2015.10.002

Gupta, S., & Rashotte, A. M. (2012). Down-stream components of cytokinin signaling and the role of cytokinin throughout the plant. *Plant Cell Reports*, *31*(5), 801–812. https://doi.org/10.1007/s00299-012-1233-0

Jagodzik, P., Tajdel-Zielinska, M., Ciesla, A., Marczak, M., & Ludwikow, A. (2018). Mitogen-activated protein kinase cascades in plant hormone signaling. *Frontiers in Plant Science*, *9*(October), 1–26. https://doi.org/10.3389/fpls.2018.01387

Kamal, M. M., Ishikawa, S., Takahashi, F., Suzuki, K., Kamo, M., Umezawa, T., Shinozaki, K.,

Kawamura, Y., & Uemura, M. (2020). Large-scale phosphoproteomic study of arabidopsis membrane proteins reveals early signaling events in response to cold. *International Journal of Molecular Sciences*, *21*(22), 1–41. https://doi.org/10.3390/ijms21228631

Karan, R., & Subudhi, P. K. (2014). Overexpression of an adenosine diphosphate-ribosylation factor gene from the halophytic grass Spartina alterniflora confers salinity and drought tolerance in transgenic Arabidopsis. *Plant Cell Reports*, *33*(2), 373–384. https://doi.org/10.1007/s00299-013-1537-8

Kieber, J. J., & Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development (Cambridge, England)*, *145*(4), 1–7. https://doi.org/10.1242/dev.149344

Kim, S. C., Kang, L., Nagaraj, S., Blancaflor, E. B., Mysore, K. S., & Chapman, K. D. (2009). Mutations in Arabidopsis fatty acid amide hydrolase reveal that catalytic activity influences growth but not sensitivityto Abscisic acid or pathogens. *Journal of Biological Chemistry*, *284*(49), 34065–34074. https://doi.org/10.1074/jbc.M109.059022

Li, S. M., Zheng, H. X., Zhang, X. S., & Sui, N. (2021). Cytokinins as central regulators during plant growth and stress response. *Plant Cell Reports*, *40*(2), 271–282. https://doi.org/10.1007/s00299-020-02612-1

Mao, J., & Li, J. (2020). Regulation of three key kinases of brassinosteroid signaling pathway. *International Journal of Molecular Sciences*, *21*(12), 1–32. https://doi.org/10.3390/ijms21124340

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., & Benková, E. (2011). Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis. *Developmental Cell*, *21*(4), 796–804. https://doi.org/10.1016/j.devcel.2011.08.014

Máthé, C., M-Hamvas, M., Freytag, C., & Garda, T. (2021). The protein phosphatase pp2a plays multiple roles in plant development by regulation of vesicle traffic—facts and questions. *International Journal of Molecular Sciences*, *22*(2), 1–19. https://doi.org/10.3390/ijms22020975

Montesinos, J. C., Abuzeineh, A., Kopf, A., Juanes-Garcia, A., Ötvös, K., Petrášek, J., Sixt, M., & Benková, E. (2020). Phytohormone cytokinin guides microtubule dynamics during cell progression from proliferative to differentiated stage. *The EMBO Journal*, *39*(17), 1–22. https://doi.org/10.15252/embj.2019104238

Nongpiur, R., Soni, P., Karan, R., Singla-Pareek, S. L., & Pareek, A. (2012). Histidine kinases in plants: Cross talk between hormone and stress responses. *Plant Signaling and Behavior*, *7*(10), 1230–1237. https://doi.org/10.4161/psb.21516

Shi, W., Zeng, Q., Kunkel, B. N., & Running, M. P. (2016). Arabidopsis rab geranylgeranyltransferases demonstrate redundancy and broad substrate specificity in vitro. *Journal of Biological Chemistry*, *291*(3), 1398–1410. https://doi.org/10.1074/jbc.M115.673491

Soma, F., Takahashi, F., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2021). Cellular Phosphorylation Signaling and Gene Expression in Drought Stress Responses: ABA-Dependent and ABA-Independent Regulatory Systems. *Plants*, *10*(4), 756. https://doi.org/10.3390/plants10040756

Tan, S., Luschnig, C., & Friml, J. (2021). Pho-view of Auxin: Reversible Protein Phosphorylation in Auxin Biosynthesis, Transport and Signaling. *Molecular Plant*, *14*(1), 151–165. https://doi.org/10.1016/j.molp.2020.11.004

Tran, L. S. P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., & Yamaguchi-

Shinozaki, K. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(51), 20623–20628. https://doi.org/10.1073/pnas.0706547105

Vega, A., Fredes, I., O'Brien, J., Shen, Z., Ötvös, K., Benkova, E., Briggs, S. P., & Gutiérrez, R. A. (2020). Phosphoproteomics after nitrate treatments reveal an important role for PIN2 phosphorylation in control of root system architecture. *BioRxiv*. https://doi.org/10.1101/2020.06.22.164640

Vicente, J., Cascón, T., Vicedo, B., García-Agustín, P., Hamberg, M., & Castresana, C. (2012). Role of 9-lipoxygenase and α-dioxygenase oxylipin pathways as modulators of local and systemic defense. *Molecular Plant*, *5*(4), 914–928. https://doi.org/10.1093/mp/ssr105

Vu, L. D., Stes, E., Van Bel, M., Nelissen, H., Maddelein, D., Inz??, D., Coppens, F., Martens, L., Gevaert, K., & De Smet, I. (2016). Up-to-Date Workflow for Plant (Phospho)proteomics Identifies Differential Drought-Responsive Phosphorylation Events in Maize Leaves. *Journal of Proteome Research*, *15*(12), 4304–4317. https://doi.org/10.1021/acs.jproteome.6b00348

Wang, P., Pleskot, R., Zang, J., Winkler, J., Wang, J., Yperman, K., Zhang, T., Wang, K., Gong, J., Guan, Y., Richardson, C., Duckney, P., Vandorpe, M., Mylle, E., Fiserova, J., Van Damme, D., & Hussey, P. J. (2019). Plant AtEH/Pan1 proteins drive autophagosome formation at ER-PM contact sites with actin and endocytic machinery. *Nature Communications*, *10*(1), 1–16. https://doi.org/10.1038/s41467-019-12782-6

Wyblouw, B., & De Rybel, B. (2019). Cytokinin – A Developing Story. *Trends in Plant Science*, *24*(2), 177–185. https://doi.org/10.1016/j.tplants.2018.10.012

VPS9a Role in Cytokinin-Governed Plant Organogenesis

VPS9a Role in Cytokinin-Governed Plant Organogenesis

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Introduction

Hormonal cross-talk between cytokinin and auxin is an essential regulation mechanism of growth and development in plants (Schaller et al., 2015). Here, we focus on the cytokinin effect on modulation of auxin fluxes by regulation of polar auxin transporters, PINs. Cytokinin affects PIN proteins on the level of transcription (Ruzicka et al., 2009; Šimášková et al., 2015). In addition, cytokinin triggers depletion and retargeting of PIN1 from the plasma membrane to the vacuole (Marhavý et al., 2011, 2014). The cytokinin-mediated PIN1 degradation was shown to be independent of transcription and translation (Marhavý et al., 2011). Therefore, in this process, cytokinin might affect the posttranslational modifications *e.g.* phosphorylation of the regulatory proteins of the endomembrane trafficking machinery.

In order to identify potential targets of this cytokinin action, we performed a global proteomic and phosphoproteomic analysis of *Arabidopsis thaliana* roots upon cytokinin treatment (for details see previous chapter). We identified a set of candidate proteins with changed phosphorylation by cytokinin with a role in vesicular trafficking, which we consider promising for further, in-detail investigation.

Vesicular trafficking in the plant cell

The complexity of eukaryotic cells is to large extent built on multiple membrane domains, which concentrate specific biochemical niches. This spatial organization is essential for division of labor and maintenance of various metabolic processes in confined environment of intracellular space. In order to carry out basic vital functions of the cell as well as to be able to react to the external stimuli, these membrane domains need to exchange material.

Therefore, there is a continuous vesicular trafficking between the membrane domains, carrying membrane lipids, membrane proteins, and soluble cargo proteins inside of the vesicles. Vesicles are formed in the process of budding from the membrane of origin, transferred along the cytoskeleton, tethered to its destination membrane, and finally docked and fused with the destination membrane. All steps of the vesicular existence must be tightly controlled. To achieve precise management of the intracellular movements in a spatio-temporal manner, cells have evolved an elaborate machinery of regulatory proteins to control various steps of the trafficking pathway (Elliott et al., 2020; Ravikumar et al., 2017; Rodriguez-Furlan et al., 2019).

One of the most important coordinators of the vesicular trafficking in eukaryotic cells are Rab GTPases (Behnia & Munro, 2005; Grosshans et al., 2006; Martinez & Goud, 1998; Minamino & Ueda, 2019; Novick & Zerial, 1997; Zerial & McBride, 2001). They serve as the interaction platform between the vesicle and other cellular structures like microtubules or tethering factors. Hence, the activity of such interaction partners, named Rab effectors, is confined in time and space to specific membrane surfaces (Zerial and McBride, 2001; Behnia and Munro, 2005; Grosshans et al., 2006; Markgraf et al., 2007, Woollard & Moore, 2008). A crucial feature of Rab function is that they dynamically cycle between GDP or GTP bound state, which allows them to switch between non-active and active state, respectively. The activation is facilitated by Rab GEFs (Guanosin Exchange Factors) by exchanging GDP for GTP (Minamino & Ueda, 2019). During the deactivation, the GTP is hydrolyzed into GDP. Rabs possess intrinsic enzymatic activity to perform the GTP hydrolysis but it is rather weak. Therefore, there are other types of regulators, Rab GAPs (GTPase Activating Proteins) that catalyze the deactivation (Lamber et al., 2019; Müller & Goody, 2018).

VPS9a, ARA6, ARA7, and RHA1 as a regulatory module of vesicular trafficking

Here, we focus on Rab GEF VPS9a, which was identified as a promising candidate in the phosphoproteomic screen upon cytokinin treatment (Table 1, previous chapter). VPS9a and it substrates, ARA6, ARA7, and RHA1 act together in the post-Golgi vesicular trafficking of the newly synthesized as well as endocytosed material.(Bolte, 2004; Ebine et al., 2011; Goh et al., 2007; Kotzer, 2004; Lee et al., 2004; Sohn et al., 2003; Ueda et al., 2001, 2004).

In the *in vitro* assays, VPS9a showed similar activation effects towards ARA6, ARA7, and RHA1, but not to other subclasses of Rabs present in *Arabidopsis* (Goh et al., 2007). Interestingly, despite ARA6, ARA7, and RHA1 share the same activator VPS9a, they exhibit distinct functions in the trafficking. Whereas ARA7 and RHA1 mediate the transport to the vacuole, ARA6 is involved in the trafficking pathway between plasma membrane and multi vesicular endosomes (Bottanelli et al., 2011, 2012; Ito et al., 2012; Kotzer, 2004; Minamino & Ueda, 2019, Ebine et al., 2011). Moreover, ARA6 is counter acting vacuolar transport by titration of the VPS9a and other factors (Ito et al., 2018).

Although single mutants of ARA6, ARA7, and RHA1 do not show any strikingly defective phenotypes, the knock-out mutation of their common activator VPS9a is embryo lethal. *vps9a-1* mutant plants, which contain insertion in the catalytic domain of the VPS9a show arrested development at the torpedo stage of the embryo. The weaker allele, *vps9a-2*, develops until adult fertile plant. However, it shows dwarfed phenotype and has strong root growth defects (Goh et al., 2007).

Altogether, VPS9a-Rab module is an essential regulatory node in promotion or attenuation of vacuolar protein transport on the interface of endocytic and biosynthetic pathways during plant growth and development. In particular, the specific activation of ARA6 or ARA7 might be the critical balancing factor deciding in favor of vesicular targeting towards the plasma membrane or the vacuole, respectively. It was shown that the C-terminus of VPS9a is essential in interaction with ARA6 (Sunada et al., 2016). However, more details about the mechanism behind regulation of Rab-VPS9a interaction are not known. It is plausible that there are endogenous cues *e.g.* plant hormones that influence this interaction, and consequently affect the direction of the vesicular trafficking.

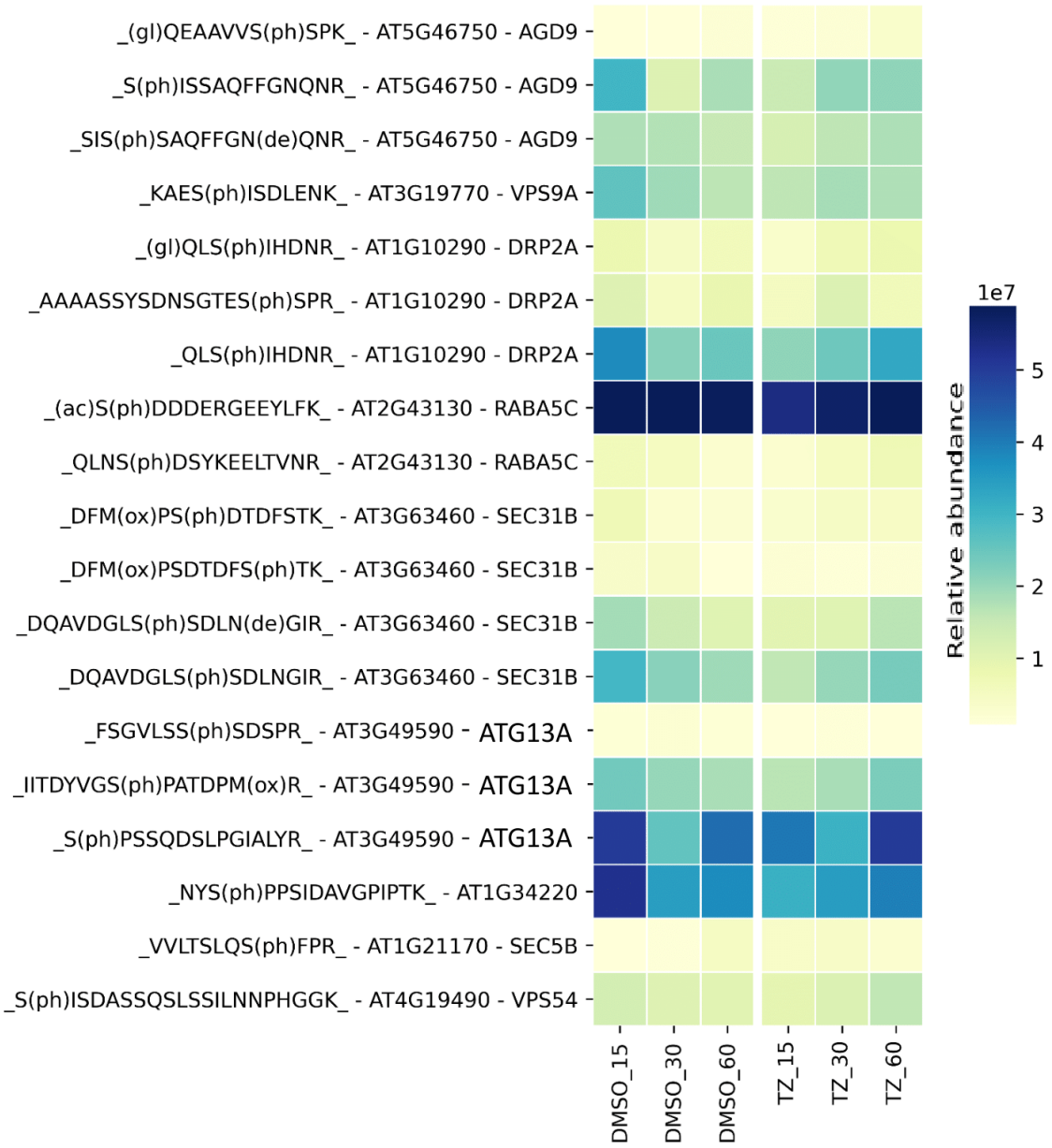


Table 1. List of candidate proteins with role in vesicular trafficking identified in the phosphoproteomic screen *The color scale represents the relative abundance of identified peptides. The squares represent the time points (15, 30, and 60 minutes) of the treatment with DMSO or 2µM trans-Zeatin.*

To investigate the pathway and molecular mechanisms that underlie cytokinin mediated targeting of PIN1 for lytic degradation to the vacuole we employed high-through put analysis of proteome and phosphoproteome (Marhavý et al., 2011; previous chapter). We hypothesized that cytokinin might modulate endomembrane trafficking by affecting the abundance and/or phosphorylation of the key trafficking regulators. The phosphoproteomic analysis revealed that several proteins involved in the vesicular trafficking show changes in their phosphorylation pattern upon cytokinin treatment. Among them, we identified VPS9, a Rab GEF for ARA6, ARA7, and RHA1 (Goh et al., 2007) to be potentially involved in cytokinin-mediated PIN1 degradation. Therefore, we decided to characterize the role of VPS9a and its phosphorylation in cytokinin-regulated plant development.

Results

VACUOLAR PROTIN SORTING 9a (VPS9a) was found in the phosphoproteomic screen

To search for putative targets of cytokinin action involved in the regulation of subcellular trafficking, we performed analysis of the global phosphoproteome of *Arabidopsis thaliana* roots (for details see previous chapter). We identified proteins involved in various steps of the vesicular trafficking *e.g*. vesicular formation (DRP2A, Taylor, 2011), regulation of the trafficking at Golgi apparatus (AGD9, Min et al., 2013) or at MVB (VPS9a, Goh et al., 2007), and autophagic degradation (ATG13, Suttangkakul et al., 2011) (Table1).

From these proteins, the VACUOLAR PROTEIN SORTING 9a (VPS9a) was selected as the most promising candidate for further characterization based on multiple reasons: 1) VPS9a shows a significant change in the phosphorylation pattern already after 15 minutes of cytokinin treatment (Figure 1). 2) VPS9a is an important regulator with established role in endocytic trafficking of proteins (including PIN1, Ebine et al., 2011; Goh et al., 2007; Ito et al., 2018; Sunada et al., 2016). 3) The *vps9a-2* mutant exhibits short primary root and root apical meristem, as well as increased lateral root density which are typical phenotypes connected to cytokinin-governed processes of the root development (Dello Ioio et al., 2007; Goh et al., 2007; Laplaze et al., 2007).

VPS9a shows altered phosphorylation pattern after 15 minutes of cytokinin (trans-Zeatin, tZ) treatment when compared to mock control. In particular, the first serine (S330) in the peptide

KAESISDLENK is significantly de-phosphorylated in response to cytokinin (Figure 1A). The phosphorylation site is not in the catalytic domain of VPS9a but in the C-terminal region, which has a regulatory function (Sunada et al., 2016, Figure 1B). Despite mass spectrometry analysis recovered S330 as the site of changed phosphorylation, the detection of phosphorylated amino acid residues that are so close to each other does not always have to be precise. Therefore, we considered the possibility that both S330 and S332 might be modified by (de)phosphorylation upon cytokinin treatment.

Using an on-line database of *Arabidopsis* phosphoproteome PhosPhAt (Durek et al., 2009; Heazlewood et al., 2008; Zulawski et al., 2013), we confirmed that the identified site is indeeda phosphorylation hotspot. Both serines in the KAESISDLENK peptide are predicted to be phosphorylated by *in silico* analysis. They were also experimentally proven to be phosphorylated by several independent studies investigating *e.g.* circadian rhythm, targets

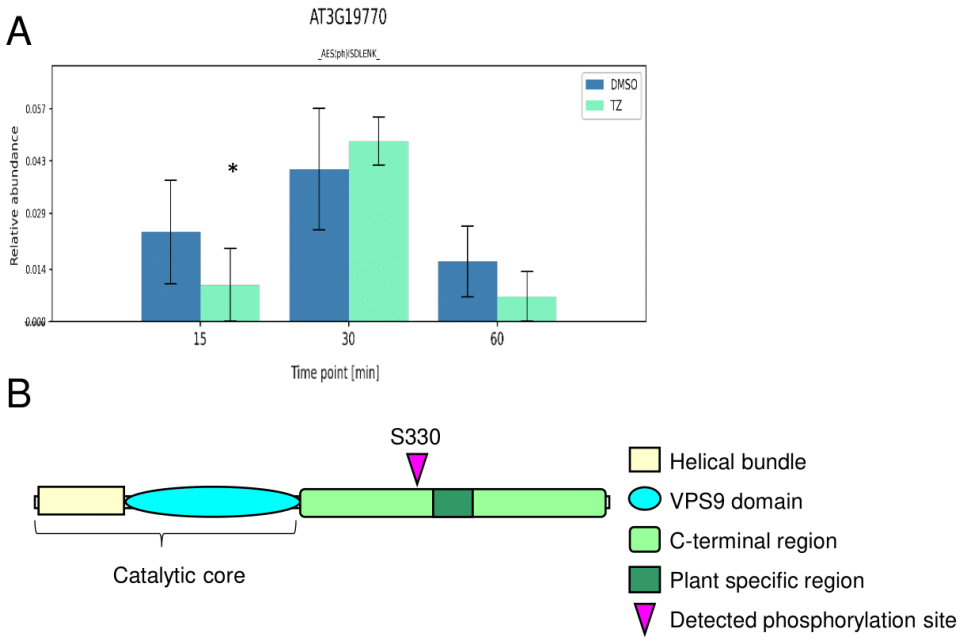
of kinases or conservancy of phosphorylation sites among species (Choudhary et al., 2015; De La Fuente Van Bentem et al., 2008; Nakagami et al., 2010; Reiland et al., 2009, 2011; Wang et al., 2013). Although these studies identified phosphorylation of both, S330 and S332, the phosphorylation of S330 was detected more often.

S330 and S332 phosphosites are conserved in other species, e.g. in rice (*O. sativa*) and in maize (*Z.mays*) (Figure 1C, Figure S1). Interestingly, the closest paralogue, Vps9b, which is considered a non-functional protein, does not share this phosphorylation site (Goh et al., 2007; Sunada et al., 2016, Figure 1C).

Altogether, this suggests function of S330 (and possibly S332) phosphorylation sites of VPS9a in plant's response to cytokinin.

vps9a-2 **mutant shows reduced sensitivity to cytokinin**

Cytokinin affects multitude of processes underlying growth and development of plants (Kieber & Schaller, 2018; Wyblouw & De Rybel, 2019). Exogenous treatment with cytokinin results in characteristic seedling phenotypes such as reduction of the primary root growth and the size of the root apical meristem, inhibition of lateral root initiation and organogenesis,



C

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AtVPS9a              -SETPMKKAESISDLENKG-ATLLKD-TEPSKVFQ-E
AtVPS9b              -SETPIKKADPITDLENKGAATLLNDRSEATKIFQ-E
OsGLUP6              ASSNPVERVQSISDLEKKGAAELLKD-DDLNKKIQ-E
S.moellendorffii     AKESSE---LSVAKLEAGGASGVVEA-DRSGQLAK-E
P.patens             ASGSSDKDTMTVAKLEALGLPDVLEA-DKTGQLAR-D
O.lucimarinus        RAAAPYVPWRTTEDVEAEGATQLTALDVAGNLTLSSD
O.tauri              SAAPAYIPWRTTEDVEAEGATALTALDVAGELALS-D
C.reinhardtii        TSQLVPLPPKSVSQLEADGVRLVLRAEAAGELRAR--
V.carteri            QKQFIPPRPKSVSELERDGIRLVIKAEAAGELRAK--
Vps9p                KFNELFSPIGEPTQEEALKSEQSNKEEDVSSLIKK--
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Figure 1: Identification of the phosphorylation site in VPS9a *(A) The relative abundance of the KAESISDLENK phosphopeptide. There is significantly less of this phosphopeptide the after 15 minutes of the treatment with 2µM tZ compared to DMSO. (B) Schematic representation of the domains of VPS9a protein (adapted from Sunada et al., 2016). Pink triangle marks the position of the identified phosphosite. (C) Alignment of the VPS9a proteins in various species shows partial conservation of the phosphorylation site (adapted from Sunada et al., 2016). Pink rectangle marks position of identified phosphosite.*

root hair initiation, and regulation of shoot growth and development (Dello Ioio et al., 2007; Kieber & Schaller, 2018; Laplaze et al., 2007; Vissenberg et al., 2020; Waldie & Leyser, 2018; Wyblouw & De Rybel, 2019). We tested whether cytokinin-mediated regulation of plant developmental processes is dependent on VPS9a.

To assess the role of VPS9a in the cytokinin effect on plant morphology, *vps9a-2* was subjected to various concentrations of hormone and the response to cytokinin (trans-Zeatin, tZ) was compared with wild type plants (Col-0). It has been reported that the *vps9a-2* mutant

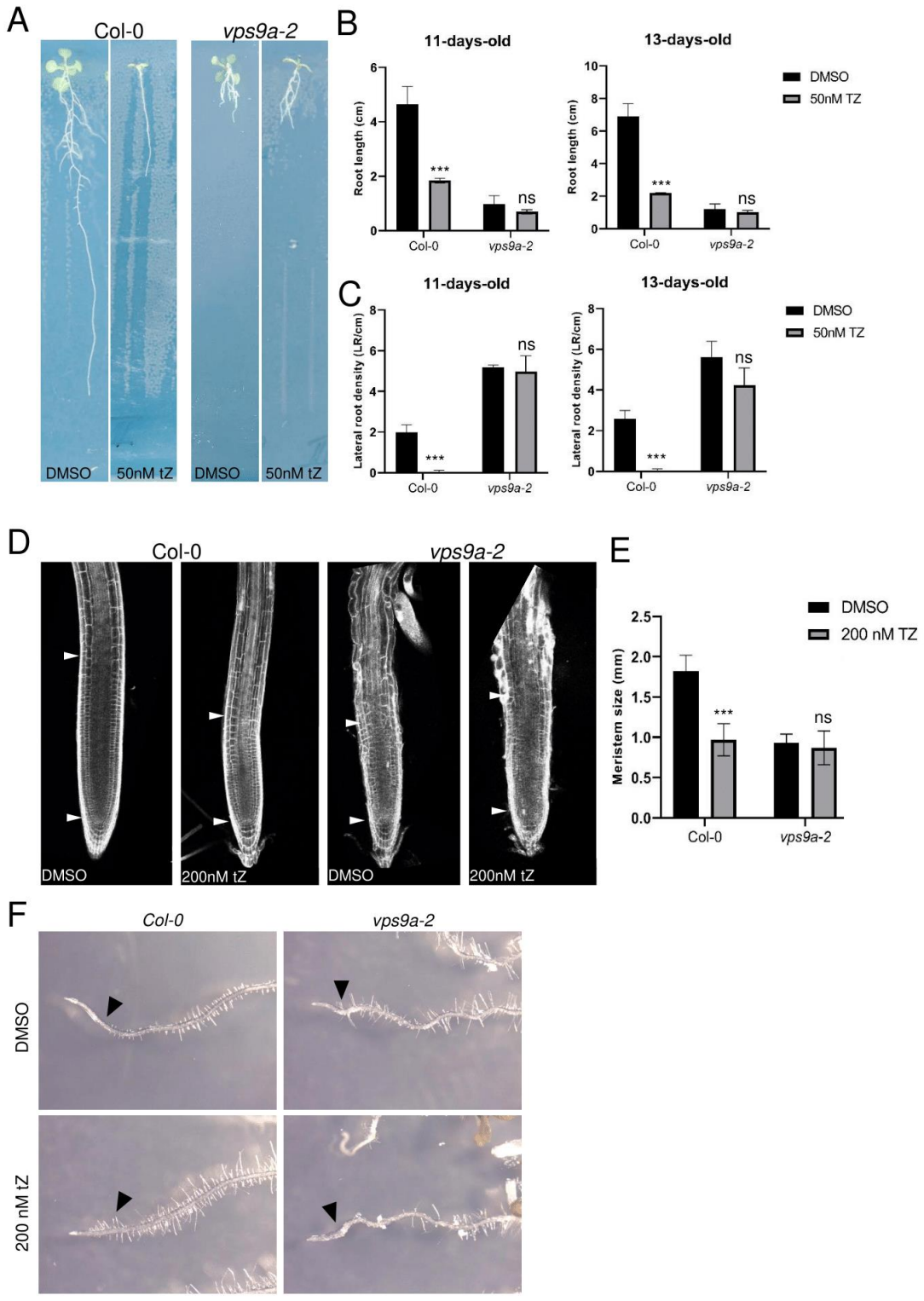

Figure 2: vps9a-2 mutant roots show reduced sensitivity to cytokinin *(description on the next page)*

Figure 2: vps9a-2 mutant roots show reduced sensitivity to cytokinin *(A) 13-day old Col-0 and vps9a-2 plants grown on the medium supplemented with DMSO or 50nM tZ. (B) Quantification of the primary root length of 11- and 13-days old plants grown on the medium supplemented with DMSO or 50nM tZ. (C) Quantification of the lateral root density in 11- and 13-days old plants grown on the medium supplemented with DMSO or 50nM tZ. (D) Root apical meristems of 9-days-old plants grown on media supplemented with DMSO or 200nM tZ. Roots were stained with propidium iodide. White arrow indicate the size of the meristem. (E) Measurements of the root apical meristem length in (D). (F) Cytokinin effect on root hair formation in Col and vps9-2. Col-0 and vps9a-2 were grown for 9 days on media supplemented with DMSO or 200nM tZ. Black arrows indicate presence/absence of root hairs. Bars represent mean ± s.d., *** = p < 0.001, n.s. = p > 0.05 by Student's t-test, n > 10. (B,C,E)*

has shorter primary root compared to Col-0 (Figure 2A, B, Goh et al., 2007). When grown on a low concentration of cytokinin (50nM), the primary root of Col-0 shows approximately 60% and 70% reduction in length compared to mock in 11- and 13-days-old plants, respectively. In contrast, the primary root of *vps9a-2* does not show significant difference in length between cytokinin and mock treatment (Figure 2A, B). Next, the short-term response of primary root growth to cytokinin was tested. 7-days-old seedlings were transferred to medium supplemented with low concentrations of tZ (10nM, 100nM) or mock and the primary root growth was monitored for 12 hours. In Col-0, a significant reduction in root growth rate on the media supplemented with cytokinin was detected compared with mock (Figure S2A). In contrast, *vps9a-2* mutant does not exhibit any significant alteration in root growth over 12 hours between media supplemented with cytokinin and with mock (Figure S2A). We also monitored the kinetics of the cytokinin response. When transferred on high concentrations of cytokinin (2µM) or mock, the Col-0 primary root growth of 7-days-old seedlings was affected by cytokinin within first few hours of the treatment. In *vps9a-2* background, cytokinin does not reduce the primary root growth. Noteworthy, after 18 hours, the roots of the *vps9a-2* mutant are significantly longer on cytokinin than in the mock conditions (Figure S2B).

vps9a-2 exhibits higher density of lateral roots compared to Col-0 in mock conditions (Figure 2A,C, Goh et al., 2007). In Col-0, we cannot observe almost any emerged lateral roots in 11- or 13-days-old seedlings grown on medium supplemented with 50nM cytokinin. In contrast, *vps9a-2* develops lateral roots despite cytokinin treatment. The density of emerged lateral roots of *vps9a-2* plants grown is not significantly different on media supplemented with cytokinin and on the mock media (Figure 2A, C).

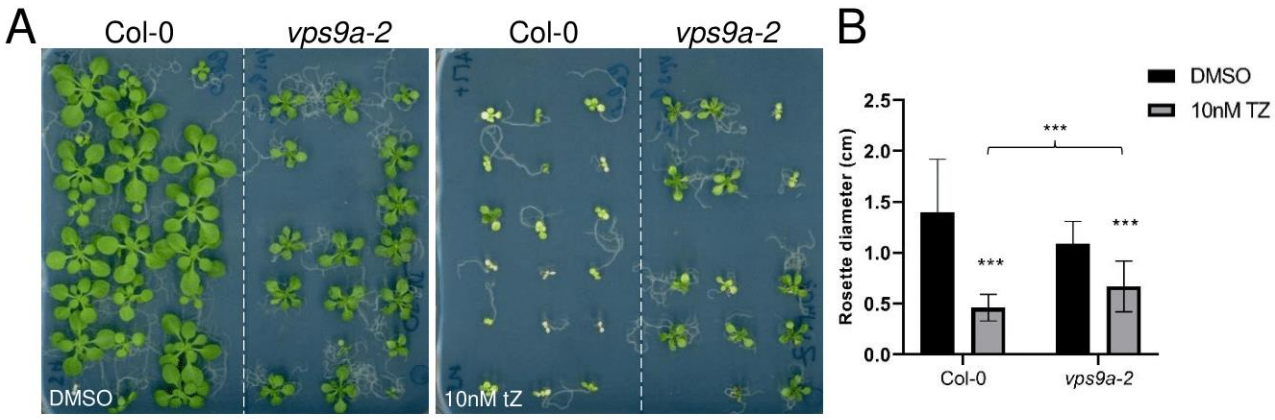

Figure 3: vps9a-2 mutant shoots show reduced sensitivity to cytokinin *(A) Col-0 and vps9a-2 plants were grown horizontally on medium supplemented with DMSO or 10nM TZ for 17 days. (B) Quantification of the rosette diameter in (A). The bars represent mean ± s.d., ** = p < 0.01, ***= p < 0.001 by Student's t-test, n > 10.(B)*

The primary root meristem of *vps9a-2* was reported to be shorter than the meristem of Col-0 (Inoue et al., 2013). *vps9a-2* and Col-0 seedlings were grown on mock or with 200nM tZ for 9 days. Roots were stained with propidium iodide and the distance from the quiescent center to the boundary between the transition and elongation zone was measured as the length of the meristem. Propidium iodide is a cationic dye that does not cross intact membranes but it penetrates throughout the meristem and binds to cell walls, visualizing an outline of living cells. In Col-0, cytokinin-grown plants have significantly shorter meristem compared to mock. The *vps9a-2* meristem does not show significant difference in length between cytokinin and mock-grown plants (Figure 2D, E).

In 9-days-old plants grown on cytokinin we also observed different response of *vps9a-2* and Col-0 to cytokinin in terms of the development of root hairs. Whereas in Col-0 plants, cytokinin promoted root hair formation when compared to mock, the root hair formation in *vps9a-2* mutant was less cytokinin-sensitive (Figure 2F).

Furthermore, we assessed the size of rosettes of 17-days-old plants horizontally grown on media supplemented with 10nM cytokinin or mock. The rosettes of Col-0 shows reduction in size on media supplemented with cytokinin compared to mock. The *vps9a-2* rosettes are smaller than those of Col-0 in the DMSO conditions, but show less reduction in size when grown on cytokinin compared to Col-0 (Figure 3A, B). Interestingly, the cytokinin-grown

vps9a-2 rosettes are significantly larger than the rosettes of Col-0 plants grown on cytokinin (Figure 3B).

To test, whether the *vps9a-2* retains sensitivity to other hormones, we performed treatment with precursor of ethylene, ACC (Bleecker & Kende, 2000). Col-0 and *vps9a-2* plants were grown on 200nM tZ, 200nM ACC, and mock media. The primary root length was monitored from the 6th to the 12th day of plant age. Both cytokinin and ACC treatment cause inhibition of the root length in Col-0 compared to mock conditions (Figure S3, Laplaze et al., 2007; Růžička et al., 2007). The difference in the root length of Col-0 on ACC or cytokinin compared to mock is significant already in the 6-days-old plants and becomes more pronounced with time (Figure S3B). While ACC causes also significant reduction of *vps9a-2* primary root length, this is not the case for cytokinin. Moreover, 11- and 12- days old *vps9a-2* plants grown on cytokinin exhibit significantly longer primary root than *vps9a-2* grown on mock media (Figure S3C). These results indicate that *vps9a-2* mutant roots maintain general capacity to react to external stimuli, such as ACC, by shortening the primary root length. Hence, they exhibit specific insensitivity to cytokinin treatment.

Altogether, these results hint at reduced sensitivity of *vps9a-2* to cytokinin compared to Col-0 in terms of root growth, lateral root density, root hair formation, and rosette size.

PIN degradation upon cytokinin treatment in *vps9a-2* mutant

One of the auxin-cytokinin cross-talk points during the root growth and the lateral root development, is cytokinin effect on the auxin transporter PIN1 (Marhavý et al., 2011, 2014; Ruzicka et al., 2009; Šimášková et al., 2015). Apart from transcriptional regulation, cytokinin also modulates auxin fluxes by affecting PIN1 vesicular trafficking. In particular, cytokinin re-routes PIN1 (but not PIN2 or other membrane proteins, see chapter 1) from the plasma membrane for vacuolar degradation (Marhavý et al., 2011, 2014).

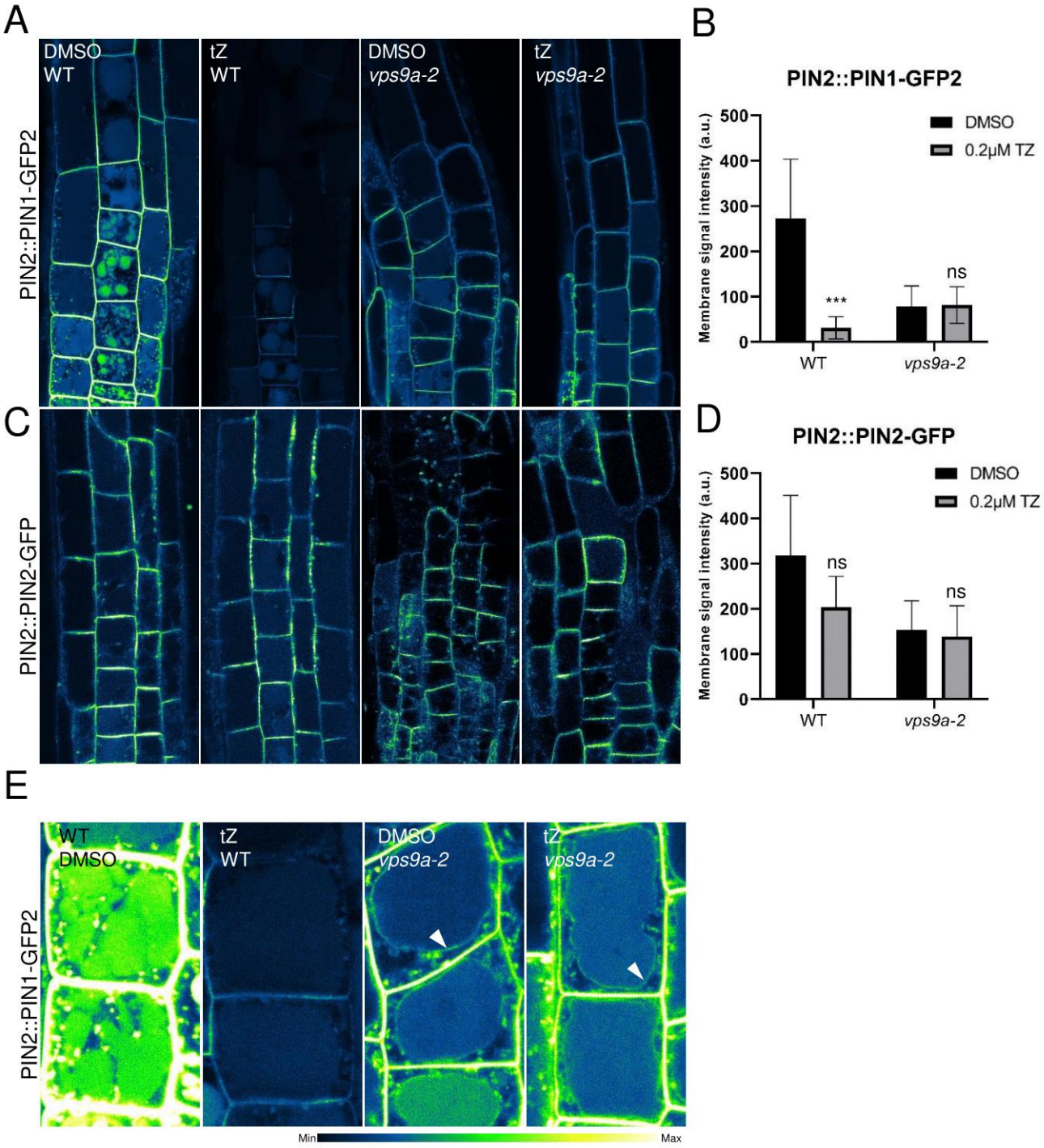

Figure 4. PIN1 is insensitive to cytokinin-mediated degradation in vps9a-2 mutant
(description on the next page)

VPS9a plays a role in the PIN1 trafficking, as *vps9a-1* mutant embryos show loss of PIN1 polar localization (Goh et al., 2007). We tested whether the cytokinin-driven PIN1 degradation is dependent on VPS9a. Multiple repetitions of crosses between *vps9a-2* and *PIN1::PIN1-GFP* (Benková et al., 2003) failed to produce homozygous *vps9a-2* population expressing PIN1-

Figure 4. PIN1 is insensitive to cytokinin-mediated degradation in vps9a-2 mutant *(A) Expression of PIN2::PIN1-GFP2 in root epidermal cells of 6-days-old seedlings after overnight treatment with DMSO or 0.2µM tZ in the darkness. (B) Quantification of PIN1-GFP plasma membrane signal in (A). (C) Expression of PIN2::PIN2-GFP in root epidermal cells of 6-days-old seedlings after overnight treatment with DMSO or 0.2µM tZ in the darkness. (D) Quantification of PIN2-GFP plasma membrane signal in (C). (E) Close-up view of the root epidermal cells in (A) expressing PIN2::PIN1-GFP2. White arrows indicate PIN1-GFP signal in the proximity/on the tonoplast. The bars represent mean ± s.d., *** = p < 0.001, n.s. = p > 0.05 by Student's t-test, n > 6 (B,D). a.u.= arbitrary units*

GFP. This might be because the PIN1-GFP insertion is on the same chromosome as *VPS9a* gene, which disables homologous recombination. Instead, we decided to use the line *PIN2::PIN1-GFP2*, with PIN1 expression in epidermal cells under *PIN2* promoter (Wisniewska et al., 2006). Using this line also allows us to take advantage of *PIN2::PIN2-GFP* (Abas et al., 2006) as a control for the transcriptional effects and for specificity of cytokinin effect on PIN1.

We transferred the 6-days-old plants on the media supplemented with DMSO/0.2µM tZ and incubated overnight in the darkness. In the *vps9a-2* background, both PIN1 and PIN2 show less plasma membrane signal compared to Col-0 in the DMSO conditions (Figure 4A). Upon treatment with cytokinin, the abundance of the PIN1 at the plasma membrane of root epidermal and stele cells is decreased in Col-0 seedlings (Figure 4A, B, Figure S4, Marhavý et al., 2011). In contrast, in the *vps9a-2* background, the decrease of the plasma membrane signal of PIN1 upon cytokinin treatment was not significant (Figure 4A, B, Figure S4).

PIN2-GFP protein expressed under the same promoter as PIN1-GFP did not show a significant decrease of the membrane signal upon cytokinin treatment in both, Col-0 (as previously shown in Marhavý et al., 2011) and *vps9a-2* background. This observation further corroborates the specificity of the cytokinin effect on the PIN1 protein and mitigates the possibility that the decrease of the PIN1 signal is a consequence of regulation of the the expression level (Figure 4C, D). Interestingly, apart from the plasma membrane, PIN1-GFP in the *vps9a-2* background seems to localize at/in close proximity to tonoplast, after both DMSO and cytokinin treatment (white arrows, Figure 4E). This might be due to the improper sorting of endomembrane vesicles, impaired ESCRT-dependent sorting to intraluminal vesicles at MVB, or inability of the vesicles to fuse with the vacuole.

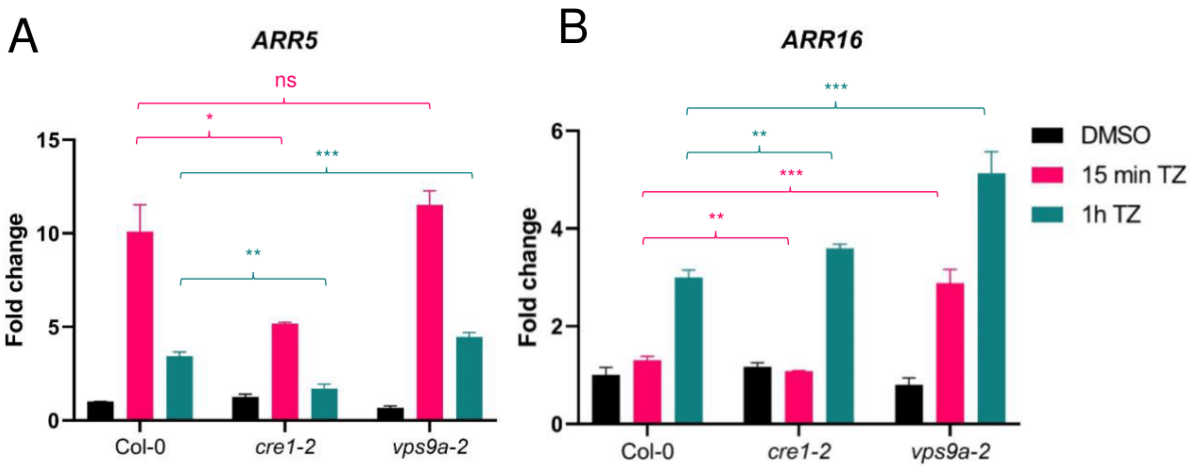

Figure 5: Cytokinin signal transduction pathway is not attenuated in vps9a-2 mutant *(A, B) Real-time Q-PCR expression analysis of the cytokinin-response gene ARR5 (A) and ARR16 (B). 7-days-old seedlings of Col-0, cre1-2 and vps9a-2 were sprayed with DMSO/5µM tZ and incubated for 15 minutes and 1 hour. The bars represent mean ± s.d., ***= p < 0.001, ** = p< 0.01, * = p< 0.05, n.s. = p > 0.05 by Student's t-test, n=3.*

Altogether, our results support involvement of VPS9a in the cytokinin-driven PIN1 trafficking to the vacuole.

Reduced cytokinin sensitivity of *vps9a-2* is not caused by deficiency in cytokinin signal transduction pathway

As a signaling molecule, cytokinin is perceived by histidine kinase receptors AHK2, AHK3, and AHK4/CRE1 localized at the ER or at the plasma membrane (Antoniadi et al., 2020; Kakimoto, 2003; Kubiasová et al., 2020; Wulfetange et al., 2011). From the AHKs, the signal is transduced to ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER proteins (AHPs), followed by translocation of AHPs to the nucleus, subsequent type-B ARR-dependent transcription of cytokinin-responsive genes, and a negative feedback regulation by the type-A ARRs (reviewed in Kakimoto, 2003; Keshishian and Rashotte, 2015; Kieber and Schaller, 2018; Li et al., 2021).

The AHK4 receptor exhibits dual localization at the ER and the plasma membrane and it is part of the BFA-sensitive trafficking pathway (Kubiasová et al., 2020). Since the VPS9a is an important regulator of vesicular trafficking, the *vps9a-2* mutant might have attenuated cytokinin perception due to the misplacement of the AHK4 cytokinin receptor.

In order to investigate whether the reduced cytokinin sensitivity of the *vps9a-2* mutant is caused by signaling deficiency, the transcriptional response to cytokinin was evaluated by

quantitative real-time PCR as the approximation of the amount signaling. We used type-A response regulators *ARR5* and *ARR16* as reporters of the early transcriptional response to cytokinin (D'Agostino et al., 2000). 7-days-old plants were sprayed with 5µM cytokinin/DMSO and the cytokinin response was measured in Col-0 and *vps9a-2* in the time point of 15 minutes and 1 hour.

In the Col-0 background, *ARR5* expression reached 10.1±1.4-fold upregulation after 15 minutes of cytokinin treatment and then decreased to 3.4±0.2-fold after 1 hour compared to DMSO (Figure 5A). The ARR16 showed mild but not significant upregulation after 15 minutes of the treatment and reached approx. 3-fold upregulation after 1h of cytokinin treatment compared to DMSO (Figure 5B). In *vps9a-2* mutant, the increase of the *ARR5* expression was comparable to this detected in the Col-0 background at 15 minutes after cytokinin application. Interestingly, the *ARR5* level was significantly higher in the *vps9a-2* than in Col-0 background after 1 hour of the cytokinin treatment (Figure 5A). Similarly, the *ARR16* level was significantly higher after 15 minutes and 1 hour of the cytokinin treatment in *vps9a-2* mutant compared to Col-0 (Figure 5B).

We used the mutant of cytokinin receptor AHK4, *cre1-2* (Tsutomu Inoue et al., 2001), as a control for decrease of cytokinin signaling due to missing plasma membrane receptor. The upregulation of *ARR5* after 15 minutes of cytokinin treatment in *cre1-2* background was significantly lower than in Col-0 (Figure 5). Therefore, if there was an impairment of cytokinin signaling due to a misplacement of the AHK4/CRE1 receptor in the *vps9a-2* mutant, we would be able to detect it in the 15 minutes time point using *ARR5* as a reporter. Although the *cre1-2* also shows upregulation of *ARR5* and *ARR16* upon cytokinin treatment, this is most likely due to the other two residual receptors (AHK2, AHK3).

Altogether, these results suggest that cytokinin signaling is not reduced in the *vps9a-2* mutant; therefore, the lower sensitivity to cytokinin is probably not caused by an impairment on the signaling level.

Monitoring of the VPS9a-GFP expression and localization upon cytokinin treatment

Next, the signal intensity and the subcellular localization pattern of *VPS9a::VPS9a-GFP* reporter line (Sunada et al., 2016) was examined upon treatment with cytokinin. We

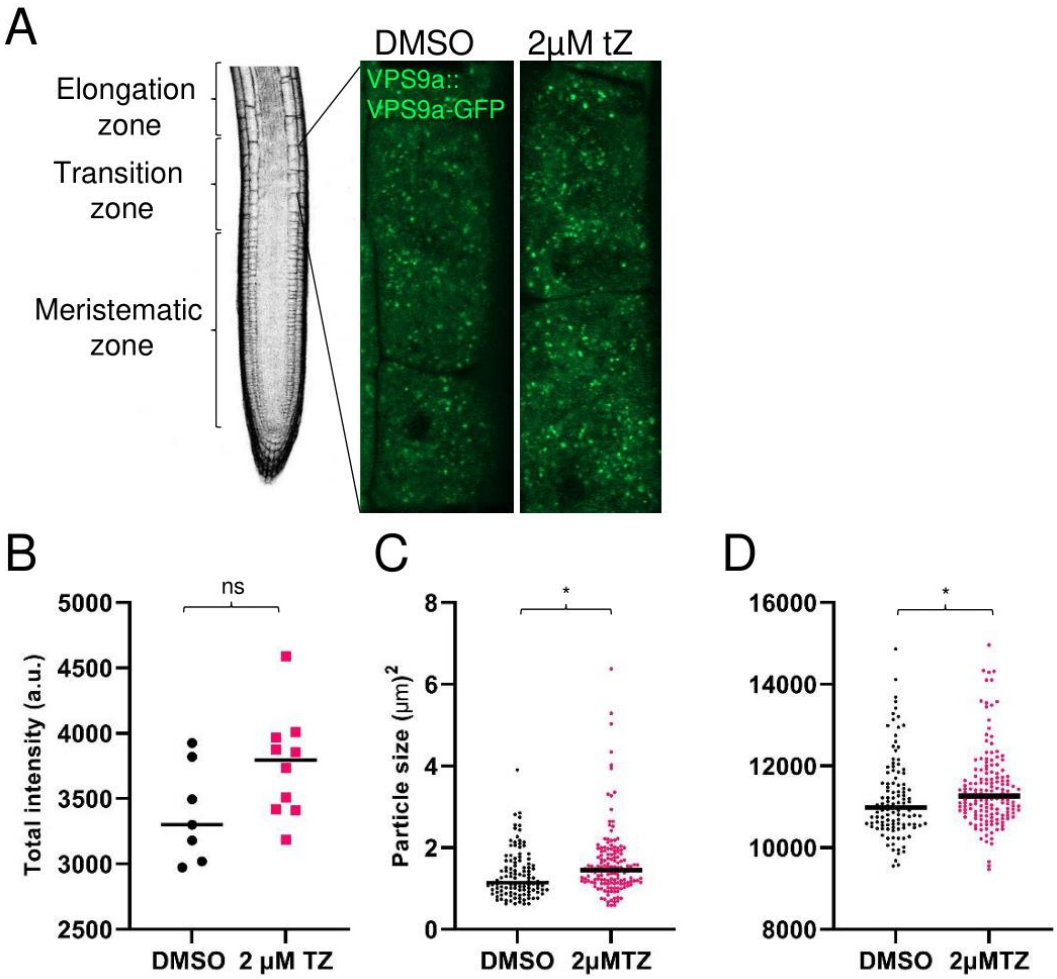

Figure 6: VPS9a-GFP expression upon cytokinin treatment *(A) Primary root meristem with indicated developmental zones (left). VPS9a::VPS9a-GFP expression in root epidermal cells at transition zone of 5-days-old seedlings after overnight incubation on media supplemented with DMSO or 2µM tZ in darkness (right). (B) Quantification of total intensity of VPS9a-GFP signal in root epidermal cells (A). (C) Quantification of particle size of the VPS9a-GFP positive membrane structures (A). (D) Quantification of particle mean intensity of structures labeled with the VPS9a-GFP in (A). The dots represent individual analyzed roots (B) or particles (C,D). The horizontal line represents median., *= p < 0.05, n.s. = p > 0.05 by Student's t-test, n >6 roots, 2 cell/root, a.u.= arbitrary units*

monitored the signal in epidermal cells at the root transition zone, where endogenous cytokinin balances the division and the differentiation of cells and thereby maintains the size of the root meristem (Dello Ioio et al., 2007, 2008; Di Mambro et al., 2017). Moreover, the transition zone of the root meristem was used for observation of cytoknin-triggered PIN1 vacuolar degradation (Figure 4).

5-days-old plants were transferred on the media supplemented with DMSO/2µM tZ and incubated overnight in darkness. In the epidermal cells at the transition zone, VPS9a-GFP localizes to membrane structures and to the cytoplasm (Figure 6A, Sunada et al., 2016). First, the overall VPS9a-GFP signal intensity was measured. Cytokinin treated plants exhibited mild but not significant increase in the overall VPS9a-GFP signal intensity compared to mock treatment (Figure 6B). Next, the size and the mean intensity of the VPS9a-GFP membrane structures was evaluated using particle analysis. Cytokinin-treated plants exhibit modest but significant increase in size and mean intensity of VPS9a-GFP positive particles compared to mock conditions (Figure 6B, C).

VPS9a activates Rab GTPases RHA1 and ARA7, which facilitate vacuolar trafficking (Goh et al., 2007; Ito et al., 2018; Kotzer, 2004; Sohn et al., 2003). The activation of Rab GTPases typically occures at the membrane surface (Bezeljak et al., 2020; Müller & Goody, 2018). Therefore, the amount of VPS9a-GFP signal at membrane compartments might be used as a proxy for determining the amount of active VPS9a. These results suggest that cytokinin might increase the amount of active VPS9a and by that promote the trafficking to the vacuole. However, cytokinin might also cause VPS9a-GFP localization to compartments of a different size or identity. Therefore, a follow up investigation using co-localization with endomembrane markers or electron microscopy analysis should be performed.

To examine further the amount of VPS9a associated with membrane compared to the amount in the cytoplasm we implemented western blot analysis of *VPS9a::VPS9a-GFP* plants (Sunada et al., 2016). Cytosolic and microsomal fractions of proteins were isolated from roots of 7-days-old plants, which were sprayed with 5µM cytokinin or DMSO and incubated overnight. Band of size ~100KDa, which corresponds to the size of the VPS9a fused to GFP was detected in both, cytosolic and microsomal fraction. In cytosolic as well as in microsomal fraction, the amount VPS9a protein increased after cytokinin treatment compared to mock (Figure 7). This suggests general increase in the VPS9a protein amount upon treatment with cytokinin. Further investigation of cytokinin effect on VPS9a transcription by QPCR will clarify this aspect.

Next, we tested phosphorylation of the VPS9a protein after cytokinin treatment. To examine, whether changes in phosphorylation of VPS9a can be detected by western blot analysis, a

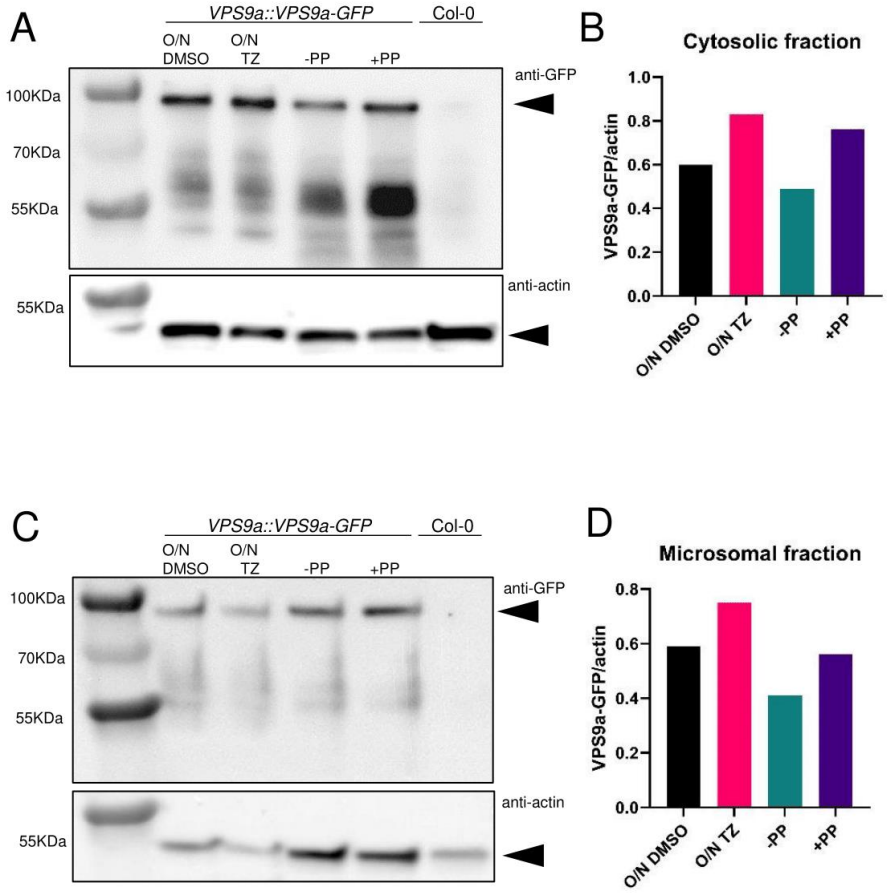

Figure 7: Expression analysis of VPS9a-GFP in roots treated with cytokinin *(A,C) Detection of VPS9-GFP in cytosolic (A) and microsomal fraction (C) of root protein extracts using Western blot analysis. 7-days-old VPS9::VPS9a-GFP seedlings were sprayed with DMSO or 5uM tZ and incubated overnight (O/N). Roots were harvested for protein isolation. -/+PP indicates lambda phosphatase treatment of the protein lysate from plants without any treatment. Black triangle marks size of the VPS9-GFP (100KDa) or actin as (42KDa) a loading control. (B,D) Quantification of the VPS9a-GFP bands relative to actin.*

lambda phosphatase treatment was performed. Phosphatase treatment causes global de-phosphorylation of proteins, which might subsequently result in changed mobility of the protein band on the western blot (personal communication). Protein extracts from roots of 7-days-old untreated plants were incubated with (+PP) or without (-PP) lambda phosphatase. The (+PP) sample shows increased amount of VPS9a protein compared to the (-PP) sample in both, cytosolic and microsomal fraction (Figure 7). This is the same trend as cytokinin treated and mock samples, respectively. However, phosphatase treatment is expected to change rather the mobility than the amount of the protein (personal communication). VPS9a-GFP band does not show a difference in the mobility after treatment with PP when compared to

untreated (-PP) protein extract. Thus, western blot analysis is not a satisfactory method for detection of the changes in phosphorylation of VPS9a protein. In further analysis we implemented anti-Phospho-Serine antibodies and the PhosTag® gel to test VPS9a phosphorylation, however, the results were not suitable for proper interpretation due to technical issues (data not shown). Altogether, cytokinin treatment causes increased association of VPS9a-GFP with membrane structures and higher VPS9a protein amount compared to mock conditions.

Cloning of phospho-mimic and phospho-dead mutant alleles of VPS9a

In the phosphoproteomic screen, we detected changes in phosphorylation of the VPS9a peptide KAESISDLENK upon cytokinin treatment (Figure 1). We decided to investigate the functional importance of the phosphorylation site by mutating both serines in the detected peptide. We substituted both of them with alanine (VPS9a^{AA}) or aspartic acid (VPS9a^{DD}) to mimic constitutively de-phosphorylated ("phospho-dead") and phosphorylated ("phospho-mimic") state, respectively (Figure 8A). To assess the impact of phosphorylation on subcellular localization, cDNAs of *VPS9a$^{WT/AA/DD}$-GFP* were transiently expressed under *CaMV35S* promoter in *Nicotiana benthamiana* leaves. VPS9a^{WT}-GFP showed patchy signal distributed in cytoplasm with occasional localization to puncta structures (Figure 8B, white arrows). We did not observe an obvious difference in the localization pattern between VPS9a^{WT}-GFP and phosphomutants VPS9a-GFP^{AA} and VPS9a-GFP^{DD} (Figure 8B).

The prevalent cytosolic localization of VPS9a^{WT}-GFP in tobacco leaves differs from the published localization of VPS9 to membrane structures in root epidermal cells (Sunada et al., 2016, Figure 6), which might be due to the different characteristics of the expression system (leaves vs roots). However, it might also reflect improper sorting and/or functional impairment of the VPS9a protein in tobacco leaves. Therefore, transient expression in tobacco might not be a suitable system to study the localization pattern of VPS9a and impact of mutations in respective phosphorylation sites.

Generation of VPS9a$^{WT/AA/DD}$ stable transformed *Arabidopsis* lines

To address the role of the identified, cytokinin responsive VPS9a phosphosite in context of plant growth and development we generated stably transformed *Arabidopsis thaliana* lines

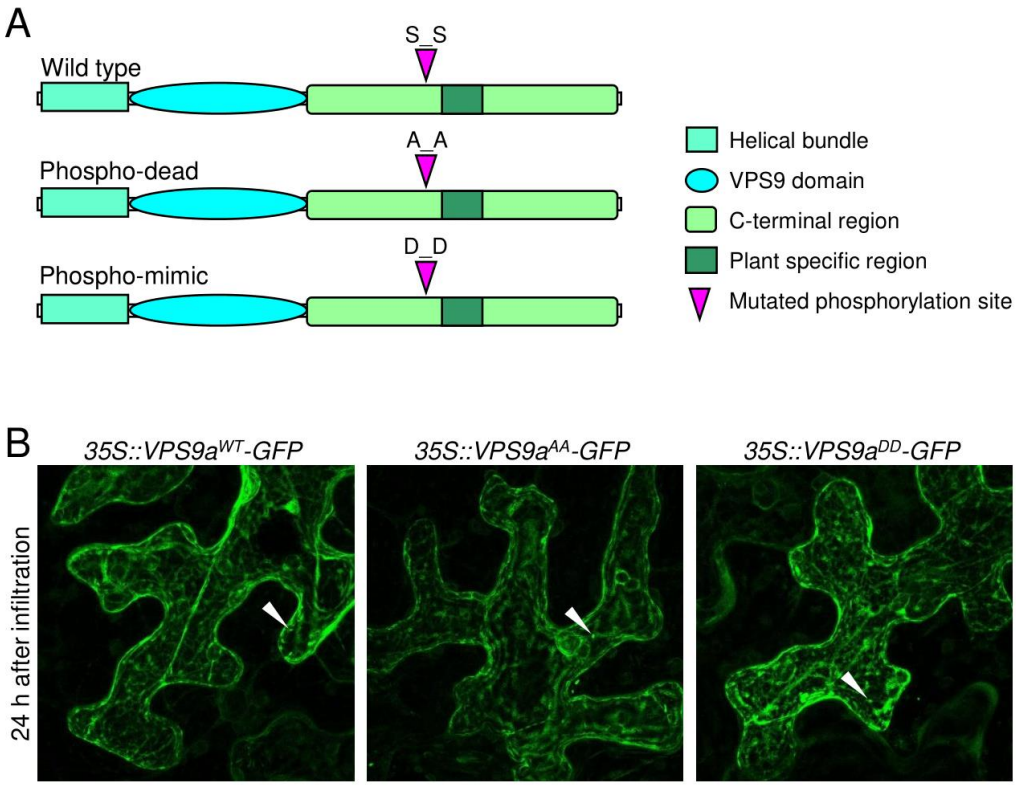

Figure 8: Generation of the phospho-dead (VPS9a^{AA}) and the phospho-mimic (VPS9a^{DD}) mutations *(A) Schematic representation of position of serin (S) in the wild-type VPS9 and the phospho-dead (A = alanine) and the phospho-mimic (D= Aspartic acid) mutations introduced into the VPS9a protein. (B) Transiently expressed 35S::VPS9a$^{WT/AA/DD}$-GFP in tobacco (Nicotiana benthamiana) leaf epidermis cells. White arrows mark puncta where VPS9a-GFP presumably associates with membrane structures.*

expressing phospho-dead *VPS9a^{AA}* and phospho-mimic *VPS9a^{DD}* mutant variants. Initially, the mutation of phosphosites was introduced into the cDNA of *VPS9a* to gain experimental flexibility in fluorescent and biochemical tagging of the VPS9a protein as well as possibility of cloning of the *VPS9a* under various promoters. We transformed *Arabidopsis thaliana* with cDNAs of *VPS9a$^{WT/AA/DD}$-GFP* driven by either endogenous 2.5kB or constitutive *CaMV35S* promoter. However, no plants showing GFP signal were recovered in the T1 generation, despite the presence of the construct was confirmed by genotyping. Since the previously reported functional *VPS9a::VPS9a-GFP* construct was cloned using a genomic DNA with endogenous promoter (Sunada et al., 2016), our results suggest that there are regulatory elements essential for expression of the *VPS9a* in the genomic fragment, which might be missing in the cDNA.

Therefore, phosphosites were mutated directly in the *VPS9a::VPS9a-GFP* construct (kind gift from T. Ueda and K. Ebine) to create *VPS9a::VPS9a^{AA}-GFP* and *VPS9a::VPS9a^{DD}-GFP*. *vps9a-1*, *vps9a-2,* and Col-0 plants were transformed with these constructs as well as with the non-mutated, wild-type version, *VPS9a::VPS9a^{WT}-GFP*, which serves as a control. Since the *vps9a-1* mutants are embryo lethal (Goh et al., 2007) the heterozygous population of this mutant was used for transformation.

In the T1 generation*, VPS9a::VPS9a^{WT}-GFP* construct rescues the *vps9a-1* embryo lethality (data not shown) and the dwarfed shoot phenotype of *vps9a-2* (Figure S9). Surprisingly, we observed various levels of phenotypic alteration of the shoot development in these transgenic lines irrespective of the genetic background. Whereas some of them were indistinguishable from Col-0 plants, other transgenic lines displayed short stems and enhanced branching or dwarfed appearance (Figure S9, data not shown). This might hint at the master regulatory role of VPS9a in fine-tuning of the vesicular trafficking machinery. Random insertions probably result in slightly different expression levels of VPS9a, which might affect the vesicular trafficking of various cargoes. The T1 generation of *VPS9a::VPS9a^{AA}-GFP and VPS9a::VPS9a^{DD}-GFP* exhibited similar distribution of shoot phenotypical defects as the *VPS9a::VPS9a^{WT}-GFP* transformants (Figure S9, data not shown).

Altogether, this increases the difficulty in choosing VPS9a^{WT}/VPS9a^{AA}/VPS9a^{DD} lines that are comparable for future examinations. Therefore, we set stringent criteria for the selection of transformants. First, plants must look WT-like to allow for successful seed propagation. Second, plants must have similar level of GFP signal, reflecting similar expression level. Third, the chosen line must be a single-insertion line, which was evaluated by observation of the segregation of the GFP signal in T2 and the significance was calculated by chi^2 test. The presence of the mutated phosphorylation site in these lines was verified by sequencing of the genomic DNA of the selected lines (Figure S10).

For simplicity, we continued only with transformants in the *vps9a-2* background, where we can clearly measure the rescue of the mutant phenotype while circumventing difficulties with evaluation of the rescue of the embryo lethal *vps9a-1* mutation. These criteria were fulfilled by one line/construct/*vps9a-2* background from WT, phosphodead and phosphomimic constructs. We are aware that for solid conclusion we need to evaluate multiple independent

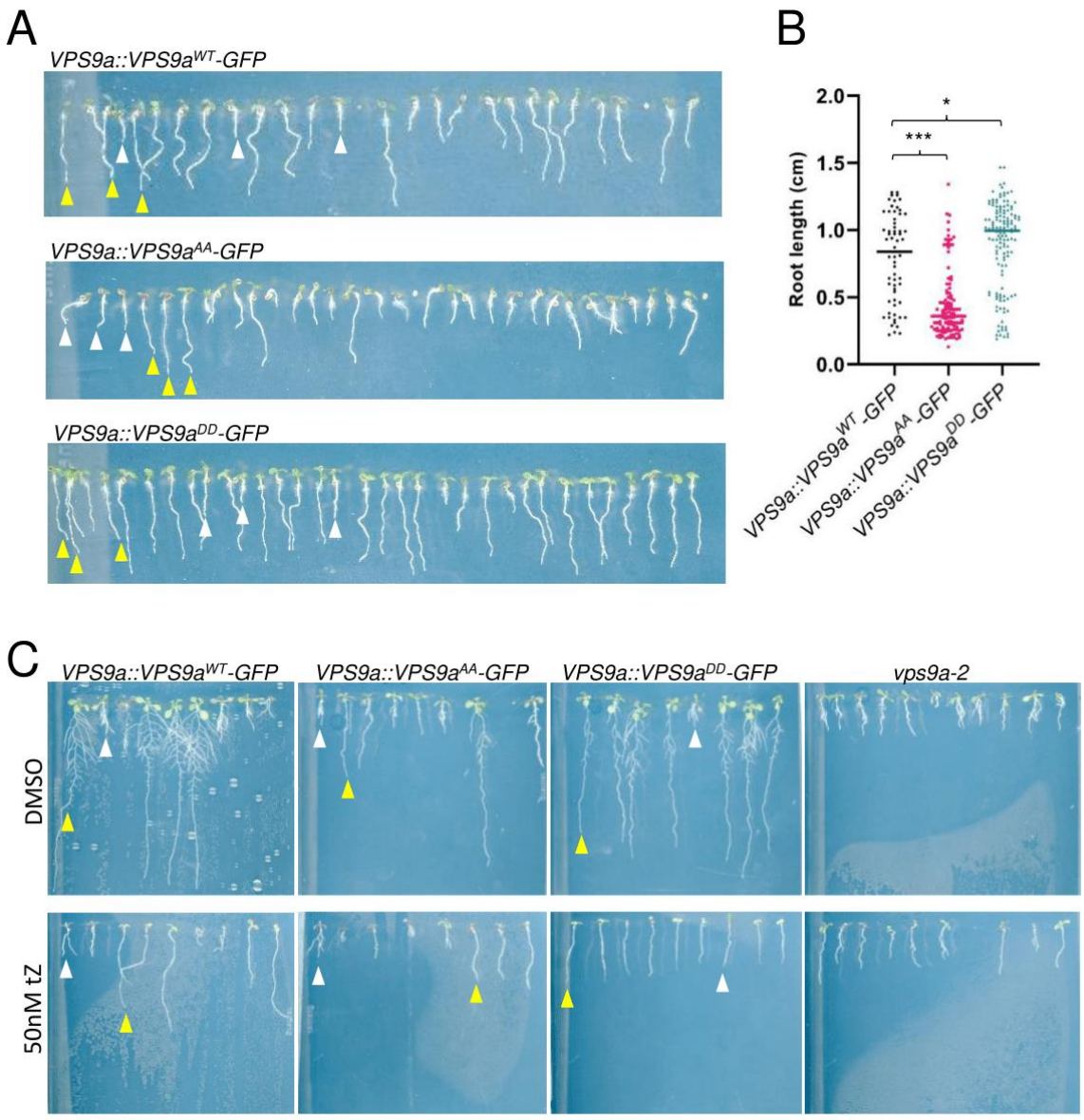

Figure 9: T2 generation of wild type (VPS9a^{WT}), phospho-dead (VPS9a^{AA}), the phospho-mimic (VPS9a^{DD}) Arabidopsis thaliana transgenic lines *(A) 5-days-old seedlings of the VPS9a::VPS9a$^{WT/AA/DD}$-GFP/vps9a-2. (B) Quantification of primary root length in (A). (C) 11-days-old VPS9a::VPS9a$^{WT/AA/DD}$-GFP/vps9a-2 plants grown on medium supplemented with DMSO/50nM tZ. White arrows mark out-segregated vps9a-2 mutant in T2 generation. Yellow arrows mark rescued plants. The dots represent lengths of individual roots. The horizontal line represents median. ***= p < 0.001, *= p < 0.05 by Student's t-test, n of roots>62 (B).*

lines. Nevertheless, these lines can be used to acquire preliminary data about role of the VPS9a phosphorylation site.

Phenotypic analysis of phospho-dead and phospho-mimic variants of VPS9a

The *vps9a-2* mutant exhibits shorter primary root than Col-0 plants (Figure 2A, Goh et al., 2007). Therefore, the length of the primary root of transformants with *vps9a-2* background was used as a quantitative measure for the level of the rescue by *VPS9a* wild type and phosphomutant constructs.

The root length of 5-days-old seedlings was assessed. Since the T2 generation was used, 75% of rescued seedlings is expected. Indeed, we observed a pool of segregated *vps9a-2* mutants exhibiting short root and a pool of rescued plants showing longer root (Figure 9A, B). The *VPS9a::VPS9a^{WT}-GFP* line shows rather continuous distribution of the root lengths, which might reflect the natural variability of the *vps9a-2* mutant. However, it might also suggest that *VPS9a::VPS9a^{WT}-GFP* does not fully rescue the mutant phenotype or causes additional root growth hindering. The *VPS9a::VPS9a^{AA}-GFP* and the *VPS9a::VPS9a^{DD}-GFP* transformants show different level of rescue from each other. Whereas the most of the *VPS9a::VPS9a^{AA}-GFP* plants exhibit short root (significantly shorter than the *VPS9a::VPS9a^{WT}-GFP* line), the majority of the *VPS9a::VPS9a^{DD}-GFP* transformants shows long root (significantly longer than *VPS9a::VPS9a^{WT}-GFP* line, Figure 9B). This hints at a distinct impact of phospho-dead and phospho-mimic version of VPS9a on the root growth.

VPS9a::VPS9a$^{WT/AA/DD}$-GFP/vps9a-2 T2 generation lines were tested for their sensitivity to cytokinin. Plants were grown for 11 days on media supplemented with DMSO/50nM tZ. On the mock media, *VPS9a::VPS9a^{WT}-GFP* and *VPS9a::VPS9a^{DD}-GFP* transformants show a rescue of the mutant phenotype in terms of the primary root growth. The *VPS9a::VPS9a^{AA}-GFP* plants exhibit partially rescued root growth with defects such as shorter main root and higher lateral root density, which is reminiscent of the *vps9a-2* mutant (Figure 9C). On cytokinin, *VPS9a::VPS9a$^{WT/AA/DD}$-GFP* constructs rescue the insensitivity of the *vps9a-2* to cytokinin in terms of inhibition of primary root growth and lateral root development (Figure 9C). In further experiments, a detailed analysis of cytokinin sensitivity of VPS9a phosphomutants in T3 homozygous population should be performed.

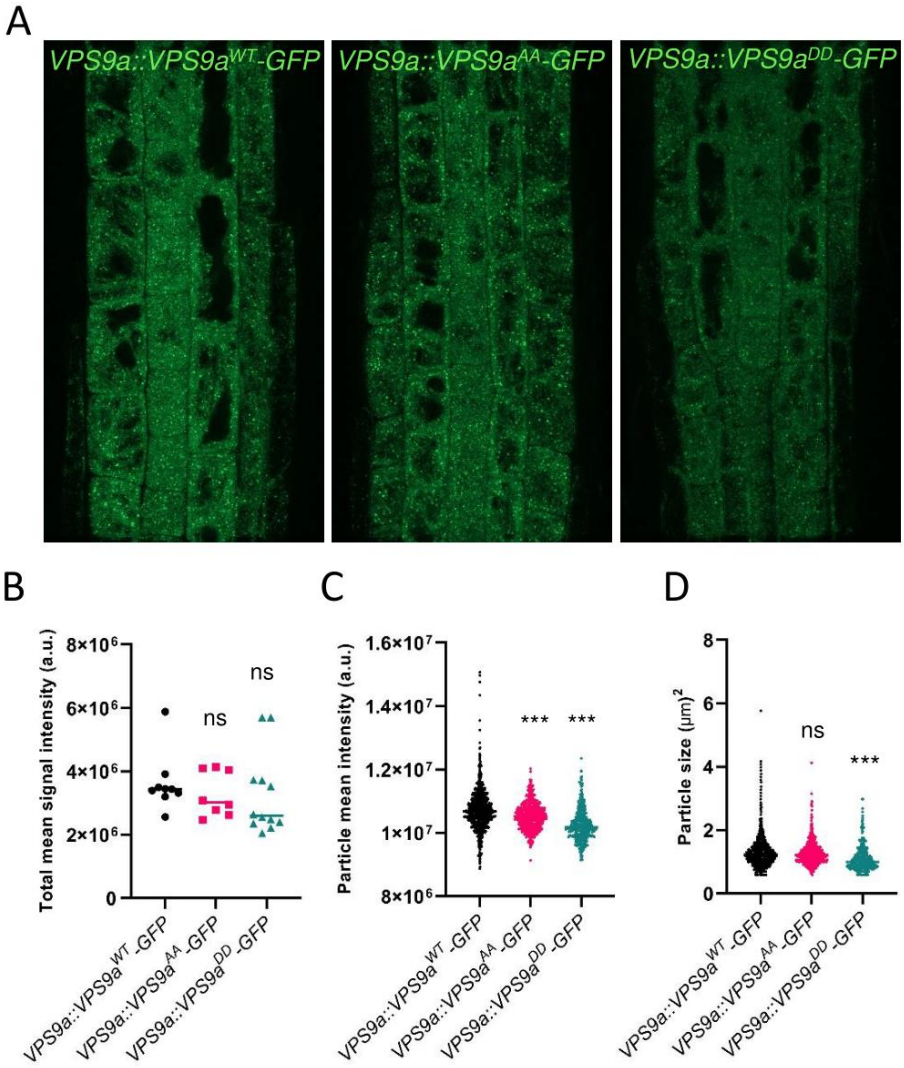

Figure 10: Subcellular localization pattern of wild type (VPS9a^{WT}-GFP), phospho-dead (VPS9a^{AA}-GFP) and the phospho-mimic (VPS9a^{DD}-GFP) in transgenic lines of T2 generation
*(A) VPS9-GFP signal in epidermal cells of transition zone of primary root meristem. Transformants are in vps9a-2 background. (B) Quantification of the total mean intensity of the fluorescent signal of (A). The dots represent single root measurements. Horizontal lines represent the mean, n.s. = p > 0.05 by Student's t-test by Student's t-test. (C) Particle analysis of mean intensity of the VPS9a::VPS9a$^{WT/AA/DD}$-GFP labeled membrane structures. (D) Particle analysis of the area of the VPS9a::VPS9a$^{WT/AA/DD}$-GFP labeled membrane structures. The dots represent single particle measurement. Horizontal lines represent the mean, ***= p < 0.001, n.s. = p > 0.05 by Student's t-test. a.u.=arbitrary units*

Next, we investigated whether phosphorylation of VPS9a might influence its subcellular localization. The total intensity of the GFP signal between wild type and phosphomutant lines was not significantly different (Figure 10B). Notably, when we performed the particle analysis, we discovered that mean intensity of membrane structures marked by both VPS9a^{AA}-GFP and VPS9a^{DD}-GFP is lower than that marked by VPS9a^{WT}-GFP (Figure 10C). Furthermore, the size

of the VPS9a^{DD}-GFP positive particles is reduced when compared to VPS9a^{WT}-GFP (Figure 10D). This observation hints at the importance of the phosphorylation site for the localization of the VPS9a to membrane structures. Importantly, the QPCR and biochemical analysis of the expression and protein levels should be performed to verify that the observed changes are not caused by differences in the abundance of the VPS9a protein.

Altogether, these results suggest that cytokinin-mediated phosphorylation might affect subcellular localization of VPS9a and fine-tune its activity in the regulation of root growth.

Rab GTPases mutant phenotypes on cytokinin

VPS9a is a Rab Guanosine Exchange Factor (Rab GEF). It activates ARA6, ARA7, and RHA1 by exchanging the GDP for GFP (Goh et al., 2007). ARA6, ARA7 and RHA1 participate in the vacuolar trafficking for both biosynthetic pathway from Golgi and endocytic pathway from the plasma membrane (Kotzer, 2004; Lee et al., 2004; Ueda et al., 2001, 2004). Whereas ARA7 and RHA1 share large similarity between each other and also other eukaryotic Rabs, ARA6 is specific to higher plants (Pereira-Leal & Seabra, 2001; Ueda et al., 2001). Recent study showed that while ARA7 and RHA1 mediate the vacuolar transport, ARA6 acts as a negative regulator of this transport by titrating the VPS9a and Rab effector PUF2 (Ito et al., 2018).

The C-terminus of VPS9a, which contains phosphosite identified in our study, is key for interaction with ARA6 and but not ARA7 or RHA1 (Sunada et al., 2016). We hypothesized that by modulating the phosphorylation of VPS9a, cytokinin might change the preference of VPS9 for interaction with its downstream Rabs. Therefore, we examined the root morphology and cytokinin sensitivity of mutants of conventional and plant-specific Rab, ARA7 and ARA6, respectively.

ara6-1, ara6-2, and ara7 plants were grown on media supplemented with DMSO/50nM tZ and the primary root length and lateral root density was measured after 11 and 13 days. In mock conditions, ara6-1 and ara7 did not significantly differ in the root length or lateral root density from Col-0 (Figure 11 A, B, C).

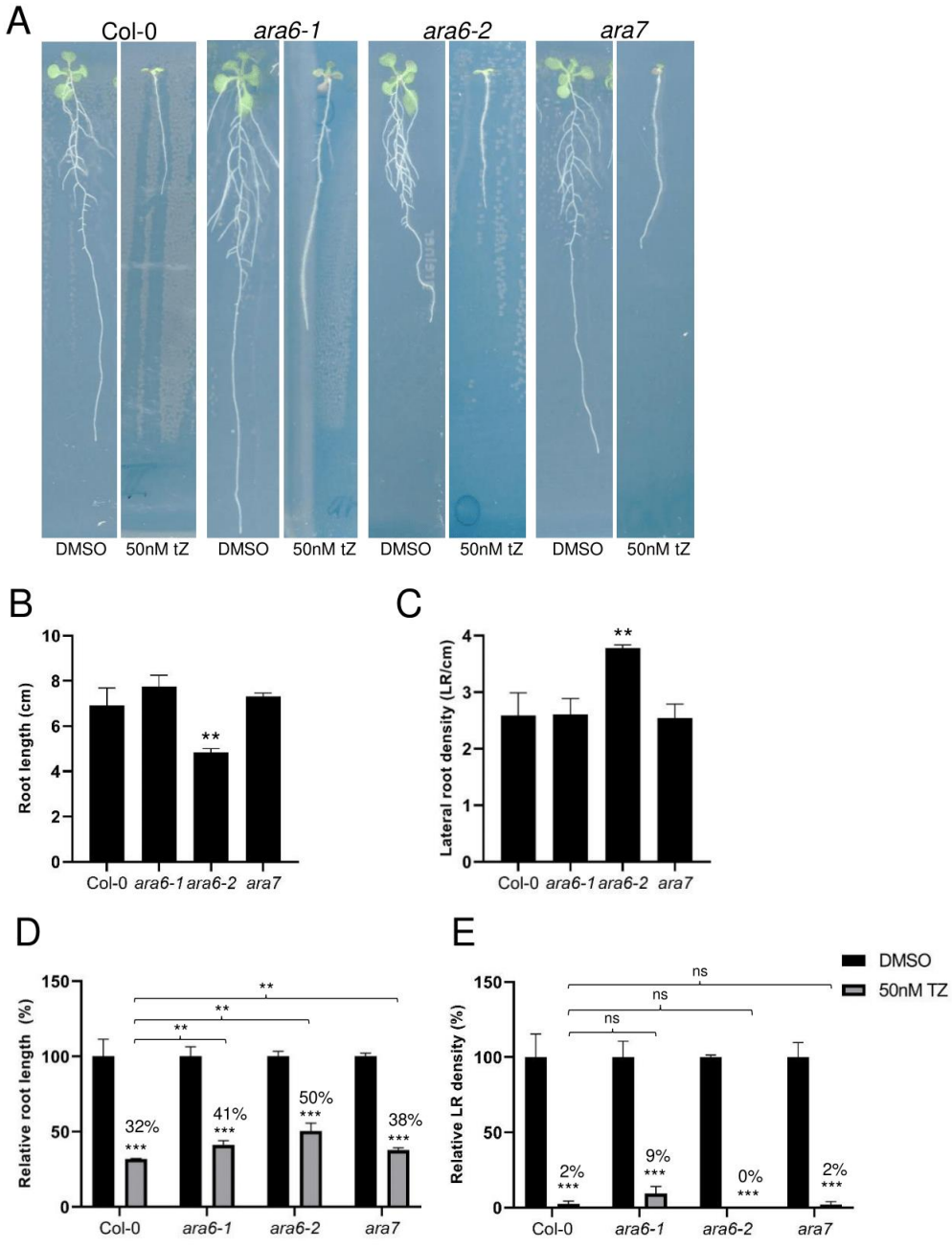

Figure 11: Cytokinin sensitivity of ara6-1, ara6-2, and ara7 *(A) Representative images of 13-days-old Col-0, ara6-1, ara6-2, and ara7 plants grown on the medium supplemented with DMSO or 50nM tZ. (B) Quantification of the primary root length in (A). (C) Quantification of lateral root density in (A). (D) Relative root length of 13-days-old Col-0, ara6-1, ara6-2, and ara7 plants grown on the medium supplemented with DMSO or 50nM tZ. (E) Relative lateral root density of 13-day old Col-0, ara6-1, ara6-2, and ara7 plants grown on the medium supplemented with DMSO or 50nM tZ. The bars represent mean ± s.d., *** = p < 0.001, ** = p < 0.01, n.s. = p > 0.05 by Student's t-test, n > 10.*

In case of ara7, this might be due to the redundancy with the other RabF2, RHA1. It has been reported that the single ara7 and rha1 mutants do not exhibit any phenotype alterations from wild-type plants, whereas, the ara7rha1 double mutant cannot be established because of the male sterility (Ebine et al., 2011). Interestingly, the ara6-2 mutant allele displays shorter root and higher lateral root density compared to Col-0 in mock conditions (Figure 11 A, B, C, Figure S11A), which is reminiscent of the vps9a-2 mutant. *ara6-2* is probably a stronger allele than *ara6-1* because it has the T-DNA insertion more upstream in the gene (Ebine et al., 2011).

In all tested Rab mutants, cytokinin significantly reduced the primary root length as well as the lateral root density compared to mock treated seedlings (Figure 11 D, E). However, closer examination reveals, that Rab mutants significantly differ from Col-0 in sensitivity of the primary root growth to cytokinin. In 13-days-old Col-0 plants, cytokinin reduces the primary root length to 32±0.5% (Figure 11D). In the *ara7* plants, it reduces the root length to 38±2%, *ara6-1* to 41±3%, and in *ara6-2* only to 50±5% of the root length of respective genotype grown in mock conditions (Figure 11D). The measurements of 11-days-old plants show similar trend (Figure S11B).

Altogether, the *ara6-1*, *ara6-2* and *ara7* mutants are less sensitive to cytokinin in terms of the primary root growth compared to Col-0. *ara6-2* exhibits shorter root and increased density of lateral roots in mock conditions and concomitantly the lowest sensitivity of the primary root growth to cytokinin. Both of these features are reminiscent of the *vps9a-2* phenotype.

Cytokinin effects on subcellular localization of ARA6-VENUS and mRFP-ARA7

By influencing the VPS9a phosphorylation, cytokinin might impact the ability of Vps9a to activate its downstream interactors, ARA6 and ARA7. Active Rabs are associated with the membrane surface (Bezeljak et al., 2020; Müller & Goody, 2018). Therefore, their subcellular localization can serve as a readout for their activity.

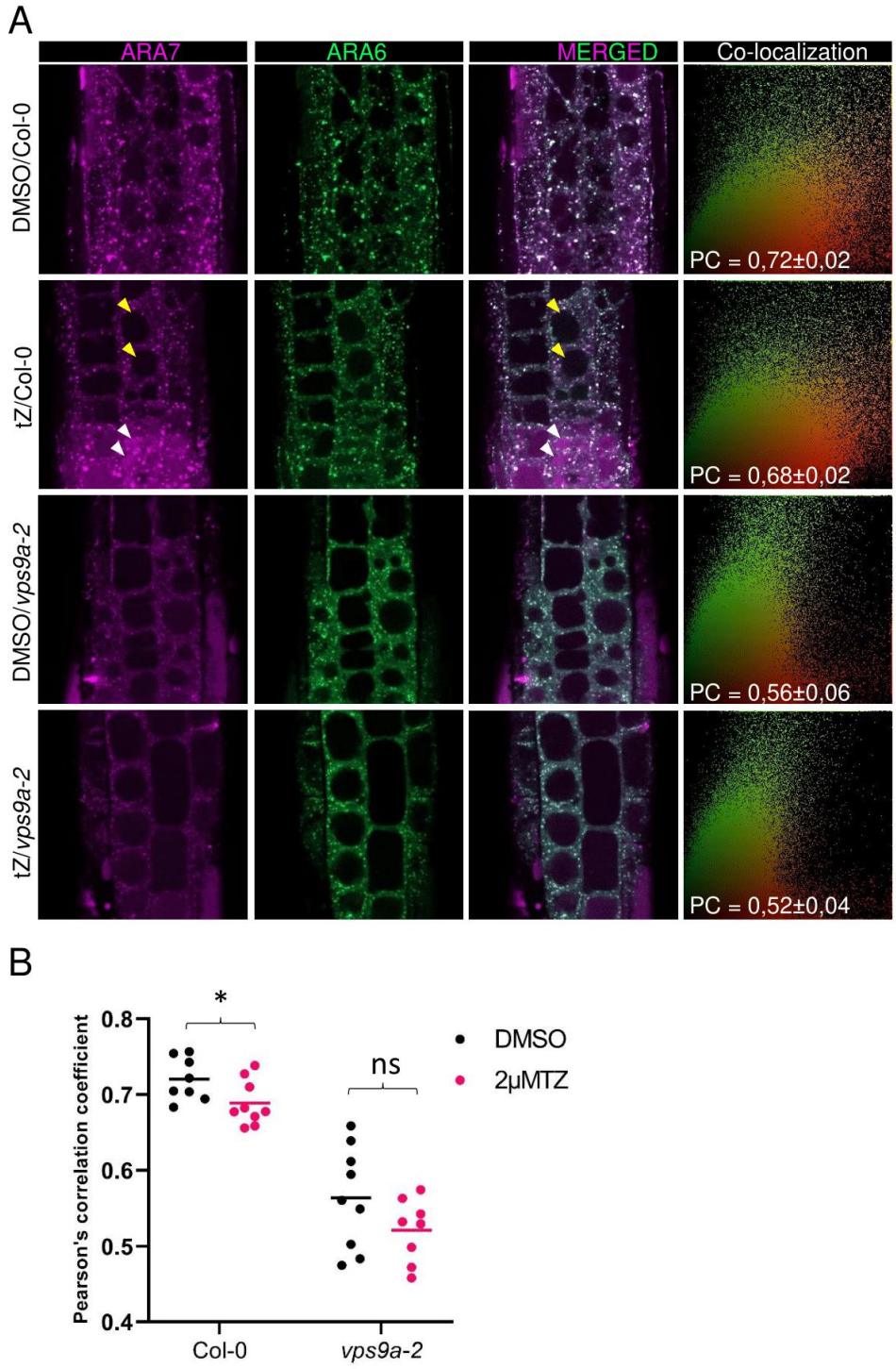

Figure 12: Subcellular localization of ARA6-VENUS and mRFP-Ara7 upon treatment with cytokinin *(description on the next page)*

Figure 12: Subcellular localization of ARA6-VENUS and mRFP-Ara7 upon treatment with cytokinin *(A) Epidermal cells in primary root meristem expressing ARA6-VENUS mRFP-ARA7 in Col-0 and vps9a-2 background. 5-days-old seedlings were transferred on media supplemented with DMSO or 2µM tZ and treated overnight in darkness. In Col-0, treatment with cytokinin promoted mRFP signal accumulation in vacuoles of cells in the meristematic zone (white arrows) but not transition zone (yellow arrows). In vps9a-2, there was no visible accumulation of mRFP signal in vacuoles upon cytokinin treatment. Co-localization between the VENUS and mRFP is quantified by Pearson's correlation coefficient (PC). (B) Quantification of the co-localization using Pearson's correlation coefficient (PC). Dots represent individual roots. Horizontal lines represent the median,* = $p < 0.05$, n.s. = $p > 0.05$ by Student's t-test, n>7*

To investigate cytokinin effect on localization and expression of ARA6 and ARA7, a dual marker line expressing *ARA6-VENUS,mRFP-ARA7* (Ebine et al., 2011) was observed after mock and cytokinin treatment.

5-days-old seedlings were transferred on the media supplemented with DMSO/2µM tZ and incubated overnight in darkness. The border between the meristematic and the transition zone of the primary root meristem was observed. In mock conditions, ARA6-VENUS and mRFP-ARA7 show partial co-localization at punctate structures in epidermal cells of the primary root meristem (Figure 12A, as reported Ebine et al., 2011). Upon cytokinin treatment, the mRFP-Ara7 signal accumulates in vacuoles. Noteworthy, the vacuolar signal of mRFP-Ara7 appears mainly in the meristematic zone (Figure 12A, white arrows), in contrast to cells in the transition zone (Figure 12A, yellow arrows). We did not observe vacuolar accumulation of ARA6-VENUS upon cytokinin treatment compared to mock as strong as in case of mRFP-ARA7 (Figure 12A). It is unlikely that this difference could be attributed to different fluorescent tags, because seedlings were incubated overnight in darkness, which allows visibility of green fluorophores in vacuoles (Kleine-Vehn et al., 2008; Tamura et al., 2003). Moreover, vacuolar GFP signal is clearly visible in the same experimental set with PIN1-GFP line (Figure 4).

To characterize the cytokinin effect on Rabs quantitatively, we performed analysis of the ARA6-VENUS and mRFP-ARA7 co-localization (French et al., 2008). The level of co-localization between the VENUS- and mRFP- tagged Rabs is expressed as the Pearson's correlation coefficient (PC) that ranges from -1 to +1, 0 indicating no discernible correlation and -1 and +1 meaning strong negative and positive correlations, respectively. ARA6 and ARA7 show

partial co-localization with PC=0.72±0.02 in mock conditions (Figure 12). In cytokinin treated samples, the co-localization significantly decreases (PC=0.68±0.02) compared to the DMSO control (Figure 12A, B). This decrease is probably caused by appearance of the mRFP but not VENUS signal in vacuoles.

Next, we tested whether cytokinin effect on mRFP-ARA7 is dependent on VPS9a. It has been already reported that the *vps9a-1* knock-out mutant shows increased cytoplasmic localization of ARA6 and ARA7 (Goh et al., 2007). Moreover, electron microscopy of *vps9a-1* embryos revealed presence of abnormal structures *e.g.* dense vesicles around the Golgi apparatus, small cup- or ring- shaped structures, and autophagosome-like structures compared to wild-type embryos (Goh et al., 2007). The *vps9a-1* mutant is embryo-lethal, hence it is not possible to use it for observations in adult primary root meristem. Therefore, a cross of *vps9a-2*, a knock-down mutant which has compromised function of VPS9a but is still viable and fertile, with dual marker line *ARA6-VENUS,mRFP-ARA7* was generated.

5-days-old *ARA6-VENUS,mRFP-ARA7*/*vps9a-2* seedlings were transferred on media supplemented with DMSO/2µM tZ and incubated overnight in darkness. In mock conditions, the subcellular pattern of ARA6-VENUS and mRFP-ARA7 is strikingly different in the *vps9a-2* background from Col-0. Firstly, the signal of mRFP-ARA7 is lower than in the Col-0 background whereas the ARA6-VENUS signal is comparable between *vps9a-2* and Col-0. Secondly, there is indeed large portion of both, ARA6 and ARA7 in the cytoplasm of *vps9a-2* when compared to the Col-0 background. Thirdly, both ARA6 and ARA7 co-localize in the ellipse-shaped structures of similar size in *vps9a-2*, whereas in Col-0 they form pattern of various sizes and only partial co-localization (Figure 12A, Figure S12 for more detail). Upon cytokinin treatment, mRFP-ARA7 does not accumulate in vacuoles in the *vps9a-2* mutant, in contrast to Col-0

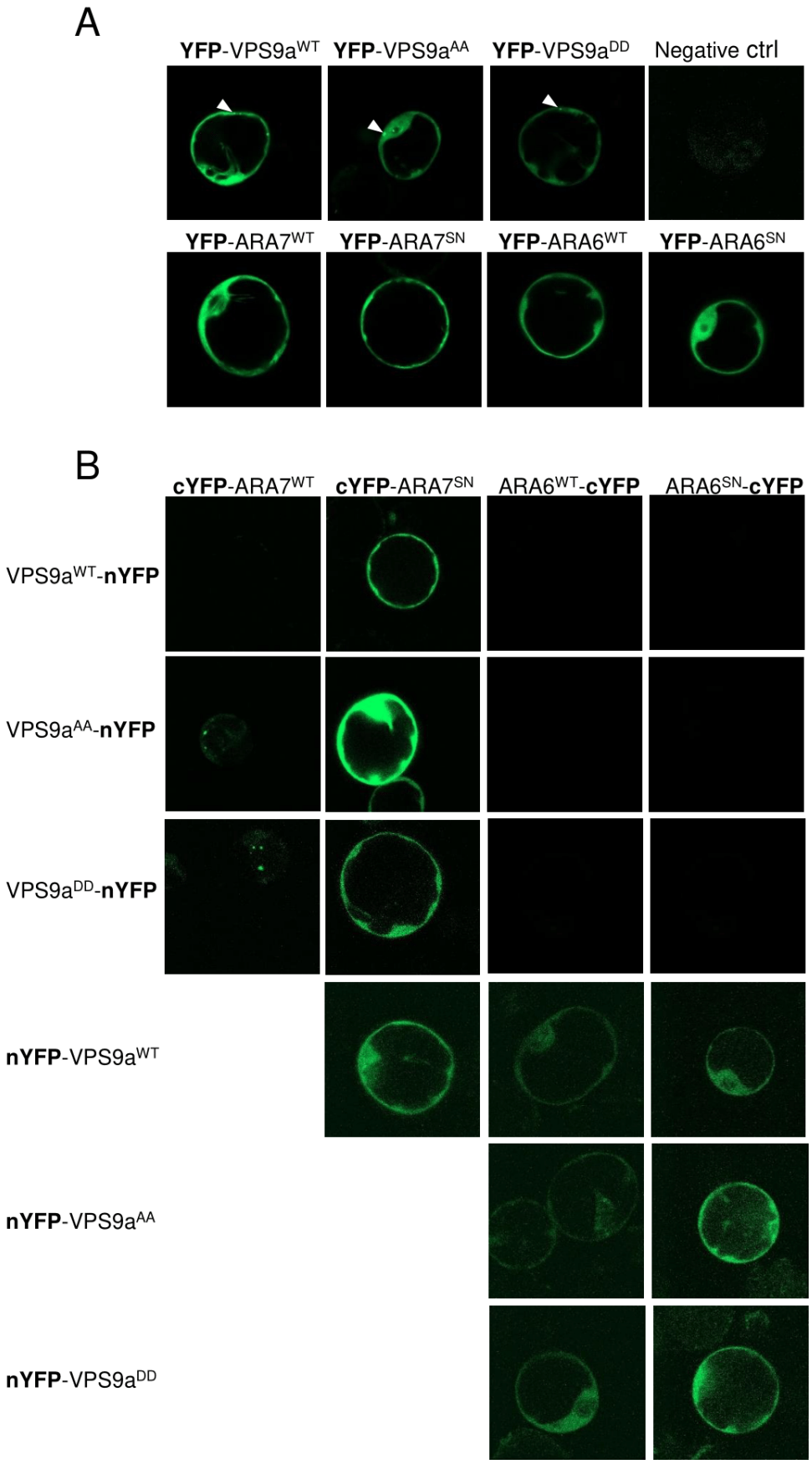

Figure 14: Bimolecular Fluorescence Complementation assay (BIFC) testing interaction of VPS9a$^{WT/AA/DD}$ with ARA6 and ARA7 *(description on the next page)*

Figure 14: Bimolecular Fluorescence Complementation assay (BIFC) testing interaction of VPS9a$^{WT/AA/DD}$ with ARA6 and ARA7 *(A) Protoplasts transformed with ARA6 and ARA7 and VPS9a$^{WT/AA/DD}$ fused with full YFP (positive controls) driven by CaMV35S promoter and transformed root protoplasts (negative control, ctrl) (B) Test of VPS9a$^{WT/AA/DD}$ with GDP-fixed ARA6^{SN} and ARA7^{SN} interaction. ARA6^{SN} interacts with nYFP-VPS9a$^{WT/AA/DD}$ but not VPS9a$^{WT/AA/DD}$-nYFP. ARA7 interacts with both nYFP-VPS9a$^{WT/AA/DD}$ and VPS9a$^{WT/AA/DD}$-nYFP.*

0.56±0.06). This is surprising given evident localization of both ARA6 and ARA7 to ellipse-shaped structures in *vps9a-2*. However, the microscopy images of ARA7 as well as the co-localization plots (red channel) show less ARA7 signal in *vps9a-2* than in Col-0, which might account for lower co-localization values in vps9a-2 mutant background compared to Col-0 (Figure 12A). Upon cytokinin treatment, the co-localization of ARA6 and ARA7 does not significantly decrease in *vps9a-2* mutant background (Figure 12B).

Altogether, in Col-0 background cytokinin treatment promotes higher accumulation of the mRFP-ARA7 than of ARA6-VENUS signal in vacuoles of root epidermal cells. This effect is more pronounced in meristematic cells than in the cells at the transition zone. In the *vps9a-2* mutant compared to Col-0, there is a larger pool of the ARA6 and ARA7 that is not recruited to any membrane, which results in increased cytoplasmic signal. Additionally, the ARA6 and ARA7 pool that is recruited to the membrane marks ellipse-shaped compartments, which are based on literature presumably cis-Golgi apparatus (Uemura et al., 2004). Finally, cytokinin does not promote accumulation of mRFP-ARA7 in vacuoles in the *vps9a-2* mutant background.

Impact of VPS9 phosphorylation on interaction with ARA6 and ARA7

VPS9a interacts with its downstream substrates in order to exchange the GDP for GTP (Goh et al., 2007). Different domains of the VPS9a protein are involved in the interaction with different Rabs. In particular, the C-terminal part of the VPS9a was shown to be important for the interaction with ARA6 but not ARA7 or RHA1 (Sunada et al., 2016). Since the phosphorylation site identified in our screen is at the C-terminus of the Vps9a protein, we hypothesized that it might affect the affinity of VPS9a to ARA6 and/or ARA7.

The Bimolecular Fluorescence Complementation (BIFC, Kudla & Bock, 2016) assay in root-derived protoplast cell culture was implemented to examine the impact of VPS9a phosphorylation status on the interaction with downstream Rabs.

As positive controls, *VPS9a^{WT}, VPS9a^{AA}, VPS9a^{DD}*, *ARA6*, and *ARA7* fused with sequence encoding full *YFP* on N-terminus were used. Their transient expression in protoplasts resulted in expected fluorescence signal in the cytoplasm with occasional localization to punctate structures (white arrows) (Ueda et al., 2001). We did not observe evident difference in subcellular localization pattern between the wild type and the phosphomutant variants of VPS9a (Figure 13A). To test the interaction, *VPS9a^{WT}/VPS9a^{AA}/VPS9a^{DD}* fused on N- and C-terminus with N-terminal part of YFP (nYFP) were co-expressed with complementary *ARA6-cYFP* and cYFP-*ARA7.*

Since VPS9a is a Rab GEF, it preferentially interacts with GDP-fixed Rabs, ARA6^{SN} and ARA7^{SN}, than with wild-type or GTP-fixed versions of these Rabs. (Goh et al., 2007; Sunada et al., 2016). We confirmed that VPS9a indeed preferentially interacts with GDP-locked Rabs (ARA6^{SN}, ARA7^{SN}) more than with their wild type variants (ARA6^{WT}, ARA7^{WT}, Figure 13B). Therefore, for testing the interactions of wild-type and phosphomutants variants of VPS9a, GDP-fixed versions of ARA6 and ARA7 were used. We found that VPS9a^{WT}-nYFP interacts with ARA7^{SN} but not with ARA6^{SN}. Interestingly, when the nYFP tag was placed on the N-terminus of VPS9a, the interaction with ARA6^{SN} was restored (Figure 13B). This corroborates the role of intact C-terminus of VPS9a in the interaction with ARA6 but not ARA7, as it was reported previously (Sunada et al., 2016). However, we did not observe apparent and consistent differences in interaction of VPS9a^{WT}, VPS9a^{AA} or VPS9a^{DD} variants with either ARA6 or ARA7.

Altogether, we observed that the interaction between VPS9a^{WT}/VPS9a^{AA}/VPS9a^{DD} and ARA6^{SN} depends on the position of the nYFP fusion with VPS9a. Although, we did not find an obvious difference between the VPS9a^{WT}/VPS9a^{AA}/VPS9a^{DD} in interaction with ARA7^{SN} or ARA6^{SN} using BIFC approach, this result is not fully conclusive. The BIFC in protoplasts is an artificial overexpression system, which might not be representative enough of the VPS9a-Rab native interactions. Therefore, further interaction assays as co-immunoprecipitation or FRET/FLIM using stably transformed plants with VPS9a and Rabs expressed under endogenous promoters should be performed.

Discussion

Cytokinin and auxin are fundamental regulators of plant growth and development (Schaller et al., 2015). One of their cross-talk points is cytokinin regulation of auxin transporters, PINs.

This regulation occurs on the transcriptional level (Ruzicka et al., 2009; Šimášková et al., 2015) but also in a transcription-independent manner where cytokinin modulates plant development by promoting vacuolar degradation of PIN1 (Marhavý et al., 2011, 2014).

In this study, we investigated the potential mechanism of cytokinin activity on a post-translational level. In the screen for proteins with altered phosphorylation upon cytokinin treatment, we identified multiple proteins involved in various steps of the vesicular trafficking. Among others, GTPase involved in endocytosis, DRP2A, or Rab GEF VPS9a and ATG13 involved in vacuolar and autophagy degradation, respectively, (Table 1, Goh et al., 2007; Suttangkakul et al., 2011; Taylor, 2011). We chose VPS9a for further characterization of its role in the cytokinin regulated plant development for multiple reasons. VPS9a is a GEF that activates Rabs on the interface of plasma membrane and vacuolar trafficking and it was previously reported to participate in the trafficking of PIN1 (Goh et al., 2007). VPS9a phosphorylation shows fast response (within 15 minutes) to cytokinin treatment (Figure1). Moreover, the *vps9a-2* knock-down mutant exhibits short root phenotype (Goh et al., 2007) with overproduction of lateral roots, indicative of auxin-cytokinin cross-talk disbalance. Therefore, VPS9a is a suitable candidate to be a player in cytokinin effect on PIN1 trafficking.

We found that VPS9a is necessary for a part of cytokinin response because *vps9a-2* plants exhibit lower sensitivity to the hormone in multiple phenotypic aspects like root growth, root meristem size, lateral root density, of root hairs abundance and rosette size (Figure2, Figure3). Moreover, in some assays, cytokinin even promotes the growth of the *vps9a-2* mutant compared to mock conditions (Figure S2B, S3C). The reduced sensitivity of the primary root growth seems to be specific to cytokinin since the *vps9a-2* roots remain sensitive to ethylene precursor (Figure S3). Importantly, *vps9a-2* mutant was resistant to cytokinin-mediated PIN1 degradation, which clearly supports the role of VPS9a in regulation of PIN1 trafficking by cytokinin (Figure 4, S4).

The reduced sensitivity of *vps9a-2* to cytokinin is not caused by a deficiency in the cytokinin signaling, because *vps9a-2* does not show impairment in expression of markers of early cytokinin response *ARR5* and *ARR16*. Actually, the *vps9a-2* mutant shows even higher levels of *ARR5* and *ARR16* induction upon cytokinin treatment than Col-0, which hints at increased

sensitivity to exogenous cytokinin treatment of the canonical cytokinin pathway in *vps9a-2* mutant.

Upon cytokinin treatment, we detected a mild increase in signal intensity or size of the membrane structures marked with *VPS9a::VPS9a-GFP* signal (Figure 6). Therefore, by rapidly changing its phosphorylation, cytokinin might promote VPS9a association with membranes which results in higher activation of the downstream Rabs, hence increased vacuolar trafficking of proteins including PIN1.

In our model, cytokinin affects the phosphorylation of VPS9a and by that it regulates VPS9a interactions with its downstream Rabs. This results in preferential activation canonical Rabs, hence promotion of vacuolar trafficking pathway. Indeed, cytokinin treatment increases the vacuolar signal of mainly mRFP-ARA7 and not ARA6-VENUS which suggests that it selectively affects ARA7- mediated trafficking pathway to vacuole. This effect is *vps9a-2* dependent, since there is no increase of the mRFP-ARA7 in the vacuole in the *vps9a-2* mutant background.

While there is general importance of the Rab GTPases in the cytokinin effect on the primary root growth (Figure 11), the protoplast assay did not reveal a function of VPS9a phosphorylation in interaction between VPS9a and ARA6 and ARA7. However, interaction of Rab GTPases with phosphodead and phospho mimic version of VPS9a should still be tested using the stably transformed *VPS9a::VPS9a*$^{WT/AA/DD}$*-GFP* lines.

Conclusion and future perspectives

In this study, we discovered that part of the cytokinin effect on plant growth and development occurs through VPS9a protein. In particular, VPS9a is essential for the process of cytokinin mediated PIN1 degradation. We found that cytokinin might influence the phosphorylation of VPS9a. There are ongoing experiments of verification of the phosphosite by independent method (PhosTag). We are working on establishing a targeted phosphoproteomic method (Rauniyar, 2015) to confirm the effect of cytokinin on the VPS9a phosphorylation.

In the future, we plan to continue with characterization of multiple VPS9a$^{AA/DD}$ transgenic lines in terms of their role in plant growth and development. Importantly, their ability to interact with ARA6 and ARA7 as well as sensitivity to cytokinin-triggered PIN1 degradation. Morphological and biochemical analysis combined with in-detail microscopy imaging of the

VPS9a$^{AA/DD}$ lines will shed more light on the cytokinin effects on the vesicular trafficking through VPS9a.

Supplementary figures

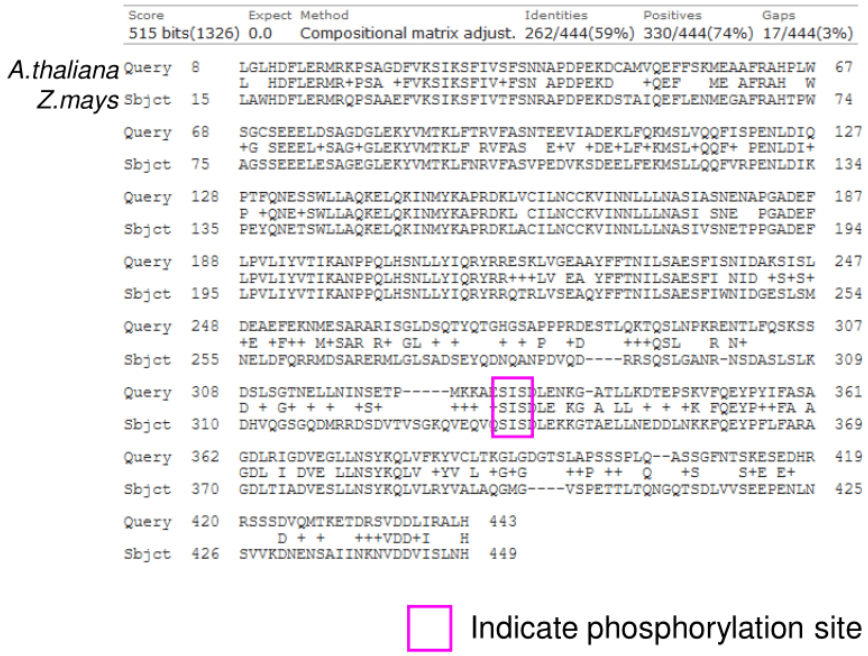

Figure S1: Alignment of VPS9a protein in Arabidopsis thaliana and Zea mays
Pink rectangle marks the peptide identified in our study. The phosphorylation site is conserved between Arabidopsis and maize. Source: https://blast.ncbi.nlm.nih.gov/Blast.cgi

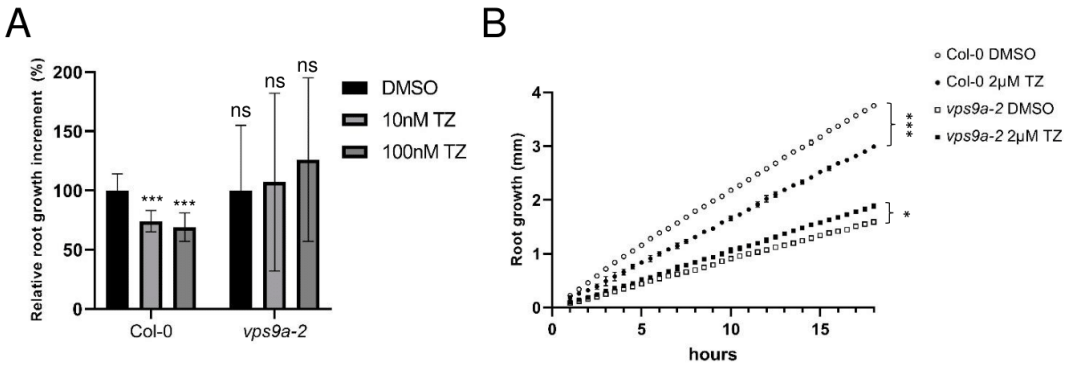

Figure S2: vps9a-2 mutant shows reduced sensitivity to cytokinin *(A) Quantification of primary root length increment of 7-days-old plants over 12 hours on media supplemented with DMSO or various concentrations of tZ (10nM, 100nM). (B) Quantification of the root growth kinetics of 7-days-old plants on media supplemented with DMSO or 2µM tZ for 18 hours. The bars and points represent mean ± s.d., *** = p < 0.001, n.s. = p > 0.05 by Student's t-test, n > 10.*

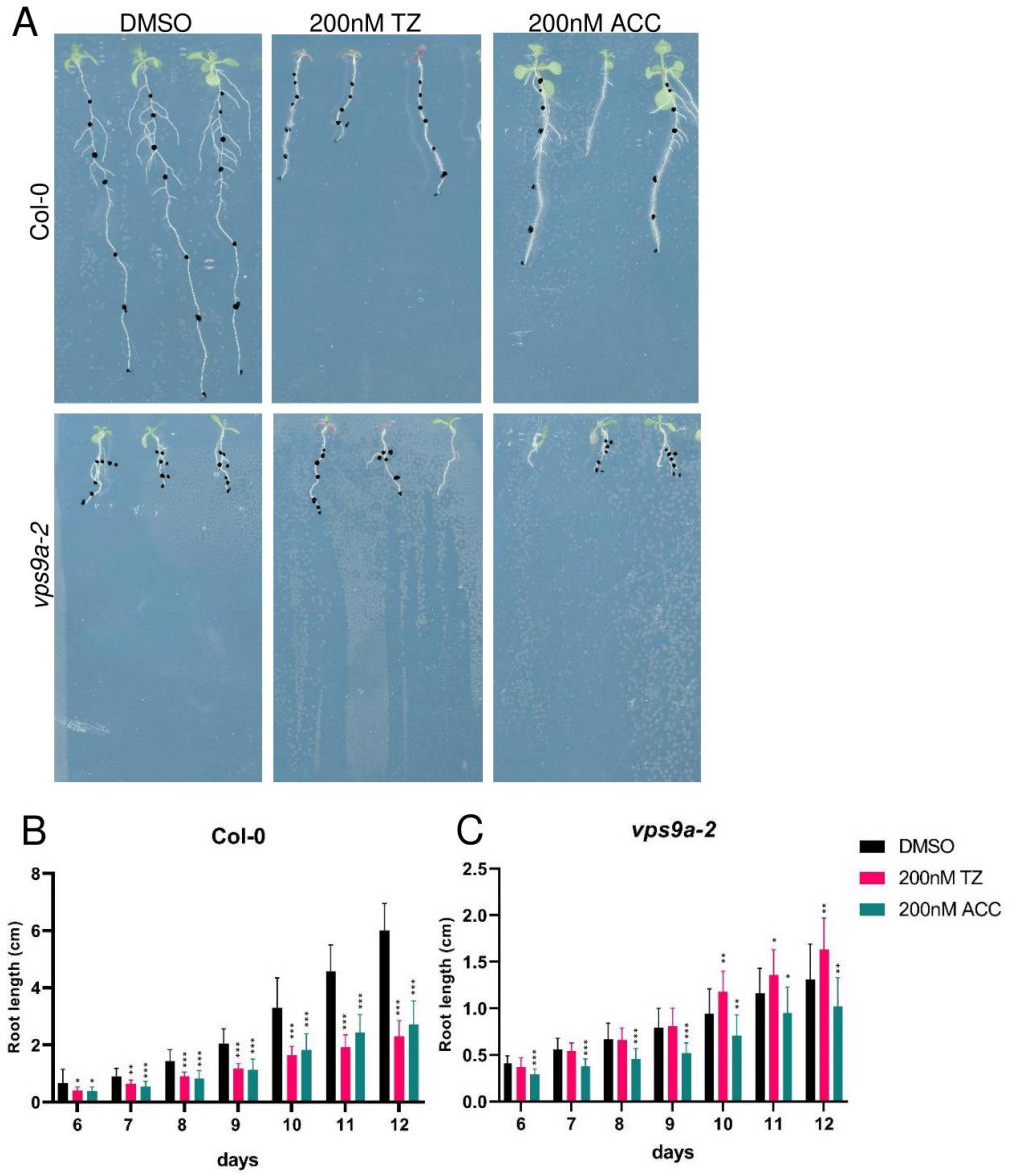

Figure S3. vps9a-2 retains sensitivity to precursor of ethylene *(A)12-days-old Col-0 and vps9a-2 plants grown on 200nM tZ and 200nM ACC. (B,C) Quantification of the primary root growth of Col-0 (B) and vps9a-2 (C) plants grown on DMSO, 200nM tZ and 200nM ACC. The bars represent mean ± s.d., *** = p < 0.001, ** = p< 0.01, * = p< 0.05, by Student's t-test, n > 10. (B, C)*

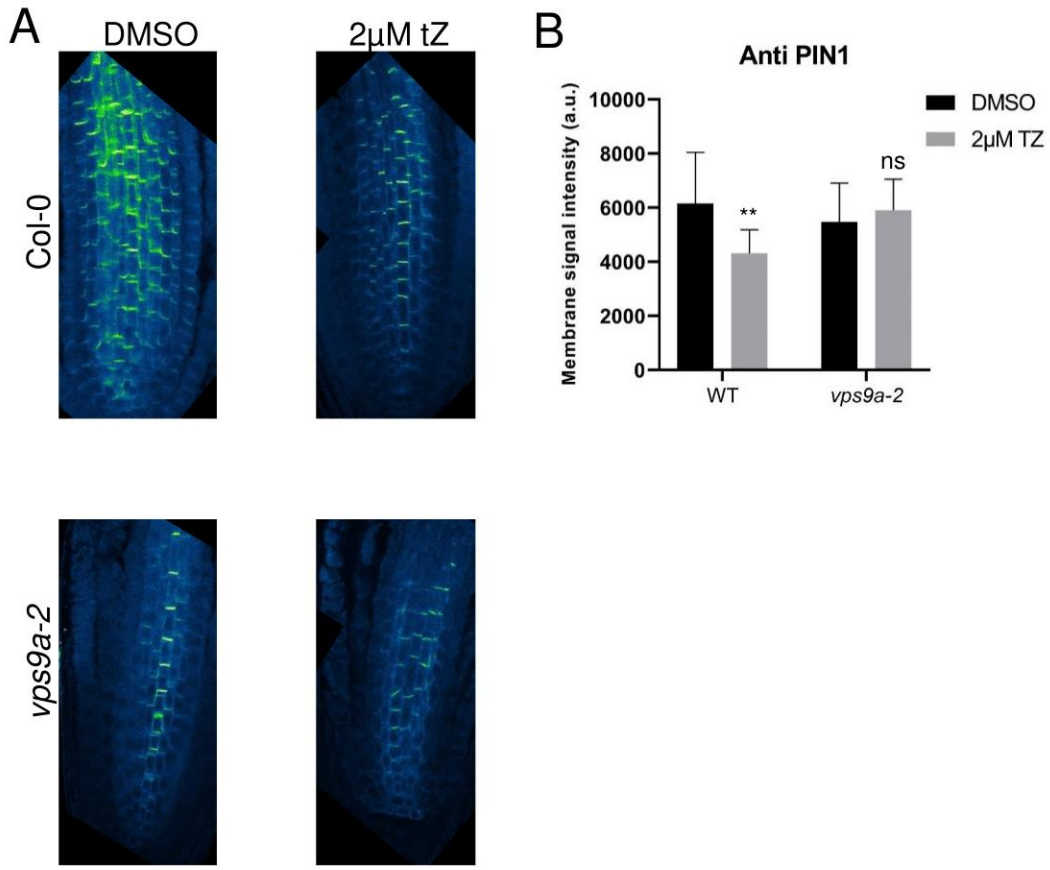

Figure S4: Anti-PIN1 immunostaining of Col-0 and vps9a-2 primary root meristem *Representative images of PIN1 in root apical meristem detected by immunostaining. 6-days-old seedlings were transferred on media containing mock or 2µM tZ for 20 hours, fixed and immunostained with anti-PIN1 antibody. (B) Quantification of the total PIN1 signal in the stele. The bars represent mean ± s.d., ** = p < 0.01, n.s. = p > 0.05 by Student's t-test, n > 11. (B), a.u.= arbitrary units*

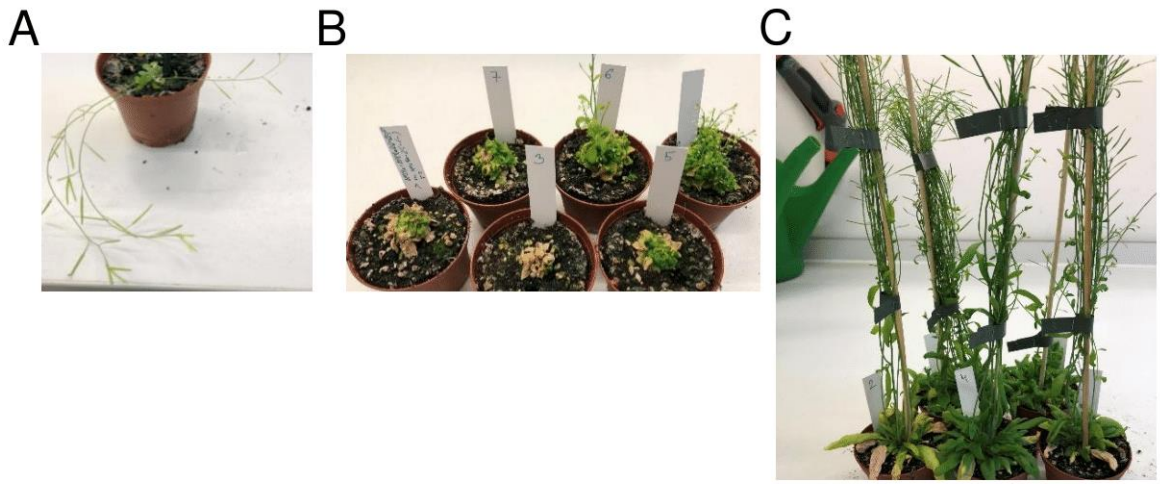

Figure S9: T1 generation of wild type (VPS9a^{WT}), phospho-dead (VPS9a^{AA}), and the phospho-mimic (VPS9a^{DD}) Arabidopsis thaliana transgenic lines *(A) vps9a-2 mutant (B)Example of T1 plants with bushy phenotype or without ability to form a stem. (C) Example of wild-type-looking T1 plants. Plants are 8-weeks-old.*

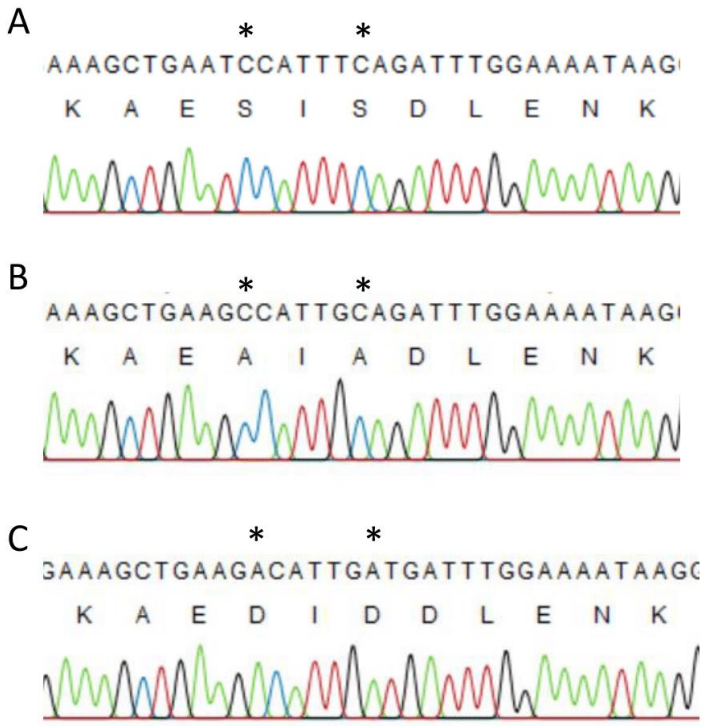

Figure S10: Sequencing reads of wild type (VPS9a^{WT}), phospho-dead (VPS9a^{AA}), and the phospho-mimic (VPS9a^{DD}) Arabidopsis thaliana transgenic lines *Close-up on the sequence for the peptide identified in the phosphoproteomics. (A) VPS9a::VPS9a^{WT}-GFP/vps9a-2 (B) VPS9a::VPS9a^{AA}-GFP/vps9a-2 (C) VPS9a::VPS9a^{DD}-GFP/vps9a-2. Stars mark the position of mutated amino acids.*

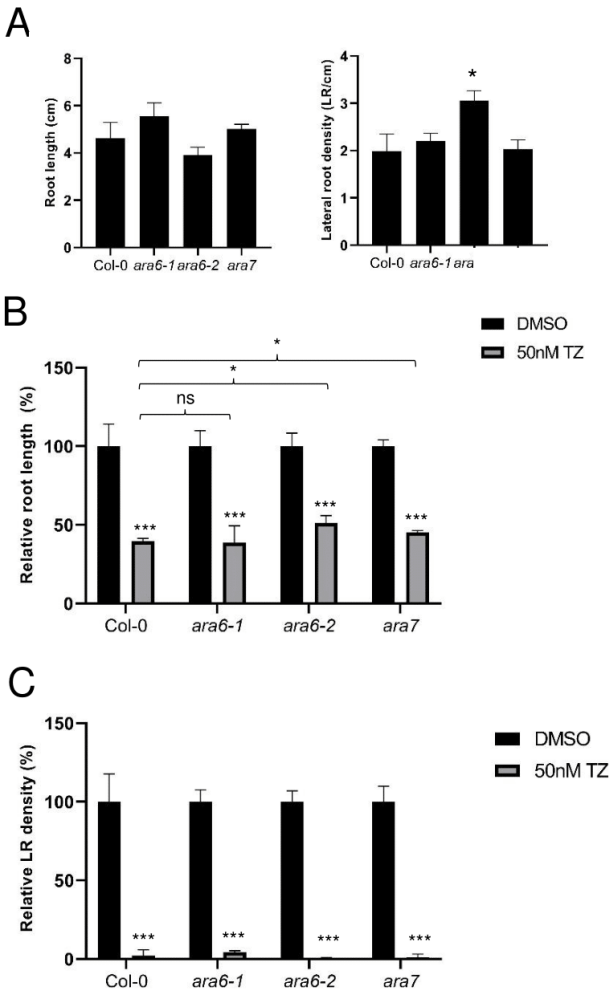

Figure S11: Cytokinin sensitivity of ara6-1, ara6-2, and ara7 *(A) Quantification of the primary root length and lateral root density of 11-days-old plants grown on the medium supplemented with DMSO or 50nM tZ. (B) Relative root length of 11-days-old Col-0, ara6-1, ara6-2, and ara7 plants grown on the medium supplemented with DMSO or 50nM tZ. (C) Relative lateral root density of 11-days-old Col-0, ara6-1, ara6-2, and ara7 plants grown on the medium supplemented with DMSO or 50nM tZ. The bars represent mean ± s.d., *** = p < 0.001, * = p < 0.05, n.s. = p > 0.05 by Student's t-test, n > 10.*

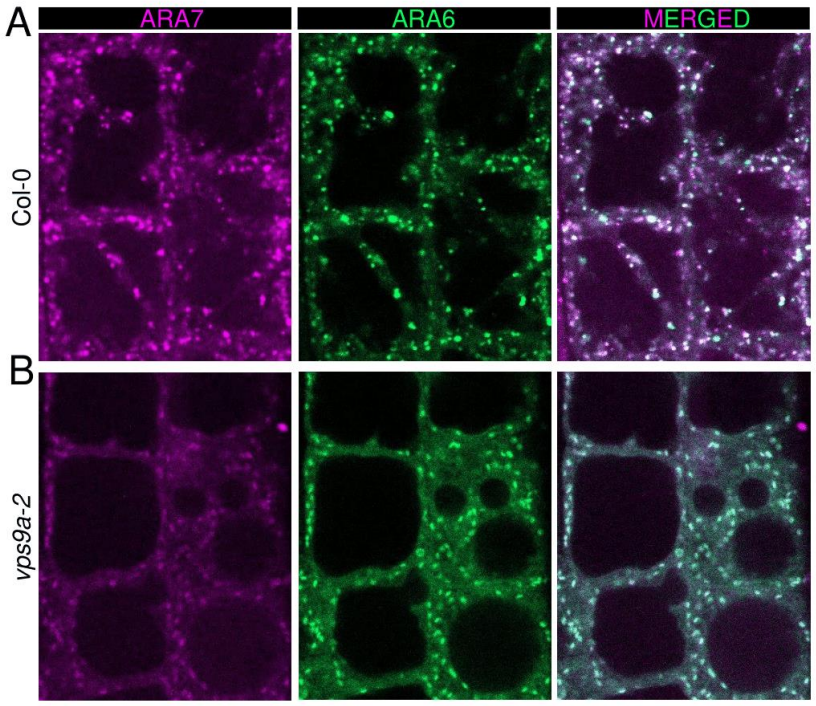

Figure S12: Subcellular localization of ARA6-VENUS and mRFP-ARA7 in the Col-0 and vps9a-2 background *Epidermal cells of primary root meristem expressing ARA6-VENUS mRFP-ARA7. (A) Col-0 background (B) vps9a-2 background*

Supplementary tables

Transformants:

Key characteristic	Project identification label	Construct	Notes to Construct	Backgroud	Generation
VPS9aWT control	T_VPS9_7_3	VPS9a::VPS9aWT-GFP	genomic DNA, from T.Ueda	vps9a-2 (-/-)	T2
VPS9aAA phoshpodead	T_VPS9_8_3	VPS9::VPS9aAA-GFP	genomic DNA, from T.Ueda, mutated by me	vps9a-2 (-/-)	T2
VPS9aDD phosphomimic	T_VPS9_12_2	VPS9a::VPS9aDD-GFP	genomic DNA, from T.Ueda, mutated by me	vps9a-2 (-/-)	T2

Crossess:

Key characteristic	Project identification label	Gene 1	Gene 2	Gene 3	Generation
vps9a-9	VPS9_35	vps9a-2(-/-)	PIN2::PIN1-GFP2 (+/+)	eir1-1(+/+)	F5
vps9a-9	VPS9_36	vps9a-2(+/+)	PIN2::PIN1-GFP2 (+/+)	eir1-1(+/+)	F4
vps9a-9	VPS9_37	vps9a-2(-/-)	PIN2::PIN2-GFP (+/+)	eir1-4(+/+)	F5
vps9a-9	VPS9_38	vps9a-2(+/+)	PIN2::PIN2-GFP (+/+)	eir1-4(+/+)	F4
vps9a-9	VPS9_10	vps9a-2(-/-)	ARA6-VENUS/mRFP-ARA7 (shine)	_	F4
vps9a-9	VPS9_11	vps9a-2(+/+)	ARA6-VENUS/mRFP-ARA7 (shine)	_	F4

Supplemental Table 1: Transformants and Crosses of *Arabidopsis thaliana* generated in this study

	Name	Sequence	Reference
Border primers			
	SALK_LBa1	TGGTTCACGTAGTGGGCCATCG	http://signal.salk.edu/tdnaprimers.2.html
	GABI_LB	ATATTGACCATCATACTCATTGC	https://www.gabi-kat.de/faq/confirmation-strategy.html
	WiscDsLox_p745	AACGTCCGCAATGTGTTATTAAGTTGTC	
	SAIL_LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC	http://signal.salk.edu/tdnaprimers.2.html
Gene specific primers			
	vps9a-2_RP	TTGGAATCACGTTCTTCATCC	SALK primer generator
	vps9a-2_LP	GCTCCTCGTGATAAGCTTGTG	SALK primer generator
	vps9a-1_RP	CTCGTTTGAAGCAATTGAAGC	SALK primer generator
	vps9a-1_LP	GTCCCATTTCTCCCTCTTTTG	SALK primer generator
	ara7_F	CCTGCGATTCTCTTCAGATCGATA	Ebine et al., 2011
	ara7_R	GCTATTACACACACATAGCTCAAC	Ebine et al., 2011
	ara6-1,-2_F	CAATTATCATCTATTCAGGGGTAAG	Ebine et al., 2011
	ara6-1,-2_R	ACGATTCAGGGCTTGTTATAT	Ebine et al., 2011
Mutagenesis primers			
Genomic DNA	QCh_VPS9a_AA_F	GAAACACCAATGAAGAAAGCTGAAGCCATTGCAGATTTGGAAAATAAGGG	
	QCh_VPS9a_AA_R	CCCTTATTTTCCAAATCTGCAATGGCTTCAGCTTTCTTCATTGGTGTTTC	
	QCh_VPS9a_DD_F	CAGTGAAACACCAATGAAGAAAGCTGAAGACATTGATGATTTGGAAAATAAGGGTGCGACGCTTC	
	QCh_VPS9a_DD_R	GAAGCGTCGCACCCTTATTTTCCAAATCATCAATGTCTTCAGCTTTCTTCATTGGTGTTTCACTG	
cDNA	II_VPS9a_AA_F	GAAGAAAGCTGAAgccATTgcaGATTTGGAAAATAAGG	
	II_VPS9a_AA_R	TATTTTCCAAATCtgcAATggcTTCAGCTTTCTTCATT	
	II_VPS9a_DD_F	GAAGAAAGCTGAAgacATTgacGATTTGGAAAATAAGG	
	II_VPS9a_DD_R	TATTTTCCAAATCgtcAATgtcTTCAGCTTTCTTCATT	

Supplemental Table 2: Primers used/generated in this study

Methods

Plant material

Arabidopsis thaliana plants, ecotype Col-0 was used as WT control in all experiments. Mutant lines *vps9a-1* (SALK_018174), *vps9a-2 (GABI_557C02), ara6-1*(SAIL_880_C07)*, ara6-2*(SAIL_98_E08)*, and ara7* (WiscDsLox355B06) were described before (Goh *et al.*, 2007; Ebine *et al.*, 2011) and were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Transgenic lines PIN2::PIN1GFP2 (Wisniewska *et al.*, 2006), PIN2::PIN2-GFP(Abas *et al.*, 2006) were found in laboratory stock. VPS9a::VPS9a-GFP (Sunada *et al.*, 2016) and ARA6-Venus mRFP-ARA7 was kindly provided by Dr.T. Ueda and Dr.K.Ebine.

The following lines were generated in this study: PIN2PIN1GFP2/*vps9a-2*, PIN2PIN1GFP2/*vps9a-2*, ARA6-Venus mRFP-ARA7/vps9a-2,VPS9a::VPS9a^{WT}-GFP/vps9a-2,vps9a-1,Col-0;VPS9a::VPS9a^{AA}-GFP/vps9a-2,vps9a-1, Col-0; VPS9a::VPS9a^{DD}-GFP/vps9a-2, vps9a-1, Col-0. All information about seeds can be found in the Supplemental table 1

Vector construction and mutagenesis

For stable transformation of *Arabidopsis thaliana*, VPS9a::VPS9a^{AA}-GFP and VPS9a::VPS9a^{DD}-GFP constructs were generated by site-directed mutagenesis of phosphorylation sites which led to amino acid substitution of serine 330 and 332 with alanine (VPS9a^{AA}) or aspartic acid (VPS9a^{DD}). The mutagenesis was performed on entry vector VPS9a::VPS9a-GFP in pENTR/D-TOPO (Sunada *et al.*, 2016, a kind gift from Dr T.Ueda and Dr. K.Ebine) using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). Using the Gateway system (Thermo Fisher Scientific), VPS9a::VPS9a^{AA}-GFP, VPS9a::VPS9a^{DD}-GFP and non-mutated VPS9a::VPS9a^{WT}-GFP were cloned into pBGW (Karimi et al., 2002) expression vector.

For transient expression in *Nicotiana benthamiana*, WT cDNA of VPS9a was subjected to site directed mutagenesis of phosphorylation sites using PCR amplification resulting in VPS9a^{AA} and VPS9a^{DD}. Using Gateway system (Thermo Fisher Scientific), VPS9a^{WT}, VPS9a^{AA}, and VPS9a^{DD} were cloned into pDONR221 and subsequently to the expression vector pB7FWG2 (Karimi et al., 2002). All mutagenesis primers are listed in Supplemental table 2.

For transient expression in protoplasts, cDNA of VPS9a^{WT}, VPS9a^{AA}, and VPS9a^{DD} were cloned into Gateway BiFC expression vectors pSAT4(A)-DEST-n(1-174)EYFP-N1 (pE3134) (C-terminal nYFP) and pSAT4-DEST-n(1-174)EYFP-C1 (pE3136) (N-terminal nYFP). As interactors, cDNAs of ARA6^{WT}, ARA6^{SN}, and ARA6^{QL} were cloned into expression vector pSAT5(A)-DEST-c(175-end)EYFP-N1 (pE3132) (C-terminal cYFP) and cDNAs of ARA7^{WT}, ARA7^{SN}, and ARA7^{QL} were cloned into expression vectors pSAT5-DEST-c(175-end)EYFP-C1(B) (pE3130) (N-terminal cYFP). All genes were also cloned with full YFP into p2YGW7 (N-terminal YFP) as a positive control.

Growth conditions

Surface sterilized *Arabidopsis thaliana* seeds were plated on half strength Murashige and Skoog (MS) media (Duchefa) with 1%(w/v) sucrose and 1% (w/v) agar (pH=5.9). Seeds were stratified for 2 days at 4°C, in the dark. Seedlings were grown vertically in long-day conditions (16h light/8h dark) at 21°C. *N. benthamiana* plants were grown at 21°C under a long-day photoperiod (16-h light/8-h dark) for 6 weeks. As light sources light emitting diode production

modules (Philips GreenPower) were used in a deep red, far red, blue combination with a photon density of 140 µmol/m^2/s ± 20%.

Plant transformation

For stable transformation of *Arabidopsis thaliana*, the constructs were transformed to Col-0, *vps9a-1*, and *vps9a-2* plants using floral dip method (Clough and Bent, 1998).

For transient transformation of *Nicotiana benthamiana, Agrobacterium tumefaciens* strain GV3101 harboring plasmids with the targeted gene and p19 strain were grown in LB broth with the appropriate antibiotics at 28°C overnight. The agrobacterium cells were harvested and resuspended in infiltration solution (10 mM of MES (Duchefa) at pH 5.6, 10 mM of $MgCl_2$, and 200 µM of acetosyringone) until OD_{600} = 0.3. Suspension of agrobacterium carrying desired plasmide was infiltrated together with the p19 strain into *N*. *benthamiana* leaf epidermal cells.

Genotyping and RT-QPCR

All primers used for genotyping are listed in Supplemental Table 2 Primers were used for genotyping and RT-PCR of *Col0, drp2a-1 and drp2b-2*. RNA was extracted from 4-d–old seedlings with Monarch Total RNA Miniprep extraction kit (NEB), and cDNA was synthetized with an iScript cDNA kit (Bio-Rad). QPCR was performed with gene-specific primers, and the housekeeping gene *PP2A* was used as the internal control. QPCR was performed with gene-specific primers for *ARR5* (ARR5_F: AGTTCGGTTGGATTTGAGGATCTG, ARR5_R: TCCAGTCATCCCAGGCATAGAG) and *ARR16* (ARR16_F: CGTAAACTCGTTGAGAGGTTGCTC, ARR16_R: GCATTCTCTGCTGTTGTCACTTTG

Hormonal treatment

All treatments were performed on half strength Murashige and Skoog (MS) media (Duchefa) with 1%(w/v) sucrose and 1% (w/v) agar (pH=5.9). For long term experiments the plants were grown on the media supplemented with indicated concentrations of trans-Zeatin (tZ**,** Sigma) or DMSO. Short term treatments were performed by transfer of plants on media supplemented with indicated concentration of tZ or DMSO.

Fluorescent imaging of transgenic seedlings by confocal microscopy

5-6 days old seedlings were mounted into a microcopy chamber (Nunc™ Lab-Tek™ Chambered Coverglass, Thermo Fisher Scientific). Confocal images were obtained with LSM 800 laser scanning confocal microscopes (Zeiss) equipped with a Plan-Apochromat 40x/1.2 water immersion objective. Fluorescence markers were excited at 488nm (GFP, YFP, Venus) or with 512nm (RFP, Propidium iodide).

Propidium iodide (PI) treatment

80 µl of PI (1mg/ml, Sigma) was applied on agar block (55x24mm) and incubated for 5 min. 5-6 days old seedlings were transferred on the agar block, placed into microscopy chamber (Nunc™ Lab-Tek™ Chambered Coverglass, Thermo Fisher Scientific), and observed (Zeiss LSM 800).

Protoplast PEG-mediated transfection

3-days-old *Arabidopsis* root suspension cell culture was treated with enzyme solution (1% cellulose (Serva), 0.2% macerozyme (Yakult), in 0.34 M glucose-mannitol (GM) solution (2.2 g MS with vitamins, 15.25 g glucose, 15.25 g mannitol, pH to 5.5 with KOH) with slight shaking for 3–4 h. The suspension was gently centrifuged (1000×*g*, 5 min). The pellet was washed with GM, concentrated on a sucrose gradient, and resuspended in GM solution to a final concentration of 4×10^6 protoplasts/ml. The plasmid DNA was isolated with E.Z.N.A.® Plasmid DNA Maxi Kit (Omega Biotek). 5-10 µg of DNA was gently mixed together with 50 µl of protoplast suspension and 150µl of PEG solution and incubated in darkness for 30 min. 140 µl of 0.275 M $Ca(NO_3)_2$ was added, centrifuged (900×g, 1min). The pellet was dissolved in 500µl of GM and incubated in darkness at room temperature for 16h.

Bimolecular Fluorescence Complementation (BiFC)

The genes encoding $VPS9a^{WT}$, $VPS9a^{AA}$, $VPS9a^{DD}$, $ARA6^{WT}$, $ARA6^{SN}$, $ARA6^{QL}$ $ARA7^{WT}$, $ARA7^{SN}$, and $ARA7^{QL}$ were cloned into BiFC Gateway vectors to generate N- or C- terminal fusion with nYFP (1-174 amino acids) or cYFP (175-end). Corresponding vectors were co-transfected to protoplasts (see above), mounted into microscopy chamber and observed (Zeiss LSM 800).

Transient expression in Nicotiana benthamiana

The leaf epidermal cells of *Nicotiana benthamiana* were infiltrated with suspension of *Agrobacterium tumefaciens* carrying desired plasmid together with the p19 strain. After 72 hours, leaf explants were mounted on a microscopy slide and observed (Zeiss LSM 800).

Protein extraction

Roots of 7-day-old seedlings of were harvested and flesh frozen in liquid nitrogen. Protein were extracted with buffer consisting of 50mM TrisHCl (Sigma), 150mM NaCl, cOMPLETE (Roche) and PhosSTOP (Sigma) tablets for the cytosolic fraction. For extraction of the microsomal fraction, the same buffer was used, with addition of 0.5% Triton (Sigma) and 0.5% NP-40 (Sigma). Extracts were separated by 10% (v/v) SDS-PAGE and bands were detected Anti-GFP-HRP (cat.no. 130-091-833, MACS Molecular) and anti-Actin (cat.no. A0480, Sigma).

Data analysis

Data were analyzed using ImagJ software (National Institute of Health, http://rsb.info.nih.gov/ij), GraphPad Prism 8, and Microsoft PowerPoint programs. Particle analysis was performed using ImageJ function “Analyze Particles.

Statistical Analysis

All data were analyzed using Student’s t test in Microsoft Excel.

Supplemental references:

Abas, L. *et al.* (2006) ‘Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism’, *Nature Cell Biology*, 8(3), pp. 249–256. doi: 10.1038/ncb1369.

Clough, S. J. and Bent, A. F. (1998) ‘Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana’, *Plant Journal*, 16(6), pp. 735–743. doi:

10.1046/j.1365-313X.1998.00343.x.

Ebine, K. *et al.* (2011) 'A membrane trafficking pathway regulated by the plant-specific RAB GTPase ARA6', *Nature Cell Biology*, 13(7), pp. 853–859. doi: 10.1038/ncb2270.

Goh, T. *et al.* (2007) 'VPS9a, the Common Activator for Two Distinct Types of Rab5 GTPases, Is Essential for the Development of Arabidopsis thaliana', *the Plant Cell Online*, 19(11), pp. 3504–3515. doi: 10.1105/tpc.107.053876.

Karimi, M., Inzé, D. and Depicker, A. (2002) 'GATEWAY vectors for Agrobacterium-mediated plant transformation.', *Trends in plant science*, 7(5), pp. 193–195. doi: 10.1016/s1360-1385(02)02251-3.

Sunada, M. *et al.* (2016) 'Functional analyses of the plant-specific C-terminal region of VPS9a: the activating factor for RAB5 in Arabidopsis thaliana', *Journal of Plant Research*, 129(1), pp. 93–102. doi: 10.1007/s10265-015-0760-5.

Wisniewska, J. *et al.* (2006) 'Polar PIN localization directs auxin flow in plants', *Science*, 312(5775), p. 883. doi: 10.1126/science.1121356.

References:

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wiřniewska, J., Moulinier–Anzola, J. C., Sieberer, T., Friml, J., & Luschnig, C. (2006). Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nature Cell Biology*, *8*(3), 249–256. https://doi.org/10.1038/ncb1369

Antoniadi, I., Novák, O., Gelová, Z., Johnson, A., Plíhal, O., Simerský, R., Mik, V., Vain, T., Mateo-Bonmatí, E., Karady, M., Pernisová, M., Plačková, L., Opassathian, K., Hejátko, J., Robert, S., Friml, J., Doležal, K., Ljung, K., & Turnbull, C. (2020). Cell-surface receptors enable perception of extracellular cytokinins. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-17700-9

Behnia, R., & Munro, S. (2005). Organelle identity and the signposts for membrane traffic. *Nature*, *438*(7068), 597–604. https://doi.org/10.1038/nature04397

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., & Friml, J. (2003). Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. *Cell*, *115*(5), 591–602. https://doi.org/10.1016/S0092-8674(03)00924-3

Bezeljak, U., Loya, H., Kaczmarek, B., Saunders, T. E., & Loose, M. (2020). Stochastic activation and bistability in a Rab GTPase regulatory network. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(12), 6504–6549. https://doi.org/10.1073/pnas.1921027117

Bleecker, A. B., & Kende, H. (2000). Ethylene: A Gaseous Signal Molecule in Plants. *Annual Review of Cell and Developmental Biology*, *16*(1), 1–18. https://doi.org/10.1146/annurev.cellbio.16.1.1

Bolte, S. (2004). The N-myristoylated Rab-GTPase m-Rabmc is involved in post-Golgi trafficking events to the lytic vacuole in plant cells. *Journal of Cell Science*, *117*(6), 943–954. https://doi.org/10.1242/jcs.00920

Bottanelli, F., Foresti, O., Hanton, S., & Denecke, J. (2011). Vacuolar transport in Tobacco leaf epidermis cells involves a single route for soluble cargo and multiple routes for membrane cargo. *Plant Cell*, *23*(8), 3007–3025. https://doi.org/10.1105/tpc.111.085480

Bottanelli, F., Gershlick, D. C., & Denecke, J. (2012). Evidence for Sequential Action of Rab5 and Rab7 GTPases in Prevacuolar Organelle Partitioning. *Traffic*, *13*(2), 338–354. https://doi.org/10.1111/j.1600-0854.2011.01303.x

Choudhary, M. K., Nomura, Y., Wang, L., Nakagami, H., & Somers, D. E. (2015). Quantitative circadian phosphoproteomic analysis of Arabidopsis reveals extensive clock control of key components in physiological, metabolic, and signaling pathways. *Molecular and Cellular Proteomics*, *14*(8), 2243–2260. https://doi.org/10.1074/mcp.M114.047183

D'Agostino, I. B., Deruere, J., & Kieber, J. J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiol*, *124*(December), 1706–1717. https://doi.org/10.1104/pp.124.4.1706

De La Fuente Van Bentem, S., Anrather, D., Dohnal, I., Roitinger, E., Csaszar, E., Joore, J., Buijnink, J., Carreri, A., Forzani, C., Lorkovic, Z. J., Barta, A., Lecourieux, D., Verhounig, A., Jonak, C., & Hirt, H. (2008). Site-specific phosphorylation profiling of arabidopsis proteins by mass spectrometry and peptide chip analysis. *Journal of Proteome Research*, *7*(6), 2458–2470. https://doi.org/10.1021/pr8000173

Dello Ioio, R., Linhares, F. S., & Sabatini, S. (2008). Emerging role of cytokinin as a regulator of cellular differentiation. In *Current Opinion in Plant Biology* (Vol. 11, Issue 1, pp. 23–27). https://doi.org/10.1016/j.pbi.2007.10.006

Dello Ioio, R., Linhares, F. S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., & Sabatini, S. (2007). Cytokinins Determine Arabidopsis Root-Meristem Size by Controlling Cell Differentiation. *Current Biology*, *17*(8), 678–682. https://doi.org/10.1016/j.cub.2007.02.047

Di Mambro, R., De Ruvo, M., Pacifici, E., Salvi, E., Sozzani, R., Benfey, P. N., Busch, W., Novak, O., Ljung, K., Di Paola, L., Marée, A. F. M., Costantino, P., Grieneisen, V. A., & Sabatini, S. (2017). Auxin minimum triggers the developmental switch from cell division to cell differentiation in the Arabidopsis root. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(36), E7641–E7649. https://doi.org/10.1073/pnas.1705833114

Durek, P., Schmidt, R., Heazlewood, J. L., Jones, A., MacLean, D., Nagel, A., Kersten, B., & Schulze, W. X. (2009). PhosPhAt: The Arabidopsis thaliana phosphorylation site database. An update. *Nucleic Acids Research*, *38*(SUPPL.1), 828–834. https://doi.org/10.1093/nar/gkp810

Ebine, K., Fujimoto, M., Okatani, Y., Nishiyama, T., Goh, T., Ito, E., Dainobu, T., Nishitani, A., Uemura, T., Sato, M. H., Thordal-Christensen, H., Tsutsumi, N., Nakano, A., & Ueda, T. (2011). A membrane trafficking pathway regulated by the plant-specific RAB GTPase

ARA6. *Nature Cell Biology*, *13*(7), 853–859. https://doi.org/10.1038/ncb2270

Elliott, L., Moore, I., & Kirchhelle, C. (2020). Spatio-temporal control of post-Golgi exocytic trafficking in plants. *Journal of Cell Science*, *133*(4), jcs237065. https://doi.org/10.1242/jcs.237065

French, A. P., Mills, S., Swarup, R., Bennett, M. J., & Pridmore, T. P. (2008). Colocalization of fluorescent markers in confocal microscope images of plant cells. *Nature Protocols*, *3*(4), 619–628. https://doi.org/10.1038/nprot.2008.31

Goh, T., Uchida, W., Arakawa, S., Ito, E., Dainobu, T., Ebine, K., Takeuchi, M., Sato, K., Ueda, T., & Nakano, a. (2007). VPS9a, the Common Activator for Two Distinct Types of Rab5 GTPases, Is Essential for the Development of Arabidopsis thaliana. *The Plant Cell Online*, *19*(11), 3504–3515. https://doi.org/10.1105/tpc.107.053876

Grosshans, B. L., Ortiz, D., & Novick, P. (2006). Rabs and their effectors: Achieving specificity in membrane traffic. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(32), 11821–11827. https://doi.org/10.1073/pnas.0601617103

Heazlewood, J. I., Durek, P., Hummel, J., Selbig, J., Weckwerth, W., Walther, D., & Schulze, W. X. (2008). PhosPhAt : A database of phosphorylation sites in Arabidopsis thaliana and a plant-specific phosphorylation site predictor. *Nucleic Acids Research*, *36*(SUPPL. 1), 1015–1021. https://doi.org/10.1093/nar/gkm812

Inoue, Takeshi, Kondo, Y., Naramoto, S., Nakano, A., & Ueda, T. (2013). RAB5 activation is required for multiple steps in arabidopsis thaliana root development. *Plant and Cell Physiology*, *54*(10), 1648–1659. https://doi.org/10.1093/pcp/pct109

Inoue, Tsutomu, Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., & Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. *Nature*, *409*(6823), 1060–1063. https://doi.org/10.1038/35059117

Ito, E., Ebine, K., Choi, S., Ichinose, S., Uemura, T., Nakano, A., & Ueda, T. (2018). Integration of two RAB5 groups during endosomal transport in plants. *ELife*, *7*, 1–22. https://doi.org/10.7554/eLife.34064

Ito, E., Fujimoto, M., Ebine, K., Uemura, T., Ueda, T., & Nakano, A. (2012). Dynamic behavior of clathrin in Arabidopsis thaliana unveiled by live imaging. *Plant Journal*, *69*(2), 204–216. https://doi.org/10.1111/j.1365-313X.2011.04782.x

Kakimoto, T. (2003). PERCEPTION AND SIGNAL TRANSDUCTION OF CYTOKININS. *Annual Review of Plant Biology*, *54*(1), 605–627. https://doi.org/10.1146/annurev.arplant.54.031902.134802

Keshishian, E. A., & Rashotte, A. M. (2015). Plant cytokinin signalling. *Essays in Biochemistry*, *58*, 13–27. https://doi.org/10.1042/BSE0580013

Kieber, J. J., & Schaller, G. E. (2018). Cytokinin signaling in plant development. *Development (Cambridge, England)*, *145*(4), 1–7. https://doi.org/10.1242/dev.149344

Kleine-Vehn, J., Leitner, J., Zwiewka, M., Sauer, M., Abas, L., Luschnig, C., & Friml, J. (2008). Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar

targeting. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(46), 17812–17817. https://doi.org/10.1073/pnas.0808073105

Kotzer, a. M. (2004). AtRabF2b (Ara7) acts on the vacuolar trafficking pathway in tobacco leaf epidermal cells. *Journal of Cell Science*, *117*(26), 6377–6389. https://doi.org/10.1242/jcs.01564

Kubiasová, K., Montesinos, J. C., Šamajová, O., Nisler, J., Mik, V., Semerádová, H., Plíhalová, L., Novák, O., Marhavý, P., Cavallari, N., Zalabák, D., Berka, K., Doležal, K., Galuszka, P., Šamaj, J., Strnad, M., Benková, E., Plíhal, O., & Spíchal, L. (2020). Cytokinin fluoroprobe reveals multiple sites of cytokinin perception at plasma membrane and endoplasmic reticulum. *Nature Communications*, *11*(1), 1–11. https://doi.org/10.1038/s41467-020-17949-0

Kudla, J., & Bock, R. (2016). Lighting the way to protein-protein interactions: Recommendations on best practices for bimolecular fluorescence complementation analyses. *Plant Cell*, *28*(5), 1002–1008. https://doi.org/10.1105/tpc.16.00043

Lamber, E. P., Siedenburg, A. C., & Barr, F. A. (2019). Rab regulation by GEFs and GAPs during membrane traffic. *Current Opinion in Cell Biology*, *59*, 34–39. https://doi.org/10.1016/j.ceb.2019.03.004

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M. B., Offringa, R., Graham, N., Doumas, P., Friml, J., Bogusz, D., Beeckman, T., & Bennett, M. (2007). Cytokinins Act Directly on Lateral Root Founder Cells to Inhibit Root Initiation. *The Plant Cell Online*, *19*(12), 3889–3900. https://doi.org/10.1105/tpc.107.055863

Lee, G. J., Eun, J. S., Myong, H. L., & Hwang, I. (2004). The Arabidopsis Rab5 homologs Rha1 and Ara7 localize to the prevacuolar compartment. *Plant and Cell Physiology*, *45*(9), 1211–1220. https://doi.org/10.1093/pcp/pch142

Li, S. M., Zheng, H. X., Zhang, X. S., & Sui, N. (2021). Cytokinins as central regulators during plant growth and stress response. *Plant Cell Reports*, *40*(2), 271–282. https://doi.org/10.1007/s00299-020-02612-1

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., & Benková, E. (2011). Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis. *Developmental Cell*, *21*(4), 796–804. https://doi.org/10.1016/j.devcel.2011.08.014

Marhavý, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J., Schwechheimer, C., Murphy, A., & Benková, E. (2014). Cytokinin Controls Polarity of PIN1-Dependent Auxin Transport during Lateral Root Organogenesis. *Current Biology*, *24*(9), 1031–1037. https://doi.org/10.1016/j.cub.2014.04.002

Markgraf, D. F., Peplowska, K., & Ungermann, C. (2007). *Rab cascades and tethering factors in the endomembrane system*. *581*, 2125–2130. https://doi.org/10.1016/j.febslet.2007.01.090

Martinez, O., & Goud, B. (1998). Rab proteins. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1404*(1–2), 101–112. https://doi.org/10.1016/S0167-4889(98)00050-0

Min, M. K., Jang, M., Lee, M., Lee, J., Song, K., Lee, Y., Choi, K. Y., Robinson, D. G., & Hwang, I. (2013). Recruitment of Arf1-GDP to Golgi by Glo3p-Type ArfGAPs Is Crucial for Golgi Maintenance and Plant Growth. *Plant Physiology*, *161*(2), 676–691. https://doi.org/10.1104/pp.112.209148

Minamino, N., & Ueda, T. (2019). RAB GTPases and their effectors in plant endosomal transport. *Current Opinion in Plant Biology*, *52*(Figure 1), 61–68. https://doi.org/10.1016/j.pbi.2019.07.007

Müller, M. P., & Goody, R. S. (2018). Molecular control of Rab activity by GEFs, GAPs and GDI. *Small GTPases*, *9*(1–2), 5–21. https://doi.org/10.1080/21541248.2016.1276999

Nakagami, H., Sugiyama, N., Mochida, K., Daudi, A., Yoshida, Y., Toyoda, T., Tomita, M., Ishihama, Y., & Shirasu, K. (2010). Large-scale comparative phosphoproteomics identifies conserved phosphorylation sites in plants. *Plant Physiology*, *153*(3), 1161–1174. https://doi.org/10.1104/pp.110.157347

Novick, P., & Zerial, M. (1997). The diversity of Rab proteins in vesicle transport. *Current Opinion in Cell Biology*, *9*(4), 496–504. https://doi.org/10.1016/S0955-0674(97)80025-7

Pereira-Leal, J. B., & Seabra, M. C. (2001). Evolution of the Rab family of small GTP-binding proteins. *Journal of Molecular Biology*, *313*(4), 889–901. https://doi.org/10.1006/jmbi.2001.5072

Rauniyar, N. (2015). Parallel reaction monitoring: A targeted experiment performed using high resolution and high mass accuracy mass spectrometry. *International Journal of Molecular Sciences*, *16*(12), 28566–28581. https://doi.org/10.3390/ijms161226120

Ravikumar, R., Steiner, A., & Assaad, F. F. (2017). Multisubunit tethering complexes in higher plants. *Current Opinion in Plant Biology*, *40*, 97–105. https://doi.org/10.1016/j.pbi.2017.08.009

Reiland, S., Finazzi, G., Endler, A., Willig, A., Baerenfaller, K., Grossmann, J., Gerrits, B., Rutishauser, D., Gruissem, W., Rochaix, J. D., & Baginsky, S. (2011). Comparative phosphoproteome profiling reveals a function of the STN8 kinase in fine-tuning of cyclic electron flow (CEF). *Proceedings of the National Academy of Sciences of the United States of America*, *108*(31), 12955–12960. https://doi.org/10.1073/pnas.1104734108

Reiland, S., Messerli, G., Baerenfaller, K., Gerrits, B., Endler, A., Grossmann, J., Gruissem, W., & Baginsky, S. (2009). Large-scale arabidopsis phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks1[w]. *Plant Physiology*, *150*(2), 889–903. https://doi.org/10.1104/pp.109.138677

Rodriguez-furlan, C., Minina, E. A., & Hicks, G. R. (2019). *Remove , Recycle , Degrade : Regulating Plasma Membrane Protein Accumulation*. *31*(December), 2833–2854. https://doi.org/10.1105/tpc.19.00433

Růžička, K., Ljung, K., Vanneste, S., Podhorská, R., Beeckman, T., Friml, J., & Benková, E. (2007). Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. *Plant Cell*, *19*(7), 2197–2212. https://doi.org/10.1105/tpc.107.052126

Ruzicka, K., Simásková, M., Duclercq, J., Petrásek, J., Zazímalová, E., Simon, S., Friml, J., Van Montagu, M. C. E., & Benková, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(11), 4284–4289. https://doi.org/10.1073/pnas.0900060106

Schaller, G. E., Bishopp, A., & Kieber, J. J. (2015). The yin-yang of hormones: Cytokinin and auxin interactions in plant development. *Plant Cell*, *27*(1), 44–63. https://doi.org/10.1105/tpc.114.133595

Šimášková, M., O'Brien, J. A., Khan, M., Van Noorden, G., Ötvös, K., Vieten, A., De Clercq, I., Van Haperen, J. M. A., Cuesta, C., Hoyerová, K., Vanneste, S., Marhavý, P., Wabnik, K., Van Breusegem, F., Nowack, M., Murphy, A., Friml, J., Weijers, D., Beeckman, T., & Benková, E. (2015). Cytokinin response factors regulate PIN-FORMED auxin transporters. *Nature Communications*, *6*, 8717. https://doi.org/10.1038/ncomms9717

Sohn, E. J., Kim, E. S., Zhao, M., Kim, S. J., Kim, H., Kim, Y. W., Lee, Y. J., Hillmer, S., Sohn, U., Jiang, L., & Hwang, I. (2003). Rha1, an Arabidopsis Rab5 homolog, plays a critical role in the vacuolar trafficking of soluble cargo proteins. *Plant Cell*, *15*(5), 1057–1070. https://doi.org/10.1105/tpc.009779

Sunada, M., Goh, T., Ueda, T., & Nakano, A. (2016). Functional analyses of the plant-specific C-terminal region of VPS9a: the activating factor for RAB5 in Arabidopsis thaliana. *Journal of Plant Research*, *129*(1), 93–102. https://doi.org/10.1007/s10265-015-0760-5

Suttangkakul, A., Li, F., Chung, T., & Vierstra, R. D. (2011). The ATG1/ATG13 Protein Kinase Complex Is Both a Regulator and a Target of Autophagic Recycling in Arabidopsis. *The Plant Cell*, *23*(10), 3761–3779. https://doi.org/10.1105/tpc.111.090993

Tamura, K., Shimada, T., Ono, E., Tanaka, Y., Nagatani, A., Higashi, S. I., Watanabe, M., Nishimura, M., & Hara-Nishimura, I. (2003). Why green fluorescent fusion proteins have not been observed in the vacuoles of higher plants. *Plant Journal*, *35*(4), 545–555. https://doi.org/10.1046/j.1365-313X.2003.01822.x

Taylor, N. G. (2011). A role for Arabidopsis dynamin related proteins DRP2A/B in endocytosis; DRP2 function is essential for plant growth. *Plant Molecular Biology*, *76*(1–2), 117–129. https://doi.org/10.1007/s11103-011-9773-1

Ueda, T., Uemura, T., Sato, M. H., & Nakano, A. (2004). Functional differentiation of endosomes in Arabidopsis cells. *Plant Journal*, *40*(5), 783–789. https://doi.org/10.1111/j.1365-313X.2004.02249.x

Ueda, T., Yamaguchi, M., Uchimiya, H., & Nakano, A. (2001). Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of Arabidopsis thaliana. *EMBO Journal*, *20*(17), 4730–4741. https://doi.org/10.1093/emboj/20.17.4730

Uemura, T., Ueda, T., Ohniwa, R. L., Nakano, A., Takeyasu, K., & Sato, M. H. (2004). Systematic analysis of SNARE molecules in Arabidopsis: Dissection of the post-Golgi network in plant cells. *Cell Structure and Function*, *29*(2), 49–65. https://doi.org/10.1247/csf.29.49

Vernoud, V., Horton, A. C., Yang, Z., & Nielsen, E. (2003). Analysis of the small GTPase gene superfamily of arabidopsis. *Plant Physiology*, *131*(3), 1191–1208.

https://doi.org/10.1104/pp.013052

Vissenberg, K., Claeijs, N., Balcerowicz, D., & Schoenaers, S. (2020). Hormonal regulation of root hair growth and responses to the environment in Arabidopsis. *Journal of Experimental Botany*, *71*(8), 2412–2427. https://doi.org/10.1093/JXB/ERAA048

Waldie, T., & Leyser, O. (2018). Cytokinin targets auxin transport to promote shoot branching. *Plant Physiology*, *177*(2), 803–818. https://doi.org/10.1104/pp.17.01691

Wang, P., Xue, L., Batelli, G., Lee, S., Hou, Y. J., Van Oosten, M. J., Zhang, H., Tao, W. A., & Zhu, J. K. (2013). Quantitative phosphoproteomics identifies SnRK2 protein kinase substrates and reveals the effectors of abscisic acid action. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(27), 11205–11210. https://doi.org/10.1073/pnas.1308974110

Wisniewska, J., Xu, J., Seifartová, D., Brewer, P. B., Růžička, K., Blilou, L., Rouquié, D., Benková, E., Scheres, B., & Friml, J. (2006). Polar PIN localization directs auxin flow in plants. *Science*, *312*(5775), 883. https://doi.org/10.1126/science.1121356

Wulfetange, K., Lomin, S. N., Romanov, G. a, Stolz, A., Heyl, A., & Schmülling, T. (2011). The cytokinin receptors of Arabidopsis are located mainly to the endoplasmic reticulum. *Plant Physiology*, *156*(August), 1808–1818. https://doi.org/10.1104/pp.111.180539

Wyblouw, B., & De Rybel, B. (2019). Cytokinin – A Developing Story. *Trends in Plant Science*, *24*(2), 177–185. https://doi.org/10.1016/j.tplants.2018.10.012

Zerial, M., & McBride, H. (2001). Rab proteins as membrane organizers. *Nature Reviews Molecular Cell Biology*, *2*(2), 107–117. https://doi.org/10.1038/35052055

Zulawski, M., Braginets, R., & Schulze, W. X. (2013). PhosPhAt goes kinases-searchable protein kinase target information in the plant phosphorylation site database PhosPhAt. *Nucleic Acids Research*, *41*(D1), 1176–1184. https://doi.org/10.1093/nar/gks1081