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Proteomic insights into antioxidant defense, mitogen activated protein kinase signalling and cytoskeleton

HABILITATION THESIS

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Contents

1. Introduction

The production of reactive oxygen species (ROS) is an unavoidable process in living systems. They have multiple signalling roles and are master regulators of plant stress response and development. ROS levels are controlled by antioxidant defense mechanisms, keeping their homeostasis in order to facilitate their signalling roles. Under harmful conditions, ROS may exceed the antioxidative defense resulting in damaging consequences on lipids, proteins and nucleic acids. Therefore, antioxidant defense must be strictly controlled via mechanisms which work in concert with the ROS production.

In this thesis, I discuss and summarize current knowledge about enzymatic antioxidant defense, its regulation and functions in plants. I focus on the roles of enzymatic antioxidants controlling ROS during plant development and I present new data about ROS regulation by mitogen activated protein kinase (MAPK)- and phosphatidylinositol kinase signalling. For better understanding of these signalling pathways, I summarize the outputs of shot-gun proteomic analyses performed on MAPK mutants and inhibitor-treated Arabidopsis seedlings. In the final section, I present recent achievements in cytoskeletal function and regulation. As described previously, both actin and microtubule cytoskeletons are sensitive to the oxidative stress and are required for the expression of stress related genes.

2. Regulation of ROS during plant response to adverse external conditions and developmental processes

2.1. State of the art

ROS as unavoidable byproducts of metabolism have important signalling roles in living organisms under both optimal and adverse environmental conditions (Takáč et al, 2003; Takáč, 2004; Apel and Hirt, 2004; Baxter et al, 2014). Currently, an attention is devoted to the roles of ROS in developmental processes of plants. ROS are produced from atmospheric oxygen by its monovalent (partial) reduction, which occurs in the presence of electron donors. Plant ROS are generated mainly by electron transport chains in chloroplasts (Kale et al, 2017) and mitochondria (Gleason et al, 2011), and during photorespiration in peroxisomes (del Rio, 2006). Apoplastic ROS are produced by plasma membrane localized NADPH oxidase (Sagi, 2006), oxalate oxidase (Voothuluru and Sharp, 2013) or by degradation of spermidine by polyamine oxidase (Geilfus et al, 2015). Apoplastic peroxidases showed ROS generating capacity as well (Bindschedler et al, 2006). Among all ROS, only four show higher abundance and stability: singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). They quickly interconvert, thus providing a high level of flexibility. However, they differ in their stability, reactivity and ability to be transported across membranes. H_2O_2 is the most stabile ROS and is transported actively across membranes by aquaporins (Miller et al, 2010).

Generation of $O_2^{\bullet -}$ is closely connected with intracellular vesicular transport. Endocytosis, a process of fundamental importance for plant development, is a prerequisite for intracellular accumulation of ROS upon salt stress in Arabidopsis. Pharmacological suppression of endocytosis leads to the reduction of intracellular ROS levels (Leshem et al, 2007). This occurs as a consequence of decreased activity and expression of NADPH oxidase (Leshem et al, 2007; Lee et al, 2008), an enzyme responsible for the generation of $O_2^{\bullet -}$. Moreover, NADPH oxidase named Respiratory burst oxidase homolog D (RbohD) forms clusters at the plasma membrane and internalizes to the cytosol during salt stress (Hao et al, 2014).

Although ROS have important signalling roles, their reactivity may cause damaging oxidative effects on lipids, nucleic acids and proteins eventually resulting in the cell death. On the other hand, they are also involved in strictly regulated programmed cell death (Petrov et al, 2015). Very important feature of the ROS signalling is that it can be propagated from cell to cell and transduce signal for long distances in a process called “ROS wave” (Miller et al, 2009). This is mediated by interplay between NADPH oxidase, calcium (Ca^{2+}) channels and oxidative stress-induced Ca^{2+} fluxes (Gilroy et al, 2016).

ROS modulate signalling through their capability to affect protein redox status via oxidation of methionine residues and thiol groups of cysteines. This oxidation activates, inactivates, or alters the function and the structure of proteins (Waszczak et al, 2015). Moreover, H_2O_2 can modulate carbonylation, which alters protein stability and might increase the susceptibility to proteolysis (Dalle-Donne et al, 2003). ROS interact with nitric oxide (NO) in a wide range of cellular processes involved in biotic stress and abiotic stresses or plant development (Piterková et al, 2015; Niu and Liao, 2016). ROS are well known inducers of NO production while NO inhibits NADPH oxidase and modulates the antioxidant capacity of the cell (del Río, 2015). On the other hand oxidative stress inhibits S-nitrosoglutathione reductase (GSNOR) activity. This enzyme controls level of S-nitrosoglutathione, a compound serving as cellular NO reservoir (Tichá et al, 2017).

In order to maintain the ROS levels under damaging concentrations, plants have evolved multiple adaptation and scavenging mechanisms. ROS scavenging is performed via enzymatic or nonenzymatic antioxidant defense mechanisms, which by strict compartmentalization provide controlled regulation of ROS levels (Figure 1; Foyer and Noctor, 2005; Mignolet-Spruyt et al, 2016). Major enzymatic antioxidants are superoxide dismutases (cytosolic, plastidial and mitochondrial; SOD), which decompose $O_2^{\cdot -}$ to H_2O_2 (Kliebenstein et al, 1998). H_2O_2 is detoxified by catalase (CAT) in peroxisomes as well as by peroxidases and enzymes of the ascorbate-glutathione cycle in the cytosol and chloroplasts (Apel and Hirt, 2004; Foyer and Noctor, 2005; Mhamdi et al, 2010). Moreover, thioredoxins, glutaredoxins and peroxiredoxins (PRX) are potent ROS scavengers (Meyer et al, 2009). Nonenzymatic

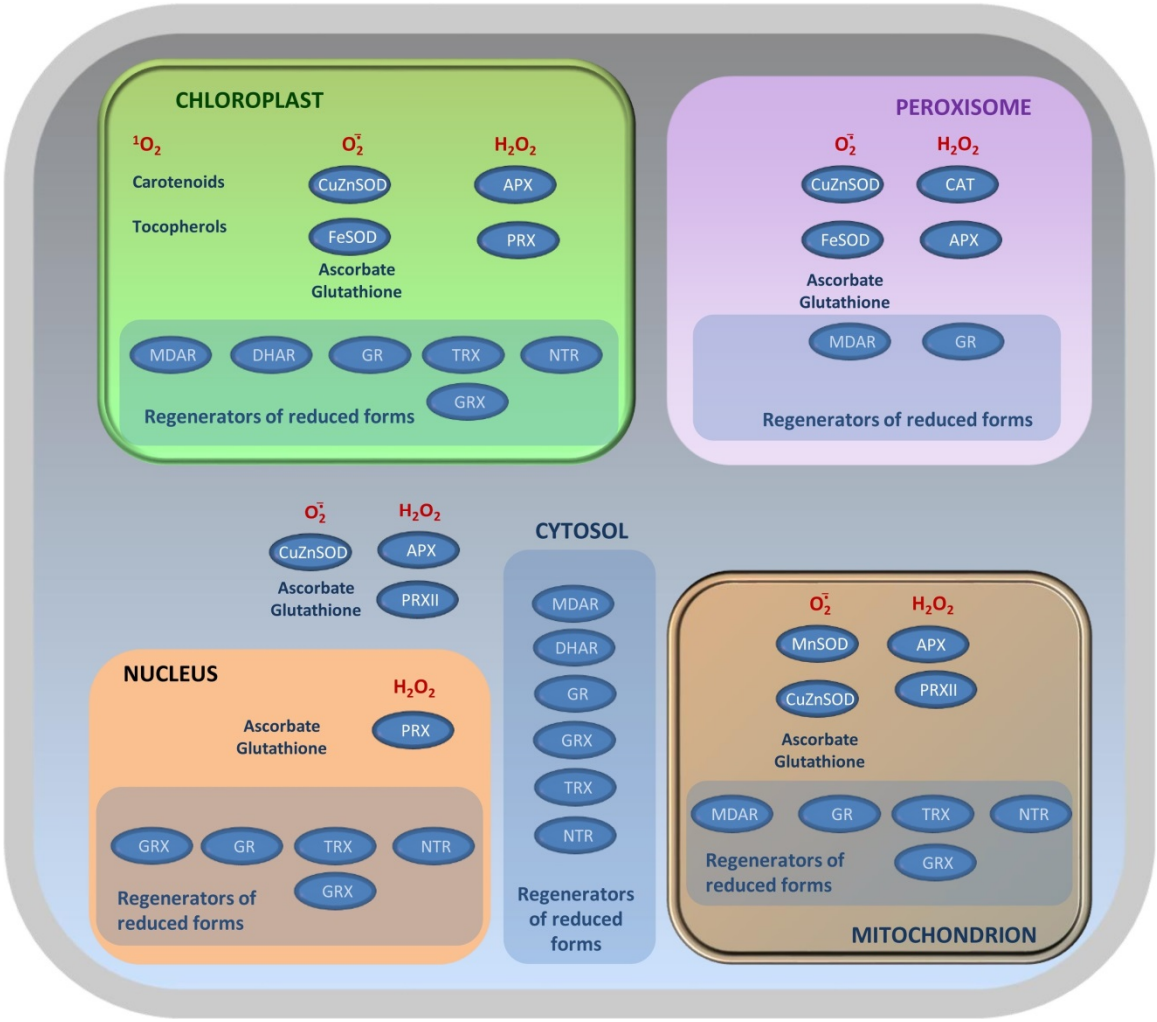

Figure 1 Principal players of plant antioxidative systems and its compartmentalisation. APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GRX, glutaredoxin; MDAR, monodehydroascorbate reductase; NTR, NADPH-thioredoxin reductase; PRX, peroxiredoxin; SOD, superoxide dismutase; TRX, thioredoxin (Noctor et al, 2017).

antioxidant defense is mainly mediated by low molecular metabolites such as ascorbate, glutathione, α-tocopherol, carotenoids and flavonoids (Foyer and Noctor, 2005).

Very important feature of ROS is their capability to activate mitogen activated protein kinases (MAPK) a key signalling proteins transducing signals triggered by many environmental and developmental factors (Colcombet and Hirt, 2008; Šamajová et al, 2013a; Smékalová et al, 2014a), see chapter 3.1 for more details. ROS have been shown to activate Arabidopsis homologues of nucleus- and

phragmoplast localized kinases (ANPs), and this activation is required for the plant immune response (Kovtun et al, 2000; Savatin et al, 2014). Such ROS triggered signal is further transduced via MPK3 and MPK6 (Kovtun et al, 2000). Another MAPK cascade activated by ROS is composed of MEKK1-MKK1/2-MPK4 (Pitzschke et al, 2009). ROS induced MAPK signalling results in transcriptional remodelling of oxidative stress related genes. So far, the only known MAPK substrate which is phosphorylated in the response to ROS is APETALA2/ethylene-responsive element binding factor 6 (ERF6), which is phosphorylated by MPK6 (Wang et al, 2013b). MAPKs modulate also abundances and activities of antioxidant enzymes (Xing et al, 2007, 2013), albeit the mechanism of this regulation is unknown.

In addition to the plant stress responses, ROS are widely involved in developmental processes (Xia et al, 2015). Thus, ROS can control cell cycle (Fehér et al, 2008), cell elongation (Liszkay et al, 2003), root hair formation (Foreman et al, 2003), lateral and adventitious root formation (Pasternak et al, 2005), root elongation (Schopfer et al, 2002), gravitropism (Joo et al, 2001) and embryogenesis (Obert et al, 2005; Rodríguez-Serrano et al, 2012). Moreover, ROS regulate these processes in the close interplay with plant phytohormones, such as auxin and cytokinins (Černý et al, 2011; Baldrianová et al, 2015; Xia et al, 2015). Phytohormones auxin and abscisic acid (ABA) are capable to promote ROS production by activating NADPH oxidase (Joo et al, 2001; Schopfer et al, 2002; Pasternak et al, 2007). Oppositely, ROS may induce perturbations in the auxin homeostasis leading to altered shoot branching and leaf rosette shape (Tognetti et al, 2010).

A little is known about the regulation of ROS levels by antioxidants during developmental processes. So far, a limited number of studies was devoted to the role of redox homeostasis and active antioxidant defense during cellular mitotic activity (Pasternak et al, 2007; Tsukagoshi, 2012), androgenesis (Żur et al, 2014), somatic embryogenesis (Libik et al, 2004) and root growth (Morgan et al, 2008). It is also known, that specific redox homeostasis mediated by glutathione is required for cell division (Diaz-Vivancos et al, 2015).

2.2. Role of antioxidant defense in the plant development

ROS interplay with auxin is required for cell cycle activation (G_0-to-G_1 transition) of plant cells (Fehér et al, 2008). By this mechanism, ROS may activate lateral and adventitious root formation (Orman-Ligeza et al, 2016). Moreover, MAPKs can link the signal from auxin and ROS to the initiation of lateral root primordia through NO production (Wang et al, 2010). This is also supported by involvement of MPK6 in the cell division of Arabidopsis root cells (Müller et al, 2010).

I investigated the auxin-ROS interplay in flax (*Linum usitatissimum*) hypocotyl segments (Takáč et al, 2016a; Supplementary material 1). Flax is an important crop for the production of oil and fiber and important target species of plant biotechnology (Millam et al, 2005). *In vitro* culture is an essential tool of flax genetic improvement and breeding. It is generally accepted that flax hypocotyl segments possess an outstanding morphogenetic capacity, thus providing a useful model for investigation of flax development (Salaj et al, 2005).

I have used two concentrations of external auxin (1-naphthaleneacetic acid; NAA) to induce adventitious root formation from the hypocotyl segments. The effect of external H_2O_2 on the adventitious root formation induced by NAA was also monitored. Histochemical analyses of H_2O_2, $O_2^{\bullet -}$ and peroxidase activities were performed on treated hypocotyl segments prior to the appearance of adventitious roots. Simultaneously, internal Indole-3-acetic acid (IAA) concentrations were examined using ultra-high performance liquid chromatography (UPLC) coupled with mass spectrometry. These data were correlated with the activities of SOD, CAT and guaiacol peroxidase in the hypocotyl segments analyzed by spectrophotometric methods (Takáč et al, 2016a, Supplementary material 1).

In this experimental system, low dose of external auxin combined with H_2O_2 positively stimulated formation of adventitious roots from flax hypocotyl segments (Takáč et al, 2016a, Supplementary material 1). They grew exclusively from the apical end of the segments. Considering the basipetal auxin flow in hypocotyls, it was assumed that adventitious roots appeared in hypocotyl areas accumulating endogenous auxin. Histochemical and fluorescent staining of hypocotyl segments revealed that H_2O_2 abundance and peroxidase activity positively correlated with root

formation. Detection of $O_2^{\bullet -}$ accumulation using nitrobluetetrazolium chloride (NBT) did not correlate with the adventitious root formation pattern (Figure 2). I found that the positive effect of H_2O_2 depends strongly on the concentration of external auxin. By using higher external auxin concentrations, H_2O_2 inhibited formation of adventitious roots. Biochemical examinations of antioxidant enzyme activities showed positive correlation of peroxidases (using guaiacol as a substrate) with adventitious root formation, supporting histochemical observations. Moreover, SOD was negatively affected by external auxin and in opposite, CAT activity increased in the response to auxin. Next, external H_2O_2 showed an ability to enhance IAA accumulation in hypocotyl segments. This is consistent with well-known perturbations of auxin homeostasis in the response to ROS (Tognetti et al, 2010).

My results contributed to the evidence that ROS and antioxidant enzymes,

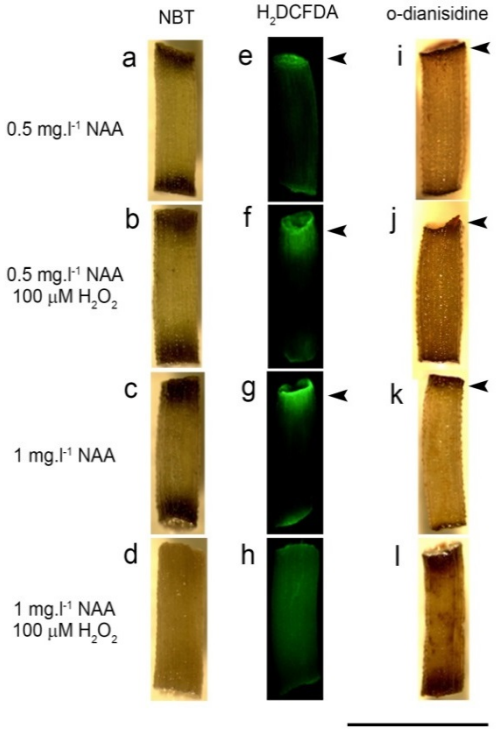

Figure 2 Histochemical localization of ROS and peroxidases in flax hypocotyl segments treated with (a, e, i) 0.5 mg.l^{-1} 1-naphthaleneacetic acid (NAA), (b, f, j) 0.5 mg.l^{-1} NAA and 100 µM H_2O_2, (c, g, k) 1 mg.l^{-1} NAA and (d, h, l) 1 mg.l^{-1} NAA and 100 µM H_2O_2 for 2 days. (a-d) Histochemical staining of superoxide in flax hypocotyl segments using nitroblue tetrazolium chloride, (e-h) fluorescent staining of ROS in hypocotyl segments using using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), (i-l) histochemical staining of peroxidases in flax hypocotyl segments using o-dianisidine. Bar represents 2mm (Takáč et al, 2016a, Supplementary material 1).

mainly peroxidases, are important modulators of adventitious root formation and they might be exploited in biotechnological crop improvement.

The formation of plant embryo is accompanied by multiple regular and irregular cell divisions regulated by various molecules including ROS (Libik et al, 2004; ten Hove et al, 2015; Winkelmann et al, 2015). Logically, contribution of ROS might be also expected during androgenesis, where microspores and young pollen grains can develop into embryos (Islam and Tuteja, 2012). Androgenesis is an important tool in genetic and breeding programs because androgenic embryos may produce homozygous double haploid plants. Switch from the gametophytic to the sporophytic development of microspores requires profound cytoplasmic and nuclear rearrangements as well as change of gene expression in order to suppress the gametophytic developmental program and induce the embryogenic one (Seguí-Simarro and Nuez, 2008). Androgenesis induction is preconditioned by appropriate developmental stage of microspores and exposure of microspores (or anthers) to abiotic stress conditions. Mostly cold and osmotic stresses are used for androgenesis induction (Maraschin et al, 2005). The molecular basis of the androgenic switch is not well understood. Therefore, we attempted to monitor changes in the maize anther proteome during cold pretreatment and after their transfer to the androgenesis-inducing media (Uváčková et al, 2012, Supplementary material 2). Anthers were collected at different stages of the experiment (together 6 samples) and proteins were extracted using phenol extraction coupled to ammonium acetate precipitation (Hurkman and Tanaka, 1986; Takáč et al, 2011; Supplementary material 3) Extracted proteins were resolved using two-dimensional (2-D) electrophoresis in three biological replicates. Gels were documented using calibrated densitometer and differences in spot densities were evaluated computationally. Spots showing statistically significant differences were cut from the gel and subjected to “in gel protein digestion” using trypsin. Proteins were identified using matrix assisted laser desorption/ionization (MALDI)-TOF/TOF mass spectrometry (using an Ultraflex II mass spectrometer, Bruker Daltonics) followed by data matching against Swissprot and TrEMBL databases. Differentially abundant proteins were classified according their functions (Figure 3) while proteins involved in the metabolism were categorized

using Mapman application (Thimm et al, 2004). This proteomic analysis was supplemented with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) staining of cultured microspores to monitor individual stages of androgenesis. Abundance changes of antioxidant enzymes were validated by specific activity staining on native polyacrylamide gels. Detailed description of the experimental procedures is in Uváčková et al, 2012 (Supplementary material 2).

Three days after incubation of microspores on the induction media we observed the onset of first sporophytic division of microspores. Therefore, we mainly focused on proteins showing increased abundance at this stage. In general, proteins

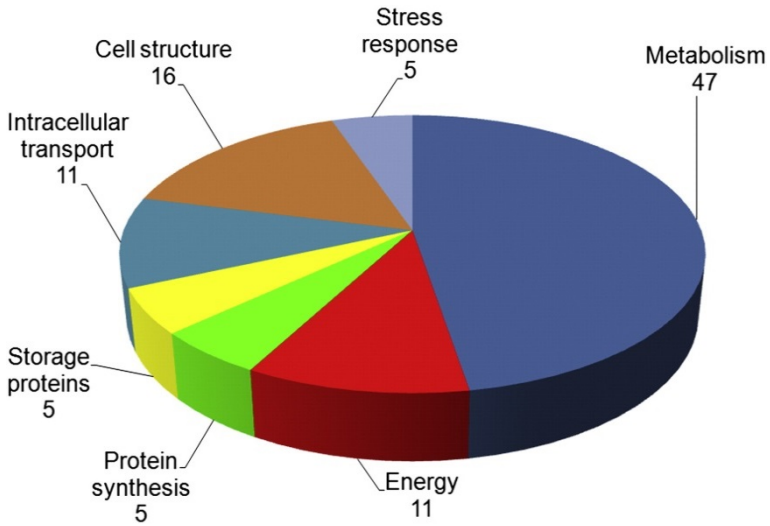

Figure 3 Functional classification of upregulated proteins identified in maize anthers during androgenic induction. The pie chart shows percent distribution of the proteins belonging to different functional classes (Uváčková et al, 2012, Supplementary material 2).

involved in metabolism and energy, stress response, cell structure, intracellular transport, protein synthesis and the storage proteins have been upregulated (Figure 3.

The androgenic switch is associated with multiple divisions of microspores leading to callus formation. Consistently, GTP binding nuclear protein Ran-2, a protein important for cell division, was threefold more abundant during the androgenic switch. Ran GTPases play important roles in mitotic spindle assembly and nuclear envelope formation (Clarke and Zhang, 2001). Thus, one can suggest that identified

Ran GTPase is involved in the cell division control in induced microspores. Such cell division acceleration during androgenesis must be also linked to cytoskeletal rearrangements and vesicular transport. Of note, an increased abundance of actin 1 was detected, likely indicating increase in demand for actin monomers. Maize androgenesis is also accompanied by increased abundance of V-type proton ATPase catalytic subunit A, a protein required for membrane fusion and transport (Dettmer et al, 2006). This might suggest a new role of endomembrane transport during induction of androgenesis.

NADPH oxidase internalizes to the cytosol upon salt stress (Hao et al, 2014) and the pharmacological suppression of endocytosis by phosphatidylinositol 3 kinase (PI3K) inhibitor 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one (LY294002) leads to the failure of intracellular ROS accumulation (Leshem et al, 2007). This must have consequences also in antioxidant defense. Therefore we focused on changes in abundances and activities of antioxidant enzymes in Arabidopsis roots treated with LY294002, a synthetic chemical compound targeting PI3K and causing inhibition of endocytosis and vacuolar transport (Takáč et al, 2013; Supplementary material 4). The experimental approach and overall proteome-wide effects of this compound on Arabidopsis roots are described in chapter 4.2.

LY294002 caused downregulation of two SOD isoforms (Cu/ZnSOD2, FeSOD1) and thioredoxin reductase and upregulation of H_2O_2 decomposing CAT 2 and peroxidases in Arabidopsis roots (Table 1; Takáč et al, 2013; Supplementary material 4). Further biochemical examinations showed that SOD activities dropped consistently with their abundance. This might be a consequence of altered ROS production upon inhibited endocytosis (Leshem et al, 2007). On the other hand, PI3K may directly affect the abundance and activities of Cu/ZnSODs via I glutamine amidotransferase domain-containing protein, also named AtDj1a, which was also downregulated in the proteomic survey. This protein is localized to the cytoplasm and the nucleus. It interacts with and activates Cu/Zn SOD in Arabidopsis (Xu and Møller, 2010). Thus, PI3K probably couples the NADPH oxidase activity with DJ1A-mediated downregulation of Cu/ZnSOD. Interestingly, antioxidant enzyme machinery behaves differently upon simultaneous inhibition of both PI3K and phosphatidylinositol 4

kinase (PI4K) by wortmannin treatment in Arabidopsis (Takáč et al, 2012; Supplementary material 5). This suggests that PI4K may affect the PI3K mediated control of antioxidant defense.

Table 1 List of oxidative stress related proteins differentially abundant in LY294002 treated Arabidopsis roots (Takáč et al, 2013; Supplementary material 4)

NCBI accession code	Protein name	fold change	P Value
NP_565666.1	copper/zinc superoxide dismutase 2	0.46	$9.02E^{-3}$
NP_001031710.1	Fe superoxide dismutase 1	0.34	$3.50E^{-2}$
NP_001031791.1	catalase 2	1.43	$1.31E^{-2}$
NP_192868.1	peroxidase, putative	1.36	$1.77E^{-2}$
NP_192897.2	glutathione peroxidase 6	4.10	$1.01E^{-2}$
NP_172018.1	rare cold inducible gene 3 (peroxidase)	1.15	$2.95E^{-2}$
NP_001030698.1	class I glutamine amidotransferase domain-containing protein (AtDJ1A)	0.61	$2.41E^{-2}$
NP_195271.2	NADPH-dependent thioredoxin reductase 1	0.52	$2.45E^{-2}$

2.3. Regulation of enzymatic antioxidant defense by MAPKs

Two MAPK cascades are activated by ROS in Arabidopsis (Kovtun et al, 2000; Pitzschke et al, 2009) while members of MAPK cascades are important mediators of antioxidant defense in plants. This was shown for MKK5 in high-light induced oxidative stress and Cu/Zn SOD (Xing et al, 2013) or salinity induced FeSOD (Xing et al, 2015) as well as for MKK1 and MPK6 in the regulation of CAT1 (Xing et al, 2008).

One MAPK cascade activated by ROS consists of ANPs (mitogen activated protein kinases kinases kinases) phosphorylating MKK4/MKK5, which leads to the subsequent phosphorylation of MPK3 and MPK6 (Kovtun et al, 2000). ANPs are required for elicitor-triggered oxidative burst as a prerequisite for efficient defense responses and elicitor-induced immunity (Savatin et al, 2014). To dissect downstream processes affected by ANP2 and ANP3 impairment, we performed a comparative shot-gun proteomic analysis on the whole seedlings of Arabidopsis *anp2anp3* double mutants (Takáč et al, 2014; Supplementary material 6). Details of experimental settings are described in Takáč et al, 2014; Supplementary material 6). Briefly, fourteen days old seedlings of these double mutants and wild type were homogenized and proteins were extracted using phenol extraction followed by

methanol/ammonium acetate precipitation. Cleaned pellets were dissolved in 6M urea and they were digested “in solution” by trypsin. Peptides were pre-cleaned on C18 gravity cartridges and separated by 2-D LC comprised of a strong cation exchange (SCX) column, followed by a C18 reverse phase column. The LCQ Deca XP Plus – electrospray ionization (ESI) ion trap mass spectrometer was programmed to operate in the data dependent mode. Triplicate raw files containing MS and MS/MS data for each biological sample were matched against both target and decoy databases. The NCBI (www.ncbi.nlm.nih.gov) Arabidopsis genus taxonomy referenced protein database served as the target database, while a reversed copy served as a decoy database. The unfiltered TurboSEQUEST result (.srf) files were subjected to statistical and label-free quantitative analysis using two independent software packages (Takáč et al, 2014; Supplementary material 6). Only proteins identified with at least three peptide hits (spectral count) for particular biological sample were considered. The representation of protein-protein interaction networks by STRING web-based application (Jensen et al, 2009) was used for proteomic data evaluation. The selected differentially abundant enzymes were validated by immunoblotting using specific primary antibodies, specific staining of enzymatic activity on native gels or spectrophotometric estimation of specific enzymatic activity. Additionally, total ascorbate, H_2O_2 and $O_2^{\bullet -}$ levels were measured spectrophotometrically. Histochemical staining of $O_2^{\bullet -}$ and H_2O_2 was conducted using NBT and 3,3'-diaminobenzidine, respectively.

Interestingly, *anp2anp3* mutant exhibits overabundances of several proteins important for antioxidative defense. Using biochemical examinations, we confirmed an increased abundance and also activity of FeSOD 1 and MnSOD in the double mutant. Enzymes of the ascorbate-glutathione cycle including ascorbate peroxidase and dehydroascorbate reductase showed higher abundances and activities. The elevated abundance of GDP-D-mannose 3′,5′-epimerase (ascorbate biosynthetic enzyme; GME) was consistent with increased ascorbate content in the double mutant. As expected, histochemical staining revealed decreased levels of H_2O_2 in the leaves of this mutant. On the other hand, $O_2^{\bullet -}$ accumulated there. This accumulation was a result of elevated NADPH oxidase activity and not of lower photosynthetic

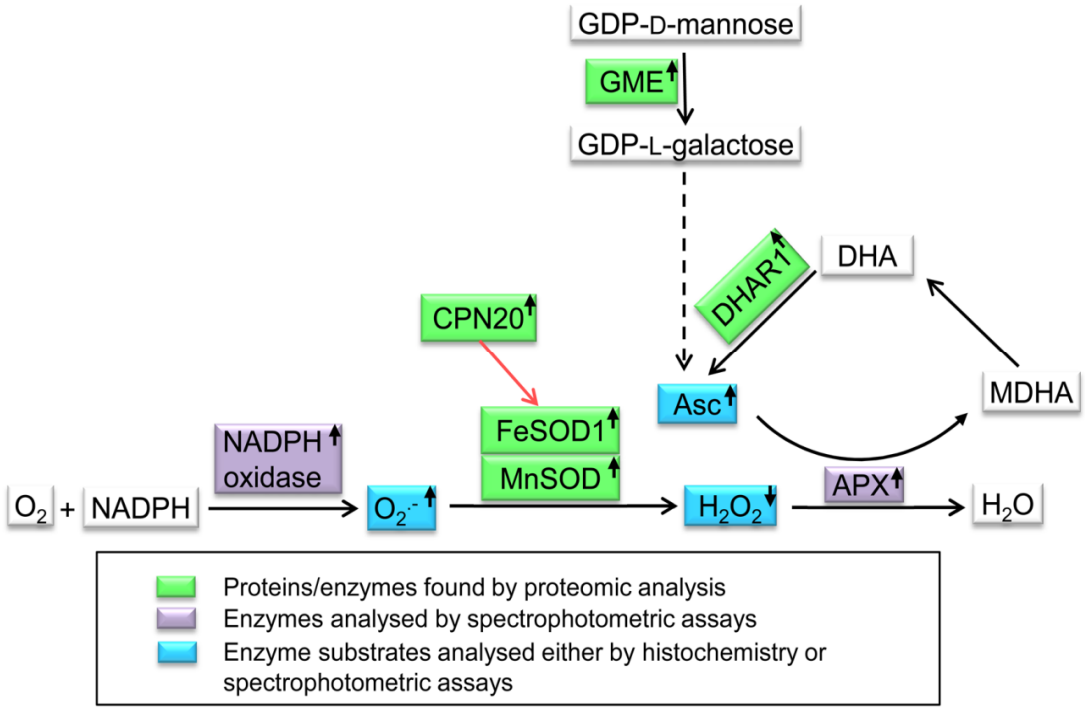

Figure 4 Functional network of proteins/enzymes, reactive oxygen species and redox-active compounds modified in the *anp2anp3* mutant. Red arrow shows activation, dashed arrow means metabolic pathway. Small arrows in boxes indicate upregulation or downregulation of individual components. Abbreviations: Asc - ascorbate, APX - ascorbate peroxidase, CPN20 - Chaperonin 20, DHAR 1 - dehydroascorbate reductase, GME - GDP-D-mannose 3',5'-epimerase, FeSOD - Fe-superoxide dismutase 1, MDHA - monodehydroascorbate, MnSOD - Mn-superoxide dismutase (Takáč et al, 2014; Supplementary material 6).

efficiency. Such accumulation of $O_2^{\bullet -}$ in the *anp2anp3* double mutant is in contrast to the elevated abundances and levels of FeSOD1, an enzyme decomposing this radical.

These data indicate possible developmental signalling role of $O_2^{\bullet -}$ which is negatively regulated by ANPs. Finally, the elevated antioxidant defense suggested that the double mutant might be more resistant to the oxidative stress which was confirmed by paraquat (inducer of $O_2^{\bullet -}$ production) treatment. It might be therefore concluded, that ANPs are activated by oxidative stress and negatively regulate tolerance of Arabidopsis to this stress (Figure 4); Takáč et al, 2014; Supplementary material 6). On the other hand, the same MAPKs confer resistance of Arabidopsis to *Botrytis cinerea* (Savatin et al, 2014), which is a clear sign of their functional divergence in Arabidopsis.

Next shot-gun proteomic analyses showed that MPK4, unlike MPK6, has potential to control enzymatic antioxidative defense in Arabidopsis roots. Such suggestion was made on the base of differential abundance of several proteins implicated in antioxidative defense of *mpk4* mutant, and in contrast to *mpk6-2* mutant, where only minor changes were obtained (Takáč et al, 2016b, Supplementary material 7). CAT enzymatic activity (decomposing H_2O_2) was lower in the *mpk4* mutant, while no pronounced changes were found in the *mpk6-2* mutant. After H_2O_2 treatment, CAT activity increased in the *mpk4* mutant, but not in the *mpk6-2* mutant. This suggests a negative control of CAT by MPK4. Surprisingly, although MPK6 participates on the oxidative stress signalling (Wang et al, 2010) the *mpk6-2* mutant exhibits only negligible changes in the abundance of proteins involved in the oxidative stress response of roots. On the other hand, our recent results (Smékalová et al, unpublished) indicate that MPK3, which works often redundantly to MPK6 under oxidative stress (Kovtun et al, 2000) might play a role in the oxidative stress by modulating the antioxidant proteins. Thus, although MPK3, MPK4 and MPK6 are activated by oxidative stress, they most likely play different roles in the regulation of enzymatic antioxidant response.

2.4. Conclusion and future prospects

ROS and antioxidants have undisputable roles in the plant development. Our studies confirmed that the control of ROS levels has crucial importance for plant developmental processes such as root organogenesis or androgenesis. Diverse developmental processes require different antioxidant enzymes. This indicates that there are specific control mechanisms, regulating their expression, abundance and activation as well as subcellular and tissue-specific localization. We have found that antioxidant defense responds specifically to PI3K inhibition which is accompanied by impaired endocytosis and vacuolar transport.

In general, current research relies on the knowledge that antioxidant enzymes are regulated by posttranslational modifications, redox regulation, miRNA and MAPK signalling pathways (Xing et al, 2007; Yamasaki et al, 2009; de Pinto et al, 2013). Further proteomic, genetic, cell biology and molecular biology investigations are

necessary to uncover precise regulations of individual antioxidant enzymes. Effort should be focused on the regulation of homeostasis and subcellular compartmentalization. Developmental light-sheet microscopic imaging of fluorescently tagged antioxidant enzymes expressed under native promoter is highly feasible for uncovering tissue and organ specific functions.

Proteomic, phosphoproteomic and redox-proteomic approaches might uncover new MAPK targets modulating antioxidant defense during oxidative stress. Employment of these techniques on transgenic plant lines with modified abundances of certain antioxidant enzymes may significantly contribute to the updated knowledge about their functions in plant development and stress response.

3. Uncovering processes downstream of MAPK signalling in plants

3.1. State of the art

Plants as sessile organisms have evolved multiple mechanisms to sense and transduce signals from external environment. MAPKs represent very important components of this system, enabling the transformation of signal from the external environment into specific responses including either the activation of defense mechanisms, or developmental and physiological reprogramming (Rodriguez et al, 2010; Šamajová et al. 2013a; Šamajová et al. 2013b; Smékalová et al, 2014a). Following perception by receptors, signal is transduced via phosphorylation of individual MAPK signalling cascade constituents, consisting of MAPK kinases kinases (MAPKKK, MAP3K), MAPK kinases (MAPKK, MAP2K) and MAPK. Activated MAPKs are then phosphorylating various target proteins (Figure 5; Šamajová et al. 2013a; Pitzschke, 2015).

Complete sequencing of the Arabidopsis genome provided identification of 20 MAPKs, 10 MAP2Ks and 80 MAP3Ks (MAPK Group, 2002). Thus, there is a significant degree of complexity while certain upstream MAP3K (or MAP2Ks) may phosphorylate more MAP2Ks (or MAPKs), respectively (Andreasson and Ellis, 2010).

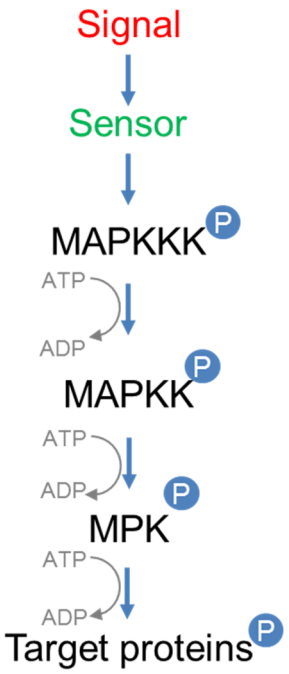

Figure 5 Simplified depiction of MAPK cascades.

Scaffold proteins ensure the control of signal specificity and duration. The first example of plant scaffold protein for MAPK cascades was identified only recently. Under immune response, the activation of MAPK cascade requires the presence of scaffold protein RACK1A (Receptor for Activated C Kinase 1), which binds all constituents of the cascade. In response to pathogen derived proteases, the phosphorylation of MAPK cascade constituents is followed by their dissociation from RACK1 and subsequent relocation (Cheng et al, 2015).

MAPKs phosphorylate transcription factors regulating the expression of genes responsible for stress response/resistance (Kim and Zhang, 2004; Meng and Zhang, 2013). For example, upon inoculation with *Botrytis cinerea*, ERF6 transcription factor is phosphorylated by MPK3 in Arabidopsis and this phosphorylation confers enhanced resistance to this pathogen (Meng et al, 2013). Transcription factor MYB44 regulates osmotic stress tolerance when it is phosphorylated by MPK3 and MKK4 (Persak and Pitzschke, 2013). MAPKs may also phosphorylate cytoskeletal proteins with crucial impact on microtubule organization and dynamics, which is important for cell division and elongation. Such phosphorylation was identified for MAP65-1 (Smertenko et al, 2006; Beck et al, 2010; Müller et al, 2010) or EB1c (Kohoutová et al, 2015) in Arabidopsis.

MAPKs phosphorylate also other proteins or enzymes, for example 1-aminocyclopropane-1-carboxylic acid synthase (ACS), which is responsible for ethylene biosynthesis. Phosphorylation of ACS by MPK6 triggers accumulation of ethylene in Arabidopsis (Liu and Zhang, 2004). Arabidopsis MAPK phosphatase 1 is phosphorylated by MPK6, which is a substrate of this phosphatase (Park et al, 2011).

MAPKs phosphorylate their targets at serine (S) or threonine (T) residues adjacent to a proline (P) and dock to their substrates on MAPK-specific docking site, so called kinase interaction motif (Pitzschke, 2015). MAPK dephosphorylation is controlled by dual-specific phosphatases. For example, PP2C-type phosphatase AP2C1 negatively regulates MPK4 and MPK6, and it is modulating innate immunity, jasmonic acid and ethylene levels in Arabidopsis (Schweighofer et al, 2007). Similar regulation was found in the case of Arabidopsis MAPK phosphatase 1 (MKP1), which negatively affects the PAMP responses and resistance against bacteria by dephosphorylation of MPK6 (Anderson et al, 2011).

MAPK signalling is fine-tuned by crosstalk with NO, Ca^{2+}, lipid and hormone signalling (Smékalová et al, 2014a), and it is also regulated by oxidation status of cysteine thiols (Liu and He, 2017). MAPK expression and MAPK-induced immune responses are also controlled by miRNA as it was recently shown in cotton (Wang et al, 2017).

The identification of new MAPK targets relies partly on biochemical and genetic studies including the detection of target protein phosphorylation by respective MAPK and protein-protein interactions using various approaches (Ishihama et al, 2014; Komis et al, 2014; Schweighofer et al, 2014). New OMICs technologies bring great progress in the identification of kinase targets. Phosphoproteomics on transgenic plants with changed expression of MAPKs provides high-throughput detection of putative MAPK target candidates *in vivo* (Hoehenwarter et al, 2013; Lassowskat et al, 2014). These studies reveal new information about phosphoproteome changes in plants with modified MAPKs expression, and uncover downstream effects of MAPKs on their target proteins. To prove the specificity of such putative MAPK phosphorylation targets they have to be validated by both bioinformatic and independent analytical methods.

Nevertheless, quantitative shot-gun proteomics performed on MAPK mutants provides also powerful tool in broadening the knowledge about MAPK signalling roles (Takáč and Šamaj, 2015; Supplementary material 8). Thorough bioinformatic tools are effective for uncovering various biochemical or physiological consequences of MAPK deficiency. Compared to phosphoproteomics, shot-gun proteomics on MAPK mutants can identify mid- and long-term complex protein profiles and networks associated with the transcriptional changes caused by deregulation of MAPK pathways, as this seems to be often the case in the mutant and overexpressor plant lines (e.g, Krysan et al. 2002). We consider this point quite important because changed complex protein profiles revealed by shot-gun proteomics might be closely linked to phenotypes of respective mutant and transgenic lines, including their characteristics such as modified stress responses or developmental defects (Takáč and Šamaj, 2015; Supplementary material 8). In our research, we conducted several shot-gun proteomic analyses of MAP3K and MAPK mutants and gained new important regulatory functions of MAPK-dependent signaling.

3.2. Proteomic analyses of Arabidopsis mutants deficient in MAPKKKs and MAPKKs

ANPs are MAP3Ks which are activated upon ROS accumulation (Kovtun et al, 2000; Krysan et al, 2002). They are required for elicitor-triggered oxidative burst which is a prerequisite of efficient defense responses and elicitor-induced immunity (Savatin et al, 2014). Furthermore, ANPs have multiple developmental roles. They regulate cytokinesis and cell expansion through microtubule organization of dividing or growing plant cells (Krysan et al, 2002; Beck et al, 2010; Takahashi et al, 2010; Beck et al. 2011). This has serious consequences for overall plant habitus exhibiting dwarf phenotype (Beck et al, 2010). Presumably, ANPs deficiency causes remodelling of the transcriptome (Krysan et al, 2002) but, according to our results, also proteome (Takáč et al, 2014; Supplementary material 6). ANPs together with downstream MKK6 and MPK4 are essential for cytokinesis in Arabidopsis. This pathway is activated by HINKEL/ NACK1 kinesin-like protein (Takahashi et al, 2010). During oxidative stress response ANPs act together with MKK4/MKK5 and MPK6 (Kovtun et al, 2000). Thus ANP signalling diverges at least into two different cascades controlling oxidative stress and immune response as well as plant cytokinesis.

In addition to the above mentioned regulation of antioxidant enzymes, our comparative shot-gun proteomic analysis of *anp2anp3* double mutant uncovered several new putative processes and proteins which are controlled by ANPs (Takáč et al, 2014; Supplementary material 6). We encountered robust changes in metabolic enzymes and proteins of photosynthetic apparatus, which is consistent with the dwarf phenotype of the seedlings (Krysan et al, 2002). ANPs negatively control the jasmonic acid biosynthetic enzyme lipoxygenase 2, concluding from dramatically increased abundance of this enzyme in the *anp2anp3* double mutant (Takáč et al, 2014; Supplementary material 6). MAPKs have the capability to regulate hormonal biosynthesis. Tobacco wound-induced protein kinase (WIPK) and salicylic acid (SA)-induced protein kinase (SIPK) positively control jasmonic acid biosynthesis (Seo et al, 2007). Moreover, MPK3 phosphorylates ACS6 and thus induces ethylene accumulation (Liu and Zhang, 2004). Our results provide the first evidence about

MAP3K likely modulating jasmonic acid biosynthesis. This is at least partially consistent with the previously published increased transcript levels of pathogenesis related genes in *anp* mutants (Krysan et al, 2002).

As mentioned above, ANPs are also implicated in the control of cytokinesis (Krysan et al, 2002; Beck et al, 2011). *Anp2anp3* double mutant exhibits severe cytokinetic defects such as incomplete cell plates and cell wall stubs, leading to bi- and multinucleate cells. In addition, it shows various abnormalities in mitotic spindle and phragmoplast rearrangements, and increased numbers of mitotic figures (Beck et al, 2011). ANP deficiency resulted in the upregulation of RabE1b, a small GTPase belonging to the RabE family. RabE proteins have function in Golgi-plasma membrane transport and are localized to the cell plate in Arabidopsis (Speth et al, 2009). We assume that RabE1b upregulation contributed to cell plate defects in the *anp2anp3* double mutant, and that this protein is negatively controlled by ANP pathway. We also found downregulation of calmodulin 4. Calmodulins are ubiquitous Ca^{2+}-binding proteins that mediate primary intercellular Ca^{2+} signaling pathways. They are required for induction of MAPK signalling upon wounding (Takahashi et al, 2011) while Ca^{2+}/calmodulin-regulated receptor-like kinase CRLK1 is interacting with MEKK1 to confer cold stress tolerance in Arabidopsis (Yang et al, 2010). We found possible positive interplay between ANPs and calmodulin 4 in Arabidopsis (Takáč et al, 2014; Supplementary material 6).

MPK3 and MPK6 are directly phosphorylated by MKK4/5, which except ANPs, may be activated by alternative MAP3Ks such as MEKK1 or YODA (Colcombet and Hirt, 2008). The YODA (MAP3K4) is required for cell fate determination during early embryogenesis (Lukowitz et al, 2004). YODA cascade transduces signal to the transcription factor SPEECHLESS (SPCH), which controls stomatal initiation and is directly suppressed by MPK3/6-mediated phosphorylation (Lampard et al, 2008). On the other hand, YODA is negatively transcriptionally regulated by transcription co-activator ANGUSTIFOLIA3. The repression of YODA leads to the higher drought tolerance of Arabidopsis (Meng and Yao, 2015).

Two classes of mutants in *MAP3K4* called *yda1* (kinase inactive) and *ΔNyda1* (a gain of function) show opposite stomatal phenotypes, with *yda1* plants having

clustered stomata and *ΔNyda1* plants having no stomata as a consequence of repressed stomatal development (Bergmann et al, 2004; Wang et al, 2007). Both mutants also exhibit pronounced shoot and root phenotypes (Figure 6; Bergmann et al, 2004; Lukowitz et al, 2004; Smékalová et al, 2014b; Supplementary material 9), which might be linked to the auxin accumulation and substantial alteration of mitotic

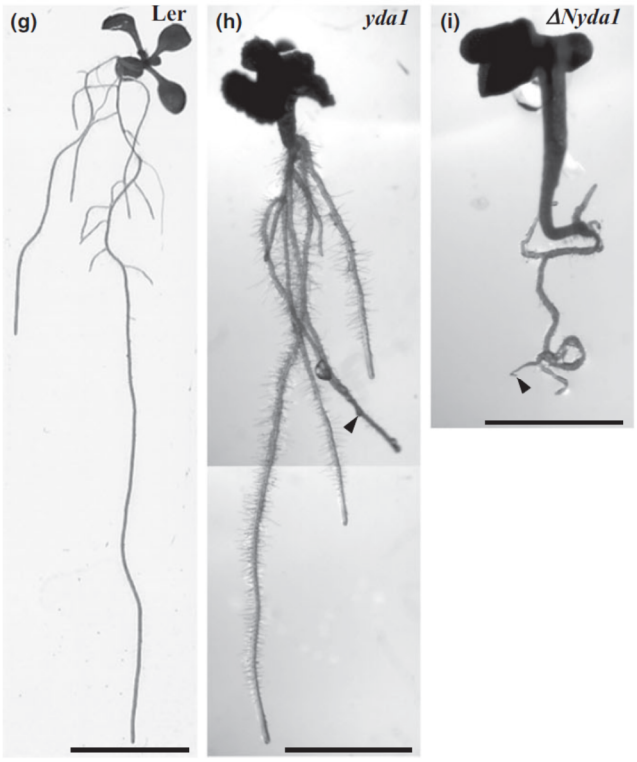

Figure 6 Representative pictures of 8 days old *Arabidopsis thaliana* (Ler ecotype) seedlings and seedlings of *yda1* and *ΔNyda1* mutants (Smékalová et al, 2014b; Supplementary material 9)**.**

microtubule arrays (Smékalová et al, 2014b; Supplementary material 9).

We conducted shot gun proteomic analyses on these mutants with main aim to uncover proteomic changes determining the above mentioned phenotypes.

The experimental approach differed only slightly from that used for the proteomic analysis of the *anp2anp3* double mutant (chapter 2.3). The preparation of plant material and peptide mixture was performed likewise. The 2-D LC-MS/MS analysis was conducted on a nanoAcquity 2-D UPLC system directly coupled to Xevo G2-S Q-TOF tandem mass spectrometer (Waters). Peptides were first separated on

an XBridge PST C18 NanoEase Column using acetonitrile (ACN) steps of 10.8%, 20.4% and 65% ACN. Each fraction was eluted onto a Symmetry C18 nanoACQUITY Trap Column (Waters) at a flow rate of 2 µl/min. Peptides in each ACN step were then separated on an ACQUITY UPLC PST C18 nanoACQUITY Column (Waters) with a 70 minutes long gradient from 3% to 40% of ACN at a flow rate of 0.45 µl/min. MS and MS/MS data were acquired in MS^E mode, with Glu-fibrinopeptide (1 pmole/µl, flow rate 0.3 µl/min, 785.8425 Da $[M+2H]^{2+}$) infused every 60s as a lock mass. The triplicate data files of each biological sample were processed and analyzed with ProteinLynx Global Server (PLGS, version 2.5.2, Waters). The processed data were searched against the NCBI Arabidopsis genus taxonomy referenced protein database along with the reversed decoy database. Quantitative analysis of identified proteins (confidence > 95%) was based on ion peak intensities observed at low collision energy mode, carried out with the Expression Analysis function in PLGS. Only the proteins with ANOVA $p \leq 0.05$ were reported as differentially expressed.

Concluding from the number of differentially abundant proteins and their functional classification, YODA appears to control a wider range of functions in comparison to ANPs (Takáč et al, 2014; Smékalová et al, 2014b; Supplementary materials 6 and 9). This may indicate divergent functions of YODA acting in multiple signalling cascades while ANPs might have more specialized functions. We have used STRING application (Jensen et al, 2009) to evaluate protein interaction networks among the differentially abundant proteins detected in *yda1* and *ΔNyda1* mutants. Both mutations caused alterations in the abundance of proteins involved in metabolism, antioxidant defense, methylation, protein synthesis and folding as well as pollen development (Figures 7 and 8). We detected an interaction cluster of proteins involved in auxin biosynthesis in both mutants (Figures 7 and 8). NITRILASE1 overaccumulated in both mutants. Since it localizes to the phragmoplast (Doskočilová et al, 2013) it might provide a new link between YODA, auxin and microtubular cytoskeleton. Overabundances of nitrilases and tryptophan synthase (responsible for biosynthesis of tryptophan as an auxin precursor) might explain elevated auxin accumulation, as found in the mutants by LC-MS/MS (Smékalová et

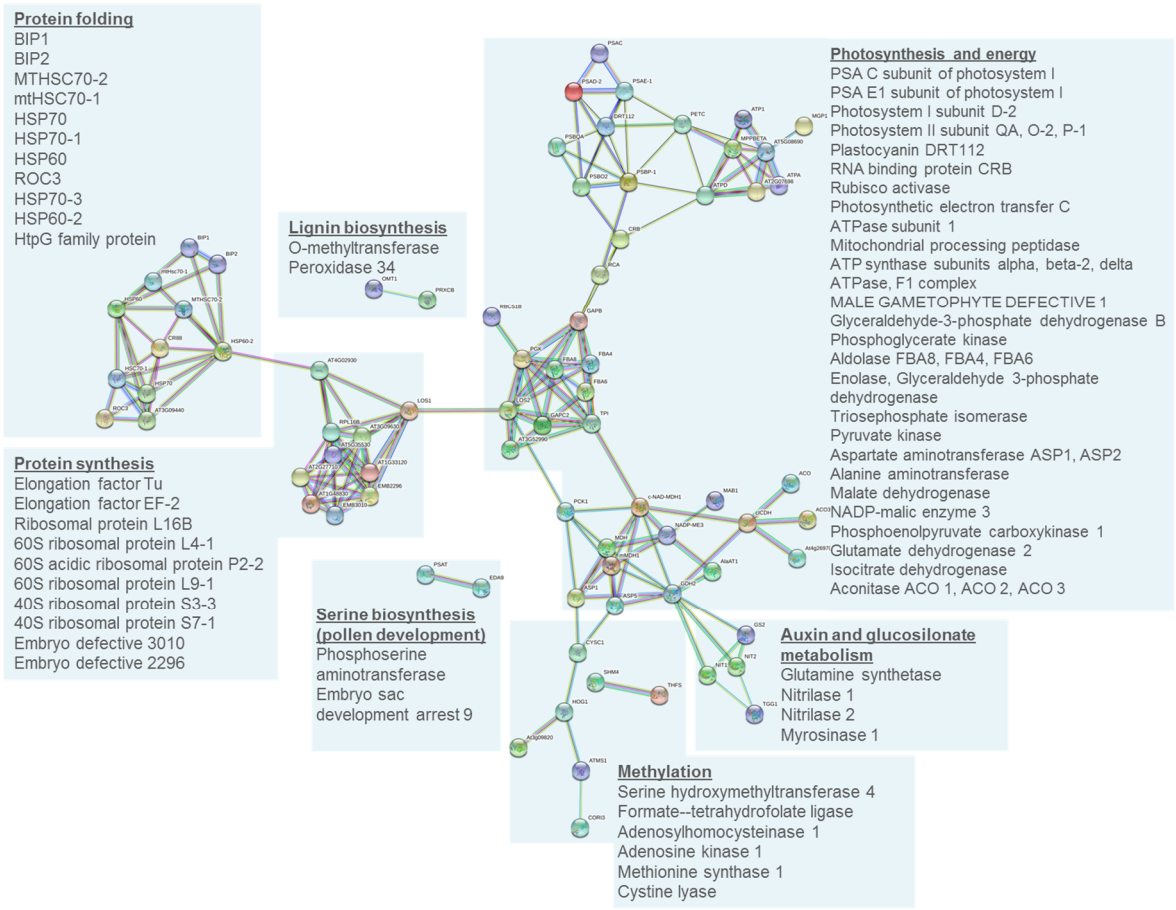

Figure 7 Depiction of protein interaction networks as predicted by STRING analysis performed on differentially regulated proteins found in the *yda1* mutant.

al, 2014b; Supplementary material 9). Thus, YODA provides another example of MAPK directed regulation of hormonal signalling in Arabidopsis.

Further, *yda1* and *ΔNyda1* mutants exhibit defects in the cell plate formation, especially in the proper orientation (Smékalová et al, 2014b; Supplementary material 9). Such reoriented cell plates might result from decreased levels of patellin 1 and patellin 2 in the mutants as compared to wild type. These proteins are involved in the cell plate formation (Peterman et al, 2004). Interestingly, patellin 2 was recently found as a phosphorylation substrate of MPK4 (Suzuki et al, 2016). Therefore our results point to the regulation of patellins by YODA pathway.

Recently published increased drought tolerance of *yda1* (Meng and Yao, 2015) is likely a consequence of overabundant proteins involved in drought response and having antioxidant activity (Smékalová et al, 2014b; Supplementary material 9). Another functional annotation occurring in the differential proteome of both mutants is

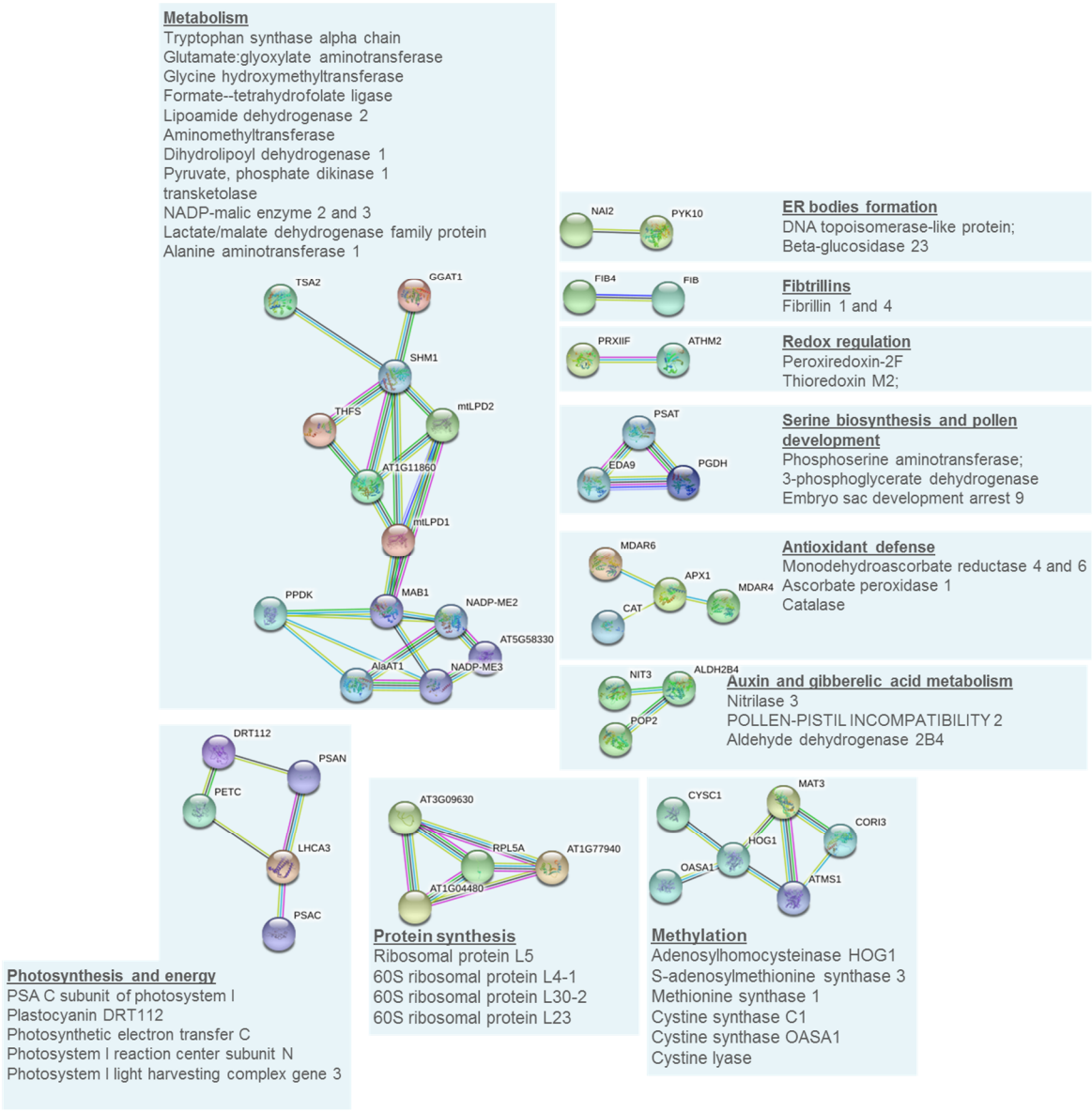

Figure 8 Depiction of protein interaction networks as predicted by STRING analysis performed on differentially regulated proteins found in the *ΔNyda1* mutant.

the response to wounding which was not reported for YODA previously. Altogether, these data favour YODA as regulator of defense responses.

In addition, intriguing upregulations of proteins involved in the protein folding were found in the *yda1* mutant. These were heat shock proteins, luminal binding proteins and protein disulfide isomerases, indicating endoplasmic reticulum (ER) stress in the *yda1* mutant. ER stress is known to activate mammalian MAPK pathways and is important for cell fate determination and apoptosis (Cipolla et al, 2017). Although activation of MAPKs after ER stress has to be experimentally verified

in plants, our findings indicate a possible new link between MAPK signalling and ER stress.

Salt stress activates a MAPK pathway in *Medicago sativa* composed of SIMKK (stress-induced MAPKK) and SIMK (Cardinale et al, 2002). In inactivated states, SIMKK and SIMK co-localize in the cytoplasm and in the nucleus. Upon salt stress, substantial parts of nuclear pools of both SIMKK and SIMK relocate to cytoplasmic compartments. Heterologous expression of chimeric SIMKK–yellow fluorescent protein (YFP) in Arabidopsis enhances an activation of Arabidopsis MPK3 and MPK6 upon salt treatment (Figure 9) and confers high sensitivity of seedlings against salt stress (Ovečka et al, 2014; Supplementary material 10). To better characterize SIMKK-YFP over ressor plants at molecular level and to explain their increased salt stress sensitivity on the proteome level, we performed a comparative shot-gun proteomic analysis using gel free approach (see details of experimental procedures in (Ovečka et al, 2014; Supplementary material 10).

Briefly, roots of 14 days old transgenic Arabidopsis seedlings (in five

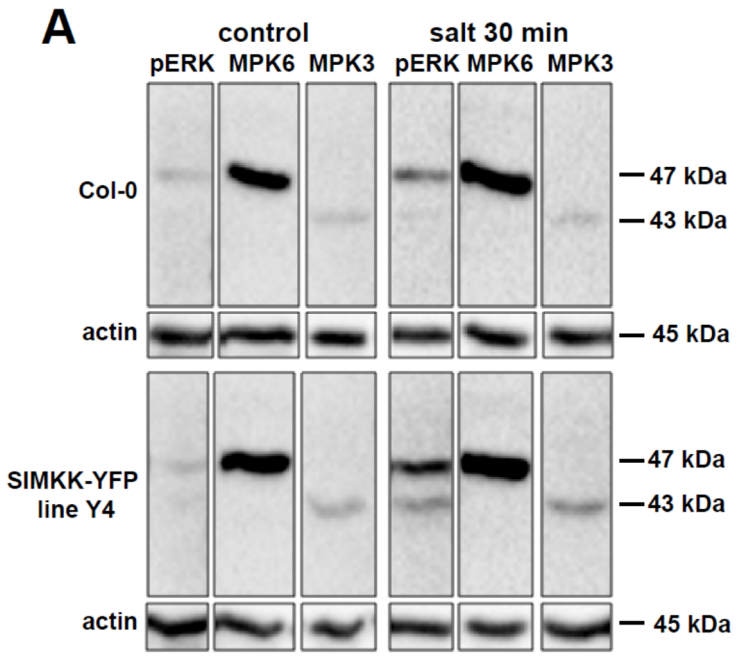

Figure 9 Salt stress (250mM NaCl, 30 min) induced MPK6 and MPK3 activation in stably transformed Arabidopsis line Y4 overexpressing SIMKK–YFP. Root protein extracts separated by SDS-PAGE were probed with antibodies against mammalian ERK1/2 (phospho-p44/42, pERK), Arabidopsis MPK6, and Arabidopsis MPK3. Molecular mass is indicated. Actin was used as a loading control (Ovečka et al, 2014, modified; Supplementary material 10).

independent biological replicates) and wild types were used for proteomic analysis. The preparation of trypsin-digested extracts was performed as described above (see chapter 2.2). For the purpose of quantification, each sample was spiked with predigested bovine hemoglobin. For sample analysis, protein aliquots were analyzed by nanoAcquity UPLC system (Waters) coupled to Premier quadrupole time-of-flight (QTOF) mass spectrometer (Waters). The peptide mixture was injected onto a reverse-phase column (nanoAcquity UPLC column BEH 130 C18) and an ACN gradient (10–50% ACN containing 0.1% formic acid in 60 min) was employed to elute the peptides into the Q-TOF. The column was connected to the PicoTip emitters (New Objective, USA) mounted into the nanospray source of the Q-TOF Premier. A multiplex MS^E approach, in which MS data are collected in an alternating, low energy (MS) and elevated energy (MS^E) mode, was used for protein identification. For MS^E quantification, the average MS signal response from bovine hemoglobin was used to determine the universal signal response factor (USRF, counts/mol of protein). This information was then used to determine the concentrations for each of the target proteins by dividing the MS by USRF. During data acquisition the quadrupole analyzer was not mass selective but operated in the radio-frequency only mode. The MS^E data were processed using PLGS v. 2.4. Each processed file was searched against the non-redundant *Arabidopsis thaliana* UniProt database with addition of internal standard HBA_BOVIN Hemoglobin subunit alpha sequence (Waters: 186002327; NCBI: P01966) using the search algorithm within the PLGS 2.4. In order to determine protein quantities the combined intensity of the multiply charged ions for the three most abundant tryptic peptides of a quantitatively added internal standard was compared with the observed response for any identified protein in a complex mixture. One way Anova statistical analysis was carried out to identify statistically significant ($p < 0.05$) differences in protein amount.

Ten differentially abundant proteins are involved in stress responses, representing the most abundant functional class. Notably, proteins involved in salt-induced oxidative stress (e.g. in H_2O_2 detoxification) such as CAT, PRX and glutathione S transferase as well as nucleoside diphosphate kinase 1, a signalling protein involved in ROS signalling and interacting with CAT (Fukamatsu et al, 2003),

were less abundant in transgenic SIMKK-YFP plants (Ovečka et al, 2014; Supplementary material 10). Next, two peroxidases, namely peroxidase 69 and peroxidase 23, belonging to the class III of peroxidase superfamily, were slightly upregulated in the transgenic plants. Aquaporin PIP2, which is involved in water transport across the plasma membrane, was more abundant in transgenic SIMKK-YFP plants, indicating the accelerated water transport followed by increased loss of water (Katsuhara et al, 2003). Germin like protein subfamily 2 member 1 (upregulated 2.6 fold in SIMKK-YFP plants) was described as plasmodesmata-localized protein, facilitating plasmodesmata permeability when overexpressed in Arabidopsis (Ham et al, 2012). In summary, we suggest that SIMKK overexpression negatively regulates the key decomposers of H_2O_2 and facilitates the water loss, which were crucial for the changed sensitivity of the transgenic plants to the salt stress (Ovečka et al, 2014; Supplementary material 10).

3.3. Proteomic analyses of Arabidopsis mutants deficient in MAPKs

MPK4 and MPK6 are commonly activated by cold and salt stresses downstream of MEKK1 (MAP3K) and MKK2 (MAP2K) in Arabidopsis (Teige et al, 2004), while MPK4 also negatively regulates salicylic acid (SA) and ROS production via this pathway (Gao et al, 2008). MEKK1 dependent activation of MPK6 (together with MPK3) via MKK4/MKK5 was reported also in the response to bacterial elicitor flagellin. This signalling cascade affects expression of defense related genes (Asai et al, 2002). The MKK4-MPK6 module also phosphorylates and activates ACS2 and ACS6 which are key enzymes for ethylene synthesis (Liu and Zhang, 2004). Additionally, MPK6 is specifically activated by MKK3 in response to jasmonic acid (JA) and negatively regulates the JA signalling pathway (Takahashi et al, 2007). MPK6 is also activated by the pathway that includes ANP1 and MKK4/MKK5 upon H_2O_2 treatment (Kovtun et al, 2000). In contrast, MPK4 is activated by MEKK1–MKK2 pathway under oxidative stress (Pitzschke et al, 2009).

Thus, MPK4 and MPK6 are MAPKs converging many signalling cascades. Respective *mpk4* and *mpk6-2* mutants exhibit marked differences in their phenotypes (Figure 10). Similarly to other analyzed MAPK mutants, shot-gun differential

proteomic analyses revealed changes in proteomes that were correlated with these different phenotypes (see also Beck et al. 2010; Mueller et al. 2010).

Peptide mixtures were prepared from roots of *mpk4* and *mpk-2* mutants as described above (see chapter 2.2). Spectral data were collected using an Orbitrap LTQ Velos mass spectrometer (Thermo Fisher Scientific) using Xcalibur version 2.1.0 united with an UltiMate 3000 nano flow HPLC system (Dionex). Protein tryptic digest were separated on reversed phase fused silica C18 column (Thermo Fisher Scientific). Peptides were eluted at a constant flow rate of 0.3 µ l.min-1 by a 170-minute long nonlinear gradient of ACN (in 0.1% formic acid) as follows: 2–55% for 125 min, 95% for 15 min, 5% for 30 min. Peptides were detected in linear trap mass spectrometer operated in a data dependent acquisition (DDA) mode. The spectral data were matched against target and decoy databases. The NCBI (www.ncbi.nlm.nih.gov) Arabidopsis genus taxonomy referenced protein database served as the target database. The relative quantitative analysis was based on sums of precursor ion intensities of filtered peptides attributed to given proteins.

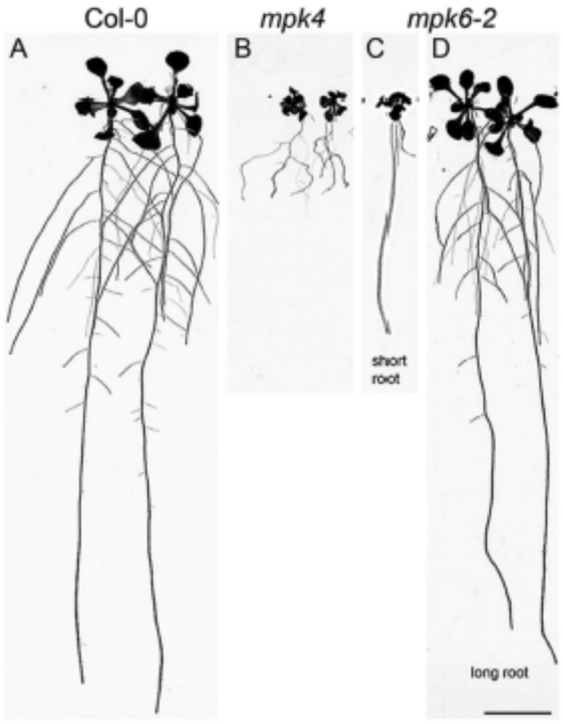

Figure 10 Representative pictures of 14 days old wild type plants Col-0 (A), *mpk4* (B) and *mpk6-2* short (C) and long root (D) mutants used for proteomic and biochemical analyses. Bar: 1 cm. (Takáč et al, 2016b, Supplementary material 7).

The numbers of differentially abundant proteins in the *mpk4* and *mpk6* mutants seemingly reflected their phenotypes (Figures 10 and 11) while only a low number of proteins was found as differentially abundant in both mutants.

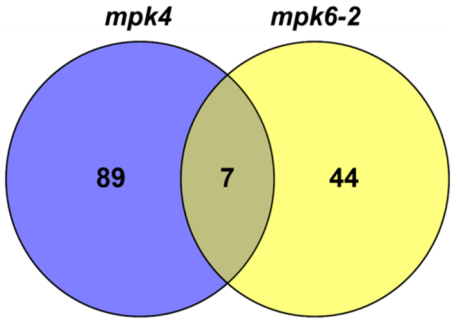

Figure 11 Venn diagram showing the numbers of differentially abundant proteins in *mpk6-2* and *mpk4* (Takáč et al, 2016b, Supplementary material 7) Arabidopsis mutants as compared to the wild type.

Impact of MPK4 and MPK6 on antioxidant defense was described above (see chapter 2.3). Interestingly, both *mpk4* and *mpk6-2* mutants possessed a number of differentially regulated proteins related to various developmental processes. Most importantly, *mpk4* mutant is likely affected in actin cytoskeleton organization, because of impaired abundances of key regulators of actin polymerization such as profilins 1 and 2 (confirmed also by immunoblotting) and actin depolymerizing factor 3. MAPK-dependent changes of the actin cytoskeleton are not well-described and our results indicate that MPK4 might regulate actin cytoskeleton.

MAPKs represent the last members of the MAPK cascades. Therefore, it is reasonable to apply bioinformatic prediction tools to find putative MPK4 and MPK6 phosphorylation targets among the differentially abundant proteins. The criteria for the prediction were the presence of MAPK-specific phosphorylation and docking sites, as well as predicted or experimentally-proved common localization of target proteins with the respective MAPK.

This analysis identified several very promising MAPK targets while two proteins were previously experimentally approved as MAPK substrates (Takáč et al, 2016b, Supplementary material 7). We have found phospholipase D alpha 1 (PLDa1) as a probable target candidate of MPK6. This indicates that PLDa1 might be

phosphorylated by MAPKs and modulates microtubule organization. MPK4 possibly phosphorylates mRNA decapping protein VARICOSE (VCS). This protein plays a crucial role in mRNA directed gene silencing. Since MPK4 negatively controls expression of genes involved in the pathogen defense (Kong et al, 2012) we suggest a possible mechanism for this negative regulation occurring through the phosphorylation of VCS. However, this needs to be experimentally tested and further validated.

We also detected a promising candidate for a new MAPK scaffold protein. A remorin family protein (AtRem1.3) showed decreased abundance in the *mpk4* mutant. AtRem1.3 is differentially phosphorylated upon treatment with bacterial elicitors and plays a role as scaffold protein in plant innate immunity (Jarsch and Ott, 2010). Our data provide a preliminary evidence for possible co-regulation of AtRem1.3 and MAPK signalling.

3.4. Conclusions and future prospects

Shot gun proteomic analysis of MAPK mutants is feasible for monitoring processes downstream of MAPK cascades. It may effectively supplement phosphoproteomic surveys. We also showed feasibility of various bioinformatic tools for the interpretation of shot-gun proteomic data. We identified new processes likely governed by MAP3Ks and MAPKs. Complementary approaches are necessary to validate our data. It is also of great interest to examine mutant proteomes under environmental stresses since conditional proteomes might differ from those under optimal developmental conditions. Furthermore, subcellular proteomics and phosphoproteomics might bring a higher proteome resolution and could diversify MAPK functions under stress-induced subcellular relocation of signalling components. More targeted approaches, such as identification of MAPK interactomes would be helpful to understand complex regulation of MAPK cascades and to identify new scaffold proteins.

4. Proteomic approaches elucidating plant vesicular trafficking

4.1. State of the art

Highly dynamic endomembrane system ensuring intracellular vesicular transport of proteins, lipids and other molecules is essential for cell division, growth, differentiation and overall physiology of living cells. Such vesicular trafficking system is subjected to strict regulation by complex network of proteins and signalling lipids (Müller et al, 2007; Blanchoin et al, 2010; Noack and Jaillais, 2017). Generally, endomembrane system is composed of membranous organelles including endoplasmic reticulum, Golgi apparatus, *trans*-Golgi network (TGN), multivesicular body (MVB) vacuole and endosomes. Most important transport routes in plant cells are illustrated on Figure 12. The vesicular transport and exchange of cargo occurs

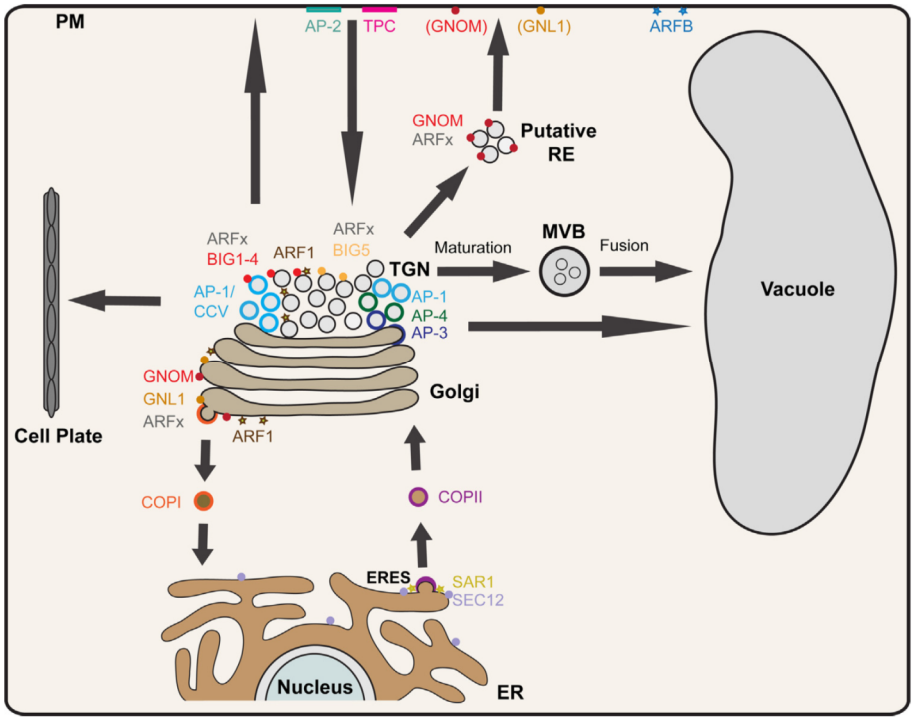

Figure 12 Major trafficking routes in Arabidopsis. The secretory pathway include the transport of newly synthetized proteins from endoplasmic reticulum to their resident compartment. In a process called endocytosis, the plasma membrane proteins are internalized into vesicles that are further transported to other destinations, including the *trans*-Golgi network (TGN) and multivesicular bodies (MVB) Some proteins go from either the TGN/EE or MVB/PVC back to the PM by a recycling route in recycling endosomes (RE) For others, the endocytic pathway ends at the vacuole, where they are degraded. Function of the key regulatory proteins are also depicted (Singh and Jürgens, 2017).

via vesicle formation, trafficking, maturation and fusion with target compartment. All of these processes are under the strict control of diverse proteins and protein complexes including ADP-ribosylation factors (ARFs), Rab and Rop (Rho of plants) GTPases, superfamily of N-ethylmaleimide-sensitive factor adaptor protein receptors (SNAREs), sorting nexins, coating and adaptor proteins, dynamins and tethering complexes, and phosphoinositide metabolizing enzymes (Norambuena and Tejos, 2017). Phospholipids are determinants of organelle identity and regulators of membrane trafficking (Noack and Jaillais, 2017). They recruit important vesicular trafficking regulators which are executing membrane fusion events.

Cargo proteins include among others components of signalling (e.g. plasma membrane-localized receptors and their ligands), transporters of ions, nutrients and hormones, and core components of the membrane fusion machinery such as SNARE proteins, integral membrane proteins that are characteristic for specific endomembrane compartments. In addition to delivering cargo proteins to their sites of action, membrane trafficking is also required to remove proteins from their sites of action and target them to a lytic compartment (vacuole or lysosome) for degradation (Singh and Jürgens, 2017).

The movement and morphology of most organelles, including endosomes, is depending on actomyosin cytoskeleton in plants (Voigt et al, 2005). Interactions between organelles and actin cytoskeleton are mediated by proteins with the simultaneous ability to bind membranes and the actin cytoskeleton. Such candidates include the NETWORKED protein family (Duckney et al, 2017), small Rop GTPases (Gu et al. 2003; Lin et al. 2015), formins and profilins (Takáč et al, 2011, Supplementry material 3; Li et al, 2017b) or membranous SYP73 which associates with the ER membrane and binds actin directly (Cao et al, 2016b). Very recently, membrane-anchored receptors and adaptors binding myosins XI have been identified (Kurth et al, 2017).

Targeted membrane transport is required for sustainable tip growth in root hairs and pollen tubes (Ovečka et al, 2012), where it ensures polarized supply of new membrane and cell wall material. Vesicular trafficking is essential for cell division, namely for cell plate formation (Dhonukshe et al, 2006). Moreover, endocytosis is

involved in the internalization of cell surface receptor kinases and their recycling (Irani and Russinova, 2009) and also in the internalization of pathogen associated molecular patterns during immune response (Gross et al, 2005). Therefore, vesicular transport is very important for biotic (Gross et al, 2005; Chinchilla et al, 2007) and abiotic stress resistance (Bolte et al, 2000; Ebine et al, 2011).

Current plant research in this field is focused on the identification of proteins regulating vesicular trafficking. Proteomics, as a method capable to identify and quantify proteins in a large scale, proved to be a suitable approach for this goal. Recent proteomic approaches on vesicular trafficking include for example proteomic analyses of affinity purified membranous proteins derived from Arabidopsis cells transfected with vesicle-localized protein fused to GFP (Drakakaki et al, 2012; Parsons et al, 2012).

4.2. Applications of chemical inhibitors for proteomic analyses of vesicular trafficking

Endomembrane vesicular trafficking might be selectively targeted by chemical compounds (Figure 13). Thus, pharmacological inhibition of certain vesicular transport routes related to TGN or endosomes leads to the intracellular accumulation of cargo and regulatory proteins transported by these routes. In this manner, the proteomic analysis of inhibitor-treated plants provides a great tool to identify new proteins involved in the vesicular trafficking. Furthermore, physiological consequences of impaired endomembrane transport might be uncovered.

Effective dissection of proteome-wide effects of small chemical molecules on biological systems is determined by the knowledge of their molecular targets and function. This allows straightforward interpretation of the obtained data. Further, to avoid secondary effects, the chemical of choice should be applicable in low doses (preferable in nanomolar or micromolar range). Notably, the output of proteomic studies investigating subcellular effects of chemical inhibitors might be substantially enriched by further cell biological observations, as recently reviewed by (Takáč et al, 2017b, Supplemental material 11). These along with other methods such as

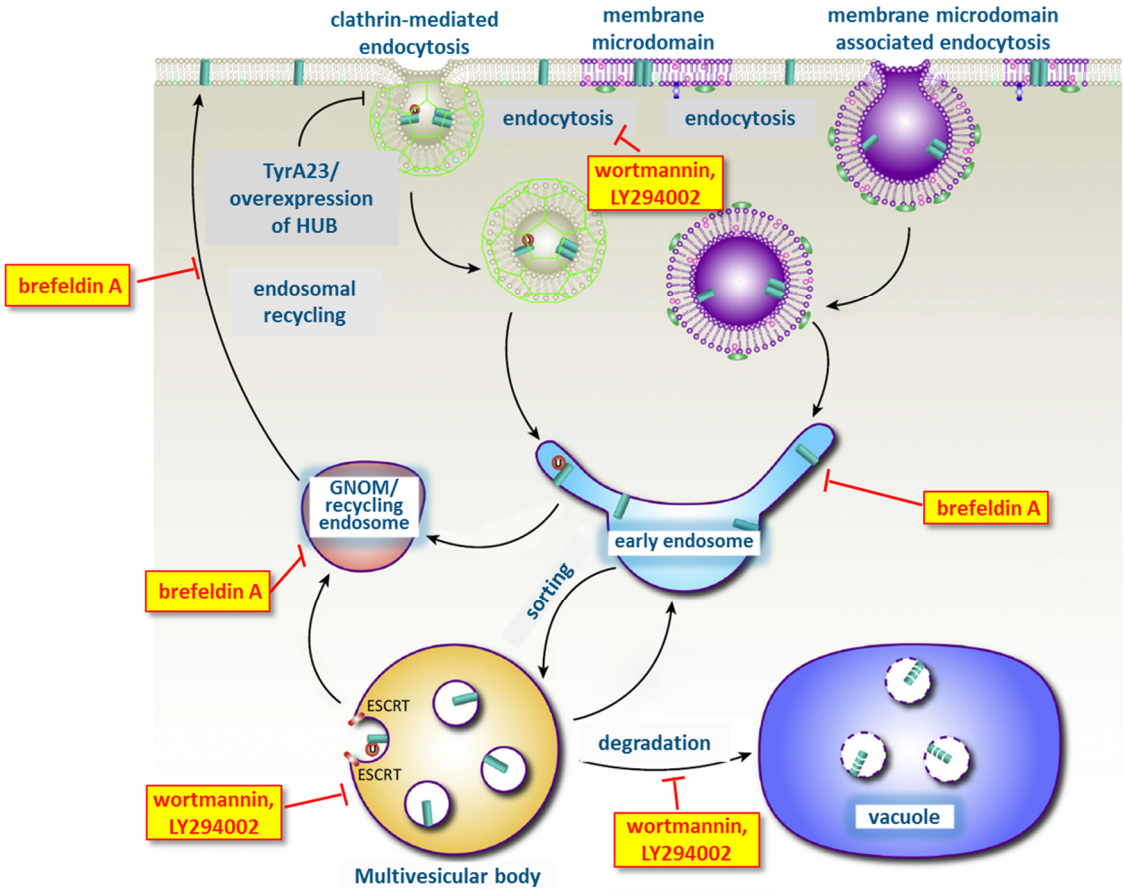

Figure 13 Depiction of brefeldin A , wortmannin and LY294002 inhibitory effects (Fan et al, 2015, modified).

biochemical analyses and physiological experiments may help to validate proteomic data.

In our research, we applied three inhibitors to target different trafficking routes in Arabidopsis roots. Roots were treated with either 50 μM BFA (Takáč et al, 2011; Supplementary material 3) or 33 μM wortmannin and 33 μM LY294004 (Takáč et al, 2012 and 2013; Supplemental materials 4 and 5). Mock controls were used for comparative proteomic analysis. To increase the resolution of the proteome, gel based and gel free proteomic approaches were applied. Within gel based approach, phenol-extracted proteins were separated using 2-D electrophoresis and the proteins were identified as described in (Uváčková et al, 2012, Supplementary material 2). The gel free analysis relied on 2-D liquid chromatographic separation of peptide mixtures, followed by MS/MS mass spectrometry and label-free relative quantification of protein abundances, as described in Takáč et al, 2011 (Supplementary material 3).

Brefeldin A (BFA) targets selected ARF-guanine nucleotide exchange factor (ARF-GEF) proteins. It causes rapid disruption of the secretory and recycling vesicular trafficking pathways (Müller et al, 2007). Subcellular responses to BFA treatment are manifold, depending on the organism and cell type/tissue under study (Robinson et al, 2008). In Arabidopsis root cells, BFA causes an accumulation of TGN secretory and recycling vesicles which aggregate together and form so-called BFA-compartments (Müller et al, 2007). Proteomic analysis of BFA treated Arabidopsis roots may identify proteins accumulating in the BFA compartments.

Other inhibitors, such as wortmannin and LY294002, may be employed to target vacuolar trafficking and dissect proteins taking part in this process (Müller et al, 2007). Wortmannin is a fungal metabolite which targets PI3K and PI4K in a dose-dependent manner (Matsuoka et al, 1995). Wortmannin at low concentrations (up to 1 μM) specifically inhibits PI3K, but at higher concentrations it inhibits both PI3K and PI4K. At the subcellular level, wortmannin affects recycling of the plant vacuolar sorting receptor BP80 from the prevacuolar compartment (PVC)/MVB to the TGN (Tse et al, 2004) thus inhibiting vacuolar trafficking. Wortmannin causes fusion, swelling, and vacuolization of MVB in Arabidopsis root cells (Jaillais et al, 2008, Takáč et al, 2012; Supplementary material 5). In addition to vacuolar sorting, wortmannin inhibits also endocytosis (Emans et al, 2002). It disrupts the spatial organization of apical F-actin in the pollen tube tip and inhibits polar targeting of tobacco pectin methylesterase NtPME1, which subsequently alters the rigidity and pectic composition of the pollen tube cell wall (Wang et al, 2013a).

LY294002 is a synthetic compound based on quercetin specific for PI3K inhibition. Like wortmannin, it competes for ATP binding site of PI3K (Walker et al, 2000) and binds to Lys-833 residue. It inhibits PI3K with an IC_{50} value (concentration required for 50% inhibition) of 0.43 μg/mL (1.40 μM) irrespective of the phosphorylation state of the enzyme (Vlahos et al, 1994). At the subcellular level, LY294002 blocks endocytosis and vacuolar trafficking in a similar manner to wortmannin.

We detected an increased abundance of RabA1d after BFA treatment (Berson et al, 2014; Takáč et al, 2011 Supplementary material 3). RabA1d is a protein belonging to the family of small Rab GTPases (Vernoud et al, 2003). Such increased abundance indicated that RabA1d protein might be accumulated specifically in the BFA compartments. This assumption was strengthened also by the fact that similar members of this protein subfamily exhibit such localization under BFA treatment (Chow et al, 2008). Indeed, detailed cell biological observations revealed that RAbA1d accumulated in the BFA compartments and partially colocalized with TGN markers (Berson et al, 2014; Supplementary material 12). Moreover, we have found that GFP-RabA1d was enriched in root hair bulges and at the apical dome of growing root hairs, indicating its function in root hair elongation. Quantitative live cell microscopy revealed that RabA1d participates on the oscillatory pattern of root hair growth. In dividing root cells, GFP-RabA1d was localized to developing cell plates, consistently with cytokinesis-related vesicular trafficking and membrane recycling. Thus, GFP-RabA1d accumulated in disc-like structures of nascent cell plates, which progressively evolved to marginal ring-like structures of the growing cell plates (Berson et al, 2014; Supplementary material 12). Unlike BFA, wortmannin and LY294002 decreased the abundance of TGN-localized Raba1d (Takáč et al, 2012, 2013; Supplementary materials 4 and 5). This implied previously unknown effect of these PI3K and PI4K inhibitors on early endosomes. Using live cell confocal laser

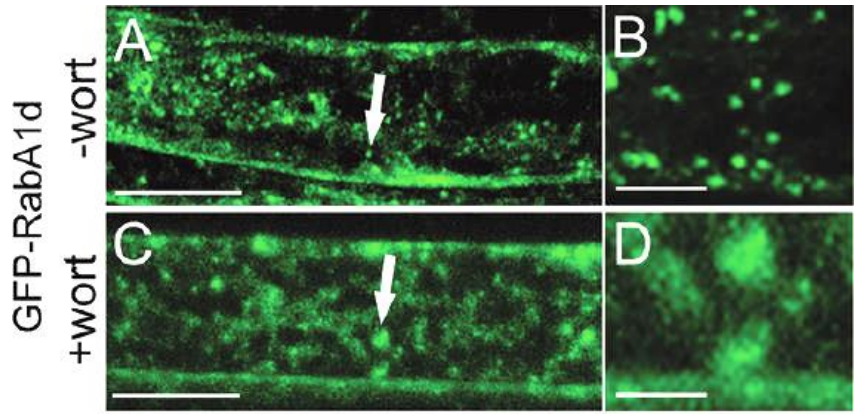

Figure 14 Subcellular distribution of GFP-RabA1d in Arabidopsis root cells treated with 33 μM wortmannin for 2 h as observed by live cell confocal laser scanning microscopy. Bars represent 10 μm (A, C) and 5 μm (B, D) (Takáč et al, 2012; Supplementary material 5).

scanning microscopy we have found that wortmannin caused an accumulation, clustering, fusion and swelling of GFP-RabA1D containing compartments (Figure 14) as well as other TGN markers (Takáč et al, 2012; Supplementary material 5). Detailed electron microscopy (EM) observations showed the significant depletion of TGN vesicles suggesting either progressive fusion of TGN with MVBs or heavy disintegration of TGN caused by 33 μM wortmannin. Consistently with the first alternative, EM analysis revealed mutual attachments and contacts of MVBs and TGN vesicles eventually leading to heterotypic fusions of MVBs with post-Golgi TGN vesicles (Figure 15; Takáč et al, 2012; Supplementary material 5).

In addition to these effects, our proteomic analysis revealed several new phenomena occurring upon BFA, wortmannin and LY294002 treatments in Arabidopsis. Accumulation of ER resident proteins in response to BFA indicated possible alterations of ER morphology. EM observations showed BFA-dependent swelling and proliferation of ER, which was not reported for Arabidopsis roots so far (Takáč et al, 2011; Supplementary material 3). We suggest that this might be caused

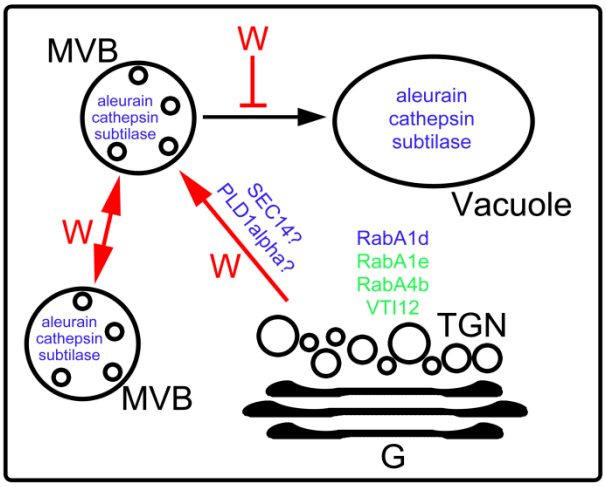

Figure 15 Schematic overview of wortmannin (abbreviated to W) effects on vesicular trafficking as revealed by this study. Red arrows indicate heterotypic fusions between trans-Golgi network (TGN) and multivesicular bodies (MVB) leading to consumption of TGN vesicles by enlarged MVBs. Bipolar red arrow indicates homotypic fusions of MVBs. Wortmannin inhibits transport from these MVB compartments to the vacuole. The putative roles of phospholipase D alpha 1 (PLDa1) and SEC14 are suggested for the transport from TGN to MVB. Proteins identified by proteomic approaches at diverse subcellular locations (TGN, MVB, and vacuole) are depicted in blue. In addition, other TGN localized small GTPases as shown by confocal microscopy are depicted in green (Takáč et al, 2012; Supplementary material 5)

by the accumulation of misfolded proteins, deducing from differential abundance of key proteins involved in unfolded protein response (Takáč et al, 2011; Supplementary material 3). Importantly, we have also identified profilin 2, a canonical actin cytoskeletal regulator, to be involved in the formation of BFA compartments. Profilin 2 accumulates in BFA compartments and might locally promote actin polymerization around and within these compartments. Such hypothesis is consistent with observation of local enrichment of actin filaments around BFA compartments (Takáč et al, 2011; Supplementary material 3).

Both wortmannin and LY294002 lowered abundances of vacuolar hydrolases, most likely due to the impaired vacuolar transport (Takáč et al, 2012, 2013; Supplementary materials 4 and 5). At the same time, we observed a robust accumulation of storage proteins in Arabidopsis roots treated with LY294002 (Takáč et al, 2013; Supplementary material 4). Whole mount immunolabelling of Arabidopsis roots with specific anti-2S albumin antibody confirmed this assumption at the subcellular level. Thus, our study provided new information about storage proteins and vacuolar hydrolases in vegetative tissues treated by LY294002.

We also found new proteins involved in regulation of vacuolar transport. ARFA1f was upregulated after LY294002 treatment (Takáč et al, 2013; Supplementary material 4). In the GTP-bound active states, ARF GTPases recruit coat proteins to patches of specific membranes to induce vesicle budding (Vernoud et al, 2003). ARFA1c, which is closely related to ARFA1f (differs only in one AA), was reported to control vacuolar trafficking and also secretion of proteins carrying vacuolar sorting signal (BP80 receptor ligands) (Pimpl et al, 2003). Our proteomic analysis suggests that ARFA1f could be involved in vacuolar trafficking in Arabidopsis roots, and its upregulation might be related to swelling and aggregation of late endosomes/MVBs upon treatment with LY294002 (Takáč et al, 2013; Supplementary material 4). We have found that a big portion of wortmannin-affected proteins are predicted as anchored in the membrane through a covalently attached glycosylphosphatidylinositol moiety (Takáč et al, 2012; Supplementary material 5). Previous studies revealed that such proteins might undergo endocytosis (Lakhan et al, 2009). Accordingly, we generated a list of candidate proteins for endocytic

transport (Takáč et al, 2012; Supplementary material 5). Our proteomic analysis also revealed that wortmannin altered proteins involved in nuclear and mitochondrial import and export, as unknown consequences of PI3K/PI4K inhibition. The nuclear import and export is likely controlled by PI4Ks, which are bearing the nuclear localization signal in their protein sequences (Takáč et al, 2012, Supplementary material 5).

4.3. Conclusions and future prospects

We showed that treatments with inhibitors of vesicular trafficking might have also unexpected consequences and should be considered in cell biology studies. Moreover, we identified several new cargo and regulatory proteins. Specific functions and trafficking routes of these proteins have to be uncovered by additional experiments. Indeed, validation by independent experiments is extremely important also in the case of proteomic analyses using inhibitor-treated plant tissues. In the future, BFA should be tested on various plant tissues, since its effect varies in tissue- but also species-dependent manner. Recently, several new inhibitors of subcellular vesicular trafficking have been developed, whose effects should be dissected by proteomic tools.

5. Detecting new cytoskeletal functions

5.1. State of the art

Cytoskeleton is a highly dynamic filamentous system composed of actin filaments and microtubules, regulated by actin-binding and microtubule-associated proteins (ABPs and MAPs). Actin filaments (F-actin) and microtubules are capable of rapid elongation or shortening driven by polymerization and depolymerization of the basic structural components (G-actin and tubulins). This, together with other mechanisms including nucleation, branching, severing and bundling determine the spatiotemporal organization of the cytoskeleton, which is crucial for plant growth, development and morphogenesis (Blancaflor et al, 2006; Wasteneys and Ambrose, 2009).

Microtubules are highly dynamic polymers of α tubulin and β tubulin heterodimers. The nucleation of microtubules requires γ-tubulin and GCP (γ-tubulin complex proteins) containing complexes. Microtubule organization and dynamics are controlled mainly by MAPs, plus-end binding (EB1) proteins, microtubule severing protein katanin, microtubule destabilizing protein 25 (MDP25), PLDa1, kinesins (motor proteins) and others (Gardiner, 2013). Some of these proteins might be regulated by signaling molecules such as MAPKs, Rop GTPases, Ca^{2+} and phosphatidic acid (PA) (Yalovsky et al, 2008; Beck et al, 2010; Zhang et al, 2012). Microtubules are under hormonal regulation, as they are reoriented upon diverse hormonal treatments (Shibaoka, 1994; Lochmanová et al, 2008). Microtubules are also sensitive to ROS. MAP65 is required for ROS signalling and transcriptional activation of antioxidative defense (Zhu et al, 2013; Livanos et al, 2014).

Assembly of actin filaments occurs through nucleation and elongation. The formation of F-actin arrays depends on the interactions of actin monomers (G-actin) and ABPs such as profilins, actin depolymerizing factors, formins, Rop GTPases, Arp2/3 complex and others (Blanchoin et al, 2010; Cao et al, 2016a). Actin is also regulated by signalling proteins and secondary messengers such as Ca^{2+} or PA (Staiger and Blanchoin, 2006, Pleskot et al, 2013). Actin itself may play signalling role and its polymerization status or reorganization is important for the expression of

proteins related to abiotic and biotic stresses (Olson and Nordheim, 2010). F-actin has a profound role in the vesicular trafficking, mainly in the endocytosis of receptor kinases (Beck et al, 2012). Actin is also affected by redox regulation and ROS (Wilson and González-Billault, 2015). After elicitation by diverse microbe or damage-associated molecular patterns, ROS serves as a common upstream signals mediating actin remodelling (Li et al, 2017a).

Such interactions with signalling molecules and proteins couple actin filaments and microtubules with external environment and mediate its developmental or conditional rearrangements.

Actin filaments and microtubules are tightly linked and co-regulated (Blancaflor, 2000; Collings, 2008). Reorganization and reassembly of actin filaments depend on microtubules (Sampathkumar et al, 2011). This interplay is likely governed by proteins which are capable to bind and modulate both microtubules and actin filaments. These are for example formins (Rosero et al, 2013), microtubule depolymerizing protein 25 (Li et al, 2011) or Rop GTPases (Fu et al, 2009).

Cytoskeleton plays crucial roles in plant growth and development. Microtubules are involved in cell elongation through the control over orientation of cellulose microfibrils in cell walls by directing movement and targeting of cellulose synthases (McFarlane et al, 2014; Kong et al, 2015;). During mitosis, microtubules form specific arrays, such as preprophase band, spindle and phragmoplast which determine cell division plane orientation and the partitioning of genetic material between two daughter cells (Müller and Jürgens, 2016). Fine F-actin delivers Golgi-derived (post-Golgi) vesicles (containing cell wall matrix components) to the plasma membrane area where exocytosis occurs. In this manner actin is required for root hair and pollen tube tip growth (Šamaj et al, 2004; Ovečka et al, 2012). It also controls stomata openings and cell elongation (Cao et al, 2016a). Actin remodelling serves as an important signalling component during plant immune response (Li et al, 2017a).

Cytoskeletal functions might be addressed by different approaches including genetic manipulations or pharmacological treatments affecting polymerization status of actin filaments and microtubules. F-actin may be targeted chemically by inhibitors such as cytochalasins, latrunculins and jasplakinolide (Peterson and Mitchison, 2002,

Sawitzky et al, 1999). Cytochalasins might inhibit polymerization by capping free barbed ends, thus preventing the addition of G-actin to actin filaments, leading to net-depolymerization (Brown, 1981). Latrunculins are natural cell permeable macrolides, which bind specifically to the ATP-binding site of actin monomer (G-actin) by hydrogen bond, thus changing its structure and preventing G-actin polymerization. As a result, latrunculin B causes shift from F-actin to G-actin (Baluška et al, 2001; Morton et al, 2000). Jasplakinolide is a natural cyclodepsipeptide inducing stabilization of F-actin (Sawitzky et al, 1999).

We attempted to study the microtubule and actin cytoskeletons in Arabidopsis using proteomics. We applied shot–gun proteomic analysis combined with cell biological observations on Arabidopsis mutants defective in the microtubule severing protein KATANIN1 (Takáč et al, 2017a; Supplementary material 13). Actin cytoskeleton was studied by shot–gun proteomic analysis of latrunculin B-treated Arabidopsis roots (Takáč et al, 2017c; Supplementary material 14).

5.2. Proteomic dissection of *KATANIN 1* mutants

KATANIN 1 is a microtubule severing AAA-ATPase assembled from a catalytic subunit of 60 kDa and a structural 80 kDa subunit (Hartman et al, 1998). At the cellular level, the severing activity of KATANIN 1 was shown to regulate plant microtubule organization (Nakamura, 2015). KATANIN 1 severing activity is induced by ROP-GTPase signalling (Lin et al, 2013) and can be modulated by other microtubule binding proteins like SPIRAL2 (Wightman et al, 2013).

KATANIN 1 is required for multiple developmental processes. Therefore, *KATANIN 1* mutants exhibit reduced root, hypocotyl, stem and leaf growth as well as stubby flower organs showing reduced anther length and fertility, defects in ovule and anther development, aberrant embryogenesis and seed formation (Bichet et al, 2001; Burk et al, 2001; Luptovčiak et al, 2017). It is generally accepted that these phenotypes are caused by reduced cell expansion (Bichet et al, 2001; Burk et al, 2001). Recently, altered cell elongation was linked with specific defects in dynamic organization of cortical microtubules in the *knt1-2* mutant (Komis et al, 2017). It was also proposed that *KATANIN 1* mutants exhibit defects in the formation and

maturation of preprophase band, rectification of cell division plane, multipolar spindles and disorientation of the cell division plane (Panteris et al, 2011; Komis et al, 2017). KATANIN 1 links hormonal signals with microtubule reorientation, because respective mutants displayed altered auxin, gibberellic acid (GA) and ethylene responses (Meier et al, 2001; Bouquin et al, 2003; Nakamura, 2015).

We performed a differential proteomic analysis comparing two Arabidopsis *KATANIN 1* mutants (*fra2* and *ktn1-2)* and the wild type Col-0 plants (Figure 16).

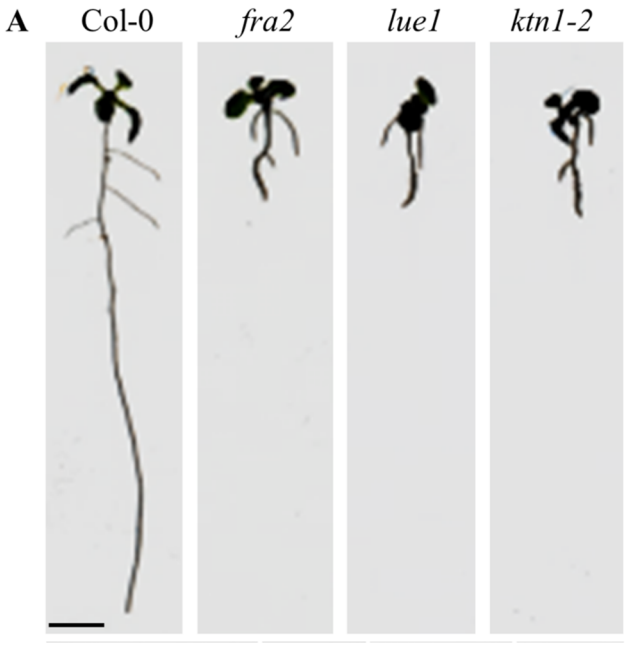

Figure 16 Representative pictures of whole Col-0 seedlings and KATANIN mutants. Note much shorter roots in the mutants (Luptovčiak et al, 2017b).

Fra2 is a single nucleotide mutant in the seventh exon of *KATANIN 1* (Burk et al, 2001). *Ktn1-2* is a knockout mutant with T-DNA inserted after the 147th nucleotide in the 5th exon of *KATANIN 1* (Nakamura, 2015). Proteomic analysis was conducted according to Takáč et al, (2016b) (Supplementary material 7). Differential proteome was evaluated using bioinformatic analyses including GO annotation and STRING. The most important findings were validated by immunoblotting, immunolabelling and quantification of transcript levels.

We have found a feedback regulation of microtubule organization by deregulation of tubulins and microtubule regulatory proteins such as MDP25 in the

KATANIN 1 mutants (Takáč et al, 2017a; Supplementary material 13**)**. This was supported by altered organization of cortical microtubules. Actin cytoskeleton was also modulated due to the altered abundances of several ABPs, again leading to reorientation and disorganization of the actin cytoskeleton. MDP25 might represent a protein linking microtubules with actin filaments.

In addition to auxin, gibberellic acid and ethylene, *KATANIN 1* mutants exhibit differential accumulation of proteins involved in ABA biosynthesis. Therefore, KATANIN 1 might be a protein integrating multiple hormonal responses in order to reorient microtubules. We also observed altered abundances of Tudor staphylococcal nuclease proteins (TSN1 and 2) which are responsible for stress-induced mRNA decapping in stress granules (Gutierrez-Beltran et al, 2015). *KATANIN 1* mutants also showed changes in the nuclear shape and size, which is likely mediated by tryptophan-proline-proline (WPP)-domain protein WPP2 upregulation. Based on qPCR analyses, expression levels of corresponding genes in the mutants were not altered (Takáč et al, 2017a; Supplementary material 13).

5.3. Applications of chemical inhibitors for proteomic dissection of cytoskeletal regulation

Previously reported studies showed that proteomic analyses of plant cells treated by cytoskeletal inhibitors provided very valuable information about cytoskeletal regulation and physiological processes controlled by cytoskeletal organization. Such pharmaco-proteomic approach was used in *Picea meyeri* pollen tubes treated with actin depolymerizing drug latrunculin B (Chen et al, 2006). Proteins involved in cytoskeletal regulation, signalling, cell wall expansion and carbohydrate metabolism were detected to be connected to the actin depolymerization.

At the subcellular level, latrunculin B perturbs actin distribution and causes depletion of F-actin. Complexes of G actin and latrunculin exert diffuse fluorescence signal and accumulate in the nuclei of Arabidopsis cells (Baluška et al, 2000). By disrupting actin cytoskeleton latrunculin B effectively alters dynamics of vesicular trafficking. Thus, latrunculin B can inhibit endosomal movements (Voigt et al, 2005).

Close subcellular investigation of growing pollen tubes revealed a dramatic effect of latrunculin B on the ultrastructure of the Golgi apparatus, mitochondria and amyloplasts (Chen et al, 2006). Finally, latrunculins severely disturb developmental processes in plants.

We aimed to decipher proteome-wide effect of latrunculin B on Arabidopsis roots. This approach can reveal differentially abundant proteins after actin depolymerization as well as actin-dependent developmental processes and downstream signalling targets sensing actin disruption.

We employed similar experimental approach as for inhibitors of vesicular trafficking with small modifications. Both gel based (according to Takáč et al, 2011)

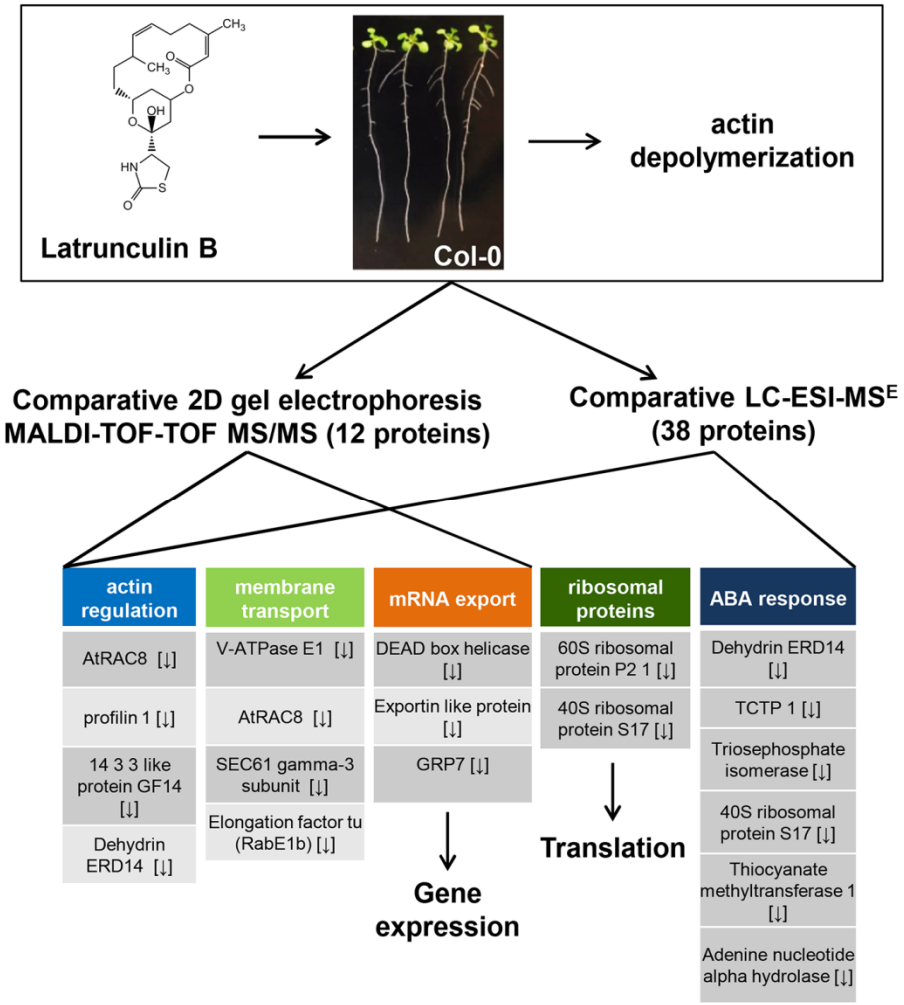

Figure 17 Schematic overviewof proteins involved in actin regulation,membrane transport, mRNA export, translation and ABA response as identified by the present proteomic analysis using latrunculin B. Arrows in brackets indicate changes in abundances after latrunculin B treatment (Takáč et al, 2017c; Supplementary material 14).

and gel free (according to Ovečka et al, 2014) proteomic analyses were performed on roots of 14 days old Arabidopsis seedlings treated with 0.5μM latrunculin B for 2 h and mock controls.

As expected, our proteomic analysis detected downregulation of actin (isoform 5) and ABPs (Takáč et al, 2017c; Supplementary material 14). This indicates a feedback mechanism regulating abundances of actin 5, profilin 1 and 2 in dependence on F-actin levels. Actin depolymerization by latrunculin B affected also abundances of proteins involved in vesicular trafficking and mRNA export, as suggested by downregulation of three such proteins. This may represent a mechanism linking actin to the expression of stress-related genes. Actin polymerization seems to be required for the homeostasis of proteins involved in ABA responses.

Thus, the status of the actin polymerization may serve as a sensing mechanism for changes in hormonal signalling, translation or gene expression (Figure 17; Takáč et al, 2017c; Supplementary material 14).

5.4. Conclusions and future prospects

In conclusion, described combination of proteomic and cell biology analyses uncovered new important functions of the plant cytoskeleton. We found important proteins linking microtubule severing to the actin cytoskeleton, cell plate formation and nuclear shape control. We also detected proteins connecting actin polymerization in Arabidopsis roots with ABA signalling and vesicular trafficking. We identified specific proteins which are sensitive to cytoskeletal changes and control gene expression either through mRNA silencing or induction of transcription. Further research is required to investigate interactions between these proteins and the cytoskeleton. Our results represent an important step in deciphering the gene expression regulation by cytoskeletal rearrangements.

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7. Abbreviations

ABA - abscisic acid

ABPs - actin-binding proteins

ACN - acetonitrile

ACS - aminocyclopropane-1-carboxylic acid synthase

ANP - *Arabidopsis thaliana* homologues of nucleus- and phragmoplast-localized kinases

APX - ascorbate peroxidase

BFA - brefeldin A

CAT - catalase

DAPI - 4',6-Diamidine-2'-phenylindole dihydrochloride

DHAR - dehydroascorbate reductase

EM - electron microscopy

ERF - ethylene-responsive element binding factor

ESI - electrospray ionization

GME - GDP-D-mannose 3′,5′-epimerase

GRX - glutaredoxin

GSNOR - S-nitrosoglutathione reductase

GEF - guanine nucleotide exchange factor

IAA - indole-3-acetic acid

LC - liquid chromatography

MALDI - matrix-assisted laser desorption/ionization

MAPs - microtubule associated proteins

MAPK - mitogen activated protein kinase

MDAR - monodehydroascorbate reductase

MDP25 - microtubule destabilizing protein 25

MAPKK, MAP2K - mitogen activated protein kinase kinase

MAPKKK, MAP3K - mitogen activated protein kinase kinase kinase

MVB - multivesicular body

NAA - 1-naphthaleneacetic acid

NBT - nitroblue tetrazolium chloride

NO - nitric oxide

PA - phosphatidic acid

PI3K - phosphatidylinositol 3 kinase

PI4K - phosphatidylinositol 4 kinase

PLDa1 - phospholipase D alpha 1

PLGS - ProteinLynx Global Server

PRX - peroxiredoxin

PVC - pre-vacuolar compartment

RbohD - respiratory burst oxidase homolog D

ROP - Rho of plants

ROS - reactive oxygen species

SCX - strong cation exchange

SIMK - stress induced MAPK

SIPK - salicylic acid induced protein kinase

SOD - superoxide dismutase

SNARE - superfamily of N-ethylmaleimide-sensitive factor adaptor protein receptor

TGN - *trans*-Golgi network

TSN - TUDOR staphylococcal nuclease

USRF - universal signal response factor

UPLC - ultra performance liquid chromatography

VCS - VARICOSE

WIPK - wound-induced protein kinase

WPP2 - tryptophan-proline-proline (WPP)-domain protein 2

8. List of publications related to this thesis (attached as supplementary materials 1-14)

1. **Takáč T**, Pechan T, Richter H, Müller J, Eck C, Böhm N, Obert B, Ren H, Niehaus K, Šamaj J (2011) Proteomics on brefeldin A-treated Arabidopsis roots reveals profilin 2 as a new protein involved in the cross-talk between vesicular trafficking and the actin cytoskeleton. Journal of Proteome Research 10, 488–501.
2. **Takáč T**, Pechan T, Šamajová O, Ovečka M, Richter H, Eck C, Niehaus K, Šamaj J (2012) Wortmannin treatment induces changes in Arabidopsis root proteome and post-Golgi compartments. Journal of Proteome Research 11, 3127–3142.
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9. Supplementary materials