Subcellular localization and characterisation of MAPKs, and cytoskeleton adaptation to stress in *Arabidopsis* roots

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Jens Müller

aus

Leverkusen

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1. Referent: PD Dr. Jozef Samaj
2. Referent: Prof. Dr. Diedrik Menzel

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1. INTRODUCTION

1.1 General background

The constantly changing environment is one of the most important challenges for each individual organism. The ability to undergo adaptation determines as to how successful an organism can cope with environmental stresses and pathogen attack. Eventually, the ability to adapt decides on the survival and propagation of a species. In contrast to animals, plants are sessile organisms, unable to move away from unfavorable changes in the environment but rather react by metabolic adaptation involving rapid sensing mechanisms followed by the appropriate responses ranging from the cellular to the systemic level.

On the cellular level, stress response starts with the perception of an extracellular stimulus and is followed by signal transduction through the cell. Each signalling event causes the formation of a specific pattern of enzymatic activities and gene expression. One general mechanism that allows propagation of a signal is the transient phosphorylation and dephosphorylation of proteins that are organized in cascade like manner. Protein phosphotransferases, also called protein kinases are found in all organisms and are highly conserved among all organisms. It is estimated that about 30% of all proteins of eukaryotic cells are phosphorylated by protein kinases.

Due to the decoding of several genomes, the high complexity of signalling mechanisms in plants appeared obvious. While the human genome encodes 518 protein kinases, Arabidopsis encodes 1019 and rice even 1429 protein kinases which represent around 5% of the plant proteins (Wang et al. 2003; Dardick et al. 2007). Around 10% of these protein kinases are part of so called mitogen activated protein kinase (MAPK) modules. MAPK modules consist of variable multi-protein complexes, whose members are phosphorylating each other in a cascade like manner, thereby transducing signals throughout the cell (MAPK group 2002).

Subunit composition of a MAPK complex, and the duration and intensity of its signalling activity are precisely regulated and give rise to a highly specific cellular response. Regulation of signalling intervenes on several levels including the assembly and composition of a given MAPK module (for review see Colcombet and Hirt 2008), deactivation of single or multiple elements of a cascade by dephosphorylating enzymes (Meskiene et al. 1998 and 2003; Ulm et al. 2002; Luan 2003; Naoi and Hashimoto 2004), and last but not least, by its
specific spatial organization (see also the following chapters). It is well known from animal cells, that MAPK modules are spatially associated with different cellular domains such as endosomal organelles (for example Howe et al. 2001, Teiss et al. 2002 and 2006, Taub et al. 2007) and cytoskeleton (reviewed by Pullikuth and Catling 2007). Additionally, MAPK members undergo shuttling between the cytosol and the nucleus, where they activate a multitude of transcription factors (TFs) and others target proteins (reviewed by Turjanski et al. 2007). However, little is known about the subcellular organization of MAPK modules in plants.

The cytoskeleton also seems to be a key player in the transduction of signals from the outside and between cells in terms of development and plant responses to the environment (for example Dhonukshe et al. 2003; Šamaj et al. 2006; Yang et al. 2008; Wasteneys and Ambrose 2009). Moreover, there appear to be mutual interactions between the cytoskeleton and MAPK modules (Nishihama et al. 2002; Limmongkon et al. 2004; Naoi and Hashimoto 2004; Šamaj et al. 2002 and 2004a; Smertenko et al. 2006).

1.2 Mitogen activated protein kinases (MAPKs)

Mitogen activated protein kinases (MAPKs) are evolutionarily conserved serine/threonine kinases, which play an essential role in signal transduction involved in the regulation of cell growth, differentiation, and stress response. Like in animals and yeast, plant MAPKs are part of a complex network of signalling modules, which consist of a core of at least two more kinases, namely MAPK kinase (MAP2K or MAPKK or MKK) and MAPK kinase kinase (MAP3K or MAPKKK or MEKK). Such modules assemble in a cascade-like manner, generally starting with MAP3K, which activates MAP2K by phosphorylation of a S/T-X_3,5-S/T motif. Subsequently, the MAP2K acts as a dual-specific kinase phosphorylating MAPK both on threonine and tyrosine within a T-X-Y motif. MAPKs, in turn, are able to phosphorylate a wide range of substrates, including cytosolic and nuclear proteins (MAPK group 2002; Feilner et al. 2005; Nakagami et al. 2005; Popescu et al. 2009).

Mammalian MAPKs are divided into different groups, including the extracellular signal-regulated kinases (ERK), the p38 MAP kinases and the c-Jun N-terminal kinases (JNK) (for review see Junttila et al. 2008). Phylogenetically, all plant MAPKs belong to the mammalian ERK subgroup of MAPKs, and they are further divided into four classes (A-D) (MAPK Group 2002; Hamel et al. 2006).
In contrast to the yeast and human genomes, which encode 6 and 13 MAPKs, respectively (Meskiene and Hirt, 2000), higher plants possess a larger number of genes coding for MAPK-related kinases. At least 20 putative MAPKs, 10 putative MAP2Ks and 80 putative MAP3Ks have been identified in the Arabidopsis thaliana genome (MAPK group 2002 and TAIR; http://www.arabidopsis.org) allowing a multitude of different combinations with diverse molecular composition of MAPK cascades.

Exceeding the role of MAPKs in animals and yeast, a big number of stimuli triggers MAPK-mediated phosphorylation of downstream substrates in plants (Feilner et al. 2005; Nakagami et al. 2005; Popescu et al. 2009). Depending on the incipient signal, one single MAPK is able to target various proteins. Recently, Popescu et al. (2009) identified about 570 potential substrates for 10 out of 20 Arabidopsis MAPKs using microarray analysis. The authors reported about a pronounced area of overlap with respect to their targets between different kinases. Several studies demonstrated such versatility of MAPKs in the recent years. For example, MPK6, one of the best characterized MAPKs in Arabidopsis, is supposed to be a part of at least six different modules, which are maintaining correct patterning of stomata (Wang et al. 2007) and are involved in responses to the bacterial elicitor flagellin (Asai et al. 2002) as well as to the phytohormones ethylene (Yoo et al. 2008) and jasmonic acid (Takahashi et al. 2007), to oxidative stress (Kovtun et al. 2000), to cold and salt (Teige et al. 2004).

So far, a number of studies suggest that the composition of a particular cascade also determines the process that is finally affected by the respective downstream MAPK in the module. Moreover, one stimulus might initiate multiple modules, and each of them can activate a specific subset of MAPKs. Thus, the perception of the bacterial elicitor flagellin causes not only the activation of Arabidopsis MPK6 and MPK3 via MEKK1 (MAP3K) and M KK4/MKK5 (MAP2Ks) (Asai et al. 2002) but also leads to the activation of Arabidopsis MPK4 by MEKK1 (MAP3K) and M KK1/MKK2 (MAP2Ks) (Suarez-Rodriguez et al. 2007).

The specificity is, however, not only provided by the module-composition, but it seems to be heavily dependent on a variety of regulatory elements. Among these, dual-specific phosphatases, so called MKPs (MAPK phosphatases), were described as specific negative regulators of MAPKs in animals, yeast and plants (Luan 2003). While mammalian cells encode ten MKPs (for review see Owens and Keyse 2007), the Arabidopsis genome encodes only five (Kerk et al. 2002), namely AtMKP1, AtMKP2, DsPTP1, PHS1 and IBR5 (for references see Lee et al. 2009). Additionally, a multitude of single protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases (PSPs) are known to regulate
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MAPK activity in animals (reviewed by Juntila et al. 2008). These phosphatases have also been described in plants (Meskiene et al. 1998 and 2003). Recently, Schweighöfer et al. (2007) reported that AP2C1, a member of the *Arabidopsis* Ser/Thr phosphatase type 2C (PP2C) family, negatively regulates MPK4 and MPK6. The PP2C family comprises 76 putative members in *Arabidopsis thaliana*, which can serve in spatio-temporal regulation of MAPK signalling-activity in plant cells.

In animals and yeast, scaffold proteins turn out to be the main organizers of MAPK modules. With their multiple binding-domains they are able to accomplish correct assembly of specific MAPK modules (reviewed by Dhanasekaran et al. 2007). Further, scaffold proteins seem to regulate MAPK signalling by recruiting regulatory proteins such as phosphatases that either regulate the scaffold itself (Ory et al. 2003) or a specific MAPK within the module (Willoughby et al. 2003). Until now, only one scaffold protein has been identified in plants, namely OMTK1 (oxidative stress activated MAPK triple kinase 1) representing a MAP3K from *Medicago sativa*. This kinase is involved in the response to oxidative, stress and it was shown to be part of a protein complex with *Medicago* MAPK 3 (MMK3) (Nakagami et al. 2004). Interestingly, MEKK1, the closest *Arabidopsis* homolog of OMTK1 has also been described as a putative scaffold for *Arabidopsis* MPK4 (Nakagami et al. 2006).

One of the most important functions of scaffold proteins is their ability to determine the specific subcellular location of MAPK modules within a cell (Dhanasekaran et al. 2007). In animal cells some MAPKs dissociate from their scaffold upon complex activation and become relocated from the cytoplasm to the nucleus (reviewed by Turjanski et al. 2007). Further, Faure et al. (1999) showed that an activated MAPK from rat liver cells localizes to the plasma membrane (PM) and to endosomes following the binding of epidermal growth factor (EGF) to its receptor (EGFR), which in turn gave rise to endocytosis of the receptor-ligand complex. Several previous studies have demonstrated that EGF binding to its receptor activates a MAPK pathway through a distinct sequence of events leading to mitosis (reviewed by van der Geer et al. 1994).

Howe et al. (2001) provided evidence for the so called “signalling endosome hypothesis”. In analogy to the previous studies these authors showed, that the complex of nerve growth factor (NGF) and it’s receptor (NGFR) in neuronal cells is sequestered into clathrin coated vesicles (CCVs) and subsequently migrates from the axon back to the cell body. They also demonstrated that signalling of the MAPK-module, activated in this way, originates from these vesicles. Further studies confirmed the localization of MAPK modules
to endosomes and thus underlined the fundamental role of scaffold proteins. In mammalian cells, it has been shown that the scaffold protein MP1 (MEK1-partner) mediates late endosomal localization of the MAPK complex Raf/MEK/ERK via binding to the small adaptor protein p14 (Teis et al. 2002). Assembly and activation of this module is known to be regulated by the small G-protein Ras, which itself is activated after binding of EGF to EGFR (van der Geer et al. 1994). Because the PM-resident p14/MP1 complex is not able to activate the MAPK complex, it is likely that endocytosis itself is essential for proper signalling (Teis et al. 2002). A strong mutual interaction between endosomal trafficking and MAPK signalling has been demonstrated in a study providing evidence that late endosomes and lysosomes are displaced to the cell periphery in homozygous p14<sup>−/−</sup> and mek1<sup>−/−</sup> knockout lines of mouse embryo fibroblasts (Teis et al. 2006). Another study showed that artificial mislocalization of late endosomes caused prolonged EGFR activity and sustained ERK signalling (Taub et al. 2007). Most recently, p18 a novel adapter protein was identified to anchor the MEK1-ERK to lipid rafts on late endosomes and it was concluded that proper function of this anchoring protein is essential for controlling endosome dynamics (Nada et al. 2009). A theoretical model of a typical signalling cascade mediated by a MAPK module is shown in Fig 1.

In summary, a well-regulated subcellular organization of MAPK modules seems to be essential for proper signalling within a cell. It is remarkable that there is little known about a putative subcellular organization of such modules within plant cells. Up to now, MAPKs are mostly described as shuttling between the cytosol and the nucleus and it has been shown in some cases that these MAPKs activate signal specific transcription factors there (Andreasson et al. 2005; Qiu et al. 2008). However, in most cases the targets of such MAPKs are unknown. One of the first studies, which provided evidence for such nuclear shuttling, described the pathogen induced translocation of a non-identified MAPK into the nucleus in parsley cells (Ligterink et al. 1997). Another example was reported by Ahlfors et al. (2004) who described the nuclear translocation of Arabidopsis MPK3 and MPK6 in ozone treated plants. Further, Coronado et al. (2002 and 2007) proposed that tobacco Ntf4 relocates to the nucleus in specific stages of pollen maturation.

One indication for a putative association of plant MAPKs with particular subcellular structures comes from a previous study on Medicago sativa roots that demonstrated that inhibition of MAPK activation by the MAPKK inhibitor UO126 results in disturbed root hair formation and tip growth accompanied by adverse effects on vesicular trafficking (Šamaj et al. 2002). Additionally, this study showed that SIMK (stress induced MAPK) from Medicago
localizes to spot-like structures within the tip of growing root hairs, and that treatment with brefeldin A (BFA), an inhibitor of secretion and membrane recycling (reviewed by Müller et al. 2007b), caused random relocation of these spots. Interestingly, Baluška et al. (2000) previously described the translocation of SIMK from the nucleus to the cell plate in dividing root cells. A similar behaviour has also been described for tobacco NPK1 (Nicotiana protein kinase 1), which resides in the nucleus during interphase (Ishikawa et al. 2002) but relocates to the phragmoplast during cytokinesis (Nishihama et al. 2002) (see also chapter 1.5)

Figure 1. Model of the putative signalling cascade mediated by a MAPK module in plants. A typical MAPK-cascade starts with a stimulus that might come from the extracellular environment (like PAMPs), the neighbouring cells (like phytohormones) or from the cell interior (like changes in the cytoskeletal architecture) (ES, E, IS). Perception may start with the recognition by a PM-resident receptor (R) (for instance FLS2, Robatzek et al. 2006) or a receptor in the cytoplasm (X) as in the case of ethylene response at the endoplasmatic reticulum (Gao et al. 2003). Once the MAP3K is activated it phosphorylates a specific MAP2K at a S/T-X,5-S/T motif. MAP2K then phosphorylates a specific downstream MAPK on a T-X-Y motif. Activated MAPK phosphorylates and therefore activates or inactivates specific target proteins. Specificity of module composition might be governed by scaffold proteins that bind module components selectively. Regulation of kinase activity might be achieved by negative regulators such as protein phosphatases (PP). Dashed line arrows indicate postulated pathways.

1.3 MAPKs in Arabidopsis thaliana

Until now, most knowledge about MAPK mediated signalling in Arabidopsis comes from characterization of three MAPKs, namely MPK3, MPK4 and MPK6. All three were shown to assemble in multiple modules that are activated by diverse sets of abiotic and biotic stimuli as well as by developmental cues (Table 1 and Fig 2). The following chapter will summarize the most important present knowledge about these three MAPKs.

A multitude of abiotic stresses were shown to activate MAPKs in Arabidopsis including heat, cold, touch, wounding, osmotic shock, UV, salt as well as the application of
reactive oxygen species. However, respective compositions of MAPK modules and putative targets of the specific cascades are only barely discovered.

One complete module consisting of MEKK1, MKK2 and MPK4/MPK6 was described for cold and salt (Teige et al. 2004). Interestingly, this stress-induced cascade is identical to the flagellin induced cascade activating MPK4 but not MPK6 (see below). The fact that identical upstream MAP3Ks and MAP2Ks activate a specific subset of MAPKs in dependence to the respective stimulus seems to be a common phenomenon. For example, both MPK3 and MPK6 are activated by upstream M KK4 and M KK5 in three pathways comprising response to flagellin via MEKK1, response to oxidative stress via ANP1 (Kovtun et al. 2000) as well as via YODA during stomata development (Wang et al. 2007).

One common component in many cascades is H\textsubscript{2}O\textsubscript{2}, which seems to act as an upstream second messenger in early response reactions to several stimuli and it has been shown to activate MPK1, MPK2, MPK3, MPK4 MPK6 and MPK7 in Arabidopsis (reviewed by Colcombet and Hirt 2008).

Early recognition and an appropriate response to pathogen attack is one of the main challenges for plants. Generally, higher plants developed the so called innate immune response representing a very effective defence mechanism. The innate immune response comprises a first non pathogen specific answer, initiated by the recognition of PAMPs (pathogen associated molecular pattern) such as structural proteins and/or cell wall components, which are typical for a large range of pathogens. Challenged by these PAMPs, plant cells change their enzymatic activity and gene expression sometimes resulting in the hypersensitive response (HR), which is characterized by local programmed cell death (PCD) in order to prevent spreading of the pathogen. Amongst others, the HR is regulated by the activity of a specific set of phytohormones, namely jasmonic acid (JA), ethylene and salicylic acid (SA). Additionally, these phytohormones are also involved in the development of a pathogen-specific long term resistance, which is called systemic acquired resistance (SAR) (for review see Jones and Dangl 2006).

In recent years, MAPKs have been recognized as important players that are involved in both, innate immune response and the development of SAR. Arabidopsis thaliana FLAGELLIN SENSING 2 (FLS2), is a membrane-integral receptor-like kinase (RLK), described as PM receptor that is known to be internalized via receptor mediated endocytosis (RME) after application of the bacterial PAMP called flagellin (Robatzek et al. 2006). FLS is a pattern-recognition receptor (PRR) that binds bacterial flagellin and initiates the immune
response, which involves activation of the MAPKs MPK3, MPK4 and MPK6 (Asai et al. 2002; Suarez-Rodriguez et al. 2007). Due to binding of flagellin, FLS dimerizes with another PM protein called BRI1-ASSOCIATED KINASE 1 (BAK1). This complex is known to be internalized into an endocytotic organelle (Chinchilla et al. 2007; Heese et al. 2007). Flagellin was shown to trigger the activation of at least two different MAPK cascades. One of them comprises the activation of MPK3 and MPK6 via MKK4 and MKK5 (Asai et al. 2002). The second cascade activates MPK4 via MKK1 and MKK2, a module that is also known to trigger cold and salt tolerance (Ichimura et al. 1998; Teige et al. 2004). Both seem to share the same upstream MAP3K, namely MEKK1.

While MPK3 activates VirE1-INTERACTING PROTEIN 1 (VIP1) a transcription factor that is known to induce the expression of defense-related genes (Djamei et al. 2007), MPK6 phosphorylates ACC (1-amino-cyclopropane-1-carboxylic acid) SYNTHASE 6 (ACS6) an enzyme that is involved in the synthesis of the phytohormone ethylene (ET) (Liu et al. 2004; Joo et al. 2008). Interestingly, MPK6 not only seems to be involved in PAMP triggered ET synthesis but also gets activated by ET via MKK9 (Novikova et al. 2000; Ouaked et al. 2003; Yoo et al. 2008).

In contrast, MPK4 was proposed to act as a negative regulator of immune response because mpk4 and mekk1 mutants show elevated levels of SA and H₂O₂, which cause PCD induced by PAMP (Ichimura et al. 1998; Petersen et al. 2000; Suarez-Rodriguez et al. 2007). MPK4 is known to target the two transcription factors (TFs), WRK25 and WRK33, as well as MPK4 substrate1 (MKS1) that is known to be involved in pathogen response (Andreasson et al. 2005). However, it is not clear, whether these targets are indeed activated by flagellin. Although, both cascades act antagonistically, they seem to be tightly connected to each other, since it was shown that MPK3 and MPK6 activity is impaired in mkk1 mutants (Meszaros et al. 2006).

Interestingly, signalling pathways activated by flagellin might represent general components of a pathogen-related response mechanism. It was shown that not only flagellin but also several other PAMPs, such as fungal chitin and harpin, activate MPK3/MPK6 or MPK4, respectively and that they trigger similar gene regulation and HR resulting in PCD (Desikan et al. 1999 and 2001; Miya et al. 2007).

A recent overview on MAPK cascades involved in plant signalling events is provided in Fig 2.
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Table 1. Stimuli activating MPK3, MPK4 and MPK6. Several stimuli activate one or more of these three MAPKs. The list of stimuli was modified from Colcombet and Hirt (2008). References are listed there. ABA=abscisic acid; ET=ethylene; PAMP=pathogen associated molecular pattern; JA=jasmonic acid.

<table>
<thead>
<tr>
<th>MAPK</th>
<th>Activating Stimuli</th>
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<tbody>
<tr>
<td>MPK3</td>
<td>osmotic stress; ( \text{H}_2\text{O}_2 ); ( \text{O}_3 ); ABA; ET; PAMPs</td>
</tr>
<tr>
<td>MPK4</td>
<td>osmotic stress; ( \text{O}_3 ); PAMPs; cold; salt</td>
</tr>
<tr>
<td>MPK6</td>
<td>osmotic stress; ( \text{H}_2\text{O}_2 ); ( \text{O}_3 ); JA; ET; PAMPs</td>
</tr>
</tbody>
</table>

for references see Colcombet and Hirt (2008)

Figure 2. Composition of known MAPK cascades involving MPK3, MPK4 and/or MPK6. (A) pathogen response: Activation of MPK3 and MPK6 in a MEKK1-MKK4/MKK5-MPK3/MPK6 module and activation of MPK4 in a MEKK1-MKK1/MKK2-MPK4 module after recognition of bacterial flagellin (flg22) by the flagellin receptor FLS2. Targets of MPK3/6 are the transcription factor (TF) VIP1, which is involved in gene activation in the course of pathogen response. Another target is ACS6, which is responsible for ethylene synthesis. MPK4-targets in flagellin response pathway are not yet identified, but it was shown elsewhere that MPK4 may target TFs involved in pathogen response. (B) morphogenesis: activation of MPK3/6 in a YODA-MKK4/MKK5-MPK3/MPK6 module, which is involved in stomatal patterning and embryo development. SPEECHLESS, a TF that is responsible for initiation of stomata was recently reported as a target of MPK3/MPK6 (Lampard et al. 2008). SHORT SUSPENSOR (SSP) was recently proposed to induce YODA-dependent signalling in the zygote and in leaves (Bayer et al. 2009) (C) oxidative stress: activation of MPK3/6 in an ANP1-MKK4/MKK5-MPK3/MPK6 module by hydrogen peroxide as the upstream stimulus. Targets are not known. (D) ethylene response: Activation of MPK3/6 by upstream MKK9. MKK9 gets activated in response to recognition of ethylene by the ER resident ethylene receptor ETR1. ETR1 also activates the CTR1, which seems to have a negative effect on MPK3/6 activation. (E) cold/salt: Activation of MPK4/6 in a MEKK1-MKK2-MPK4/MPK6 module. Targets are unknown. (F) jasmonic acid (JA): Activation of MPK6 by JA might be a secondary effect, whether there is a direct activation of MPK6 by MKK3, is not clear. The pathways presented in this figure are created on the basis of the review article by Colcombet and Hirt (2008). Updated elements are supplemented with references, and all other references can be found in this article.
1.4 Endocytosis in plants

The first indications for the existence of endocytosis in plants have been obtained two decades ago. For instance, electron-dense tracers have been applied to prove uptake via the plasma membrane into membrane bound intracellular structures (Tanchak and Fowke, 1987; Hillmer et al., 1986 and 1988; Galway et al., 1993) and clathrin coated pits associated with the plasma membrane (PM) have been identified in plant cells (Robinson and Hillmer, 1990). Nevertheless, unambiguous and conclusive evidence for the operation of endocytosis and its vital role in plant cells have been missing for a long time.

In the past years, however, a broad range of molecular markers was developed (for example Ueda et al., 2004; Uemura et al., 2004; Voigt et al. 2005; Jaillais et al., 2006; Ortiz-Zapater et al., 2006; Lam et al., 2007a,b) and, together with vital marker-dyes such as FM1-43 and FM4-64 (Vida and Emr, 1995; Emans et al. 2002; Bolte et al., 2004), widely used to analyse PM-recycling and endocytosis, as well as to identify and characterize corresponding endomembrane compartments. It is now firmly established that the uptake of extracellular cargo by endocytosis and recycling of diverse membrane-bound and cell-wall-associated components not only exist, but are highly organized and crucial for plants. Endocytosis is also regarded as essential for the plant development and for plant adaptation to the environment.

One of the most striking discoveries of the last years was the demonstration of receptor mediated endocytosis (RME) in plants. Although, it has long been speculated about the existence of RME (Horn et al., 1990), and a set of candidate receptors have been proposed to reside in the PM and in endosomal structures (for references see Geldner and Jürgens, 2006), the clear evidence for RME was provided by Robatzek et al. (2006) only recently. These authors showed that the Arabidopsis LRR (leucine rich repeat) receptor FLS2 (Flagellin sensitive 2) resides at the PM and gets internalized into endosomal compartments upon specific binding of its ligand flg22, a small flagellin peptide, which is known to elicit basal defence responses (Gomez-Gomez et al., 2001; Gomez-Gomez and Boller, 2002) (see also chapter 1.3). There are more candidates possibly involved in induced endo- and exocytotic events mediating plant immune-responses (Robatzek, 2007) and future work will show if RME is, like in animals, a key regulator in the plant’s specific recognition of putative pathogens.
1.4.1 Identification of endosomal compartments – markers and reporters

One straightforward approach to analyse the organization of the endosomal system within plants is to identify the intracellular compartments, which are involved in this trafficking route. A relatively well-established way to identify and characterize endosomes is to use specific molecular markers, which are known to act predominantly at one specific endomembrane compartment. Like in all eukaryotic cells, plant cells possess a broad range of highly conserved protein families known to be essential for proper protein sorting, directed vesicular trafficking and fusion of endosomal/PM derived vesicles with the target membranes. All of these protein families can be divided into sub-classes, which often are associated specifically with only one endosomal compartment. The most common protein families used in this context are t-SNAREs/syntaxins (soluble N-ethyl maleimide sensitive factor attachment protein receptors), small GTPases and a set of diverse endosomal sorting receptors (for detailed description see Müller et al. 2007b). Additionally, there are some molecular markers which do not occur in plants but are used as heterologous markers, because they are known to define specific compartments or trafficking pathways in other eukaryotic cells. One actual example is the human transferrin receptor (hTfR) (Ortiz-Zapater et al., 2006).

Using ultrastructural analysis as well as different molecular markers, a couple of endosomal compartments have been described during the past years in plants. They partially resemble those known from animals and yeast. Initially, it seemed to be difficult to integrate these structures into a functional endocytotic/secretory pathway. Nevertheless, recent studies provide first important insights into their functions, organization and interconnections. A model of the plant endosomal system based on recent findings, including a specification of the known endosomal markers is shown in Fig 3.
1.4.2 Pharmacological studies

Drugs affecting vesicular trafficking turned out to be very useful tools in determining the origin, assignment, and functional interconnections of distinct endosomal compartments within the endocytotic network. In addition to diverse drugs that are disturbing the dynamics of cytoskeletal elements and therefore affect the vesicular/organellar motility and organization, there are mainly two inhibitors extensively used in plants, which directly interfere with vesicular trafficking and fusion events, namely Brefeldin A (BFA) and Wortmannin.

Brefeldin A (BFA) is a fungal toxin from *Eupenicillium brefeldianum*, and it is commonly used as an inhibitor of membrane recycling by effectively inhibiting secretion (reviewed by Müller et al. 2007b). Whereas secretion is inhibited endocytosis not only

Figure 3. Endocytotic routes and compartments in plant cells. Components internalized by clathrin-dependent endocytosis (coated pits, CCP) are delivered to an early endosome (EE) that was proposed to be identical to the TGN (Dettmer et al., 2006; Lam et al., 2007). Internalized cargo is either recycled back to the PM or delivered to the MVBs/PVC, which are assumed to function as late endosomes (LE). From there, vacuolar proteins as well as proteins designated for degradation are transported to the vacuole, while cargo receptors may be recycled back to the TGN (Oliviusson et al., 2006). Additionally, there is at least one endosomal compartment that is not identified yet. This compartment is labeled by SNX1 (sorting nexin 1) and might be a late endosome, since structural homologs of AtSNX1 in yeast are known to be part of a retromer complex residing on the MVB/PVC compartment (Oliviusson et al., 2006). Both TGN and MVBs are labeled by FM4-64 and therefore clearly of endosomal nature. Molecular markers are depicted in different colors. Rab-GTPases: blue, SNAREs: green, VSRs: black, other molecules: yellow. Inhibitors with their inhibitory effects are shown in purple. Black arrows indicate established endocytotic routes, dashed arrows indicate putative pathways. The green colored compartment is not identified at the ultrastructural level, but it is labeled by endosomal markers visualized by confocal laser scanning microscopy. MVB = multivesicular body; PVC = prevacuolar compartment.
continues, but rather increases under BFA treatment (Emans et al. 2002; Wang et al. 2005). BFA was shown to inhibit ARF-GTPases by targeting ARF-GEF proteins resulting in disruption of the formation of COPI-coated vesicles, thereby stopping the membrane replenishment from the ER to the Golgi. There is at least one other ARF-GEF member called GNOM that is also sensitive to BFA. It is localized on an endosomal compartment and was proposed to be involved in membrane trafficking from endosomes to the PM (Geldner et al., 2003). Secretion is stopped, whereas endocytosis may continue and produce a surplus of internalized membranes, which aggregate and form so-called BFA compartments in plants. In Arabidopsis roots, these BFA compartments consist mostly of accumulated TGN vesicles, which are surrounded by Golgi-stacks (Grebe et al. 2003; Šamaj et al. 2004a).

Wortmannin is the second inhibitor extensively used in studies on endosomal organization and trafficking. It is known to target phosphoinositide 3 kinase (Ui et al., 1995) and it is inhibiting vacuolar/lysosomal trafficking at late endosomes in both mammalian (Bright et al., 2001) and plant cells (Matsuoka et al., 1995) in a dose-dependent manner. On the subcellular level, it has been shown that Wortmannin causes multivesicular bodies (MVB) / prevacuolar compartments (PVC) to form vacuolated structures, but it does not affect the Golgi (Tse et al., 2004). However, results from Wortmannin treatments always have to be interpreted with some caution, because it is known to inhibit at higher concentrations also phosphoinositide 4 kinase (Matsuoka et al., 1995).

In summary, BFA and Wortmannin can be used as valuable pharmacological tools for the study of secretory and endocytotic pathways. Because of the characteristic morphological changes which they cause at the level of TGN and MVB/PVC morphology and function, they can be further used to identify the localization of proteins within the endocytotic/secretory network.

1.4.3 Trans-Golgi network (TGN), an early endosomal compartment in plants

In animals, the TGN is described as a structure tightly associated with the trans-side of the Golgi apparatus and primarily responsible for the sorting of secretory and lysosomal proteins. However, in plants it seems that the TGN serves also, in addition to its role in the secretory pathway, as an early endosome in the endocytotic pathway.

One of the first molecular markers described as specific for the TGN was the Arabidopsis SNARE protein SYP41 (Uemura et al., 2004). It has been shown, that a GFP-SYP41 fusion construct did not co-localize with Golgi- or PVC markers but aggregated under
the influence of BFA. Later, it was shown that the *Arabidopsis* membrane-bound V-ATPase subunit VHA-a1 is co-localized with SYP41 and resides in the TGN (Dettmer *et al*., 2006). Interestingly, the endocytotic marker FM4-64 rapidly co-localizes with VHA-a1 in *Arabidopsis* root cells. This observation led to the conclusion that the TGN also serves as an early endosomal compartment. Moreover, it has been shown that the V-ATPase inhibitor Concanamycin A inhibits the endocytotic trafficking of FM4-64 to late endosomes and to the vacuole. Upon Concanamycin A treatment, FM4-64 and VHA-a1 were restricted to the TGN. Dettmer *et al.* (2006) also showed that BFA causes the VHA-a1 positive structures to aggregate within BFA-compartment. This clearly suggested that the TGN serves both, within the secretory/vacuolar and the endocytotic pathway.

A recently published work describing the localization of secretory carrier membrane protein1 (SCAMP1) to the TGN (Lam *et al*., 2007) supported and further strengthened previous conclusions. SCAMPs are endocytosis-mediating proteins in animals. Lam *et al.* (2007) localized the rice SCAMP1 in transgenic tobacco BY-2 cells both at the PM and within highly mobile, punctuated cytoplasmatic structures. Co-localization of SCAMP1 with V-ATPase, suggested that this organelle constitutes the TGN. Indeed, immunogold EM experiments revealed that SCAMP1 localizes to vesicular structures of TGN. Furthermore, it was shown that FM4-64 labels SCAMP1-positive organelles rapidly, and prior to the MVB/PVC. Together, these data provided evidence that SCAMP1 is localized to the TGN, an organelle which is serving as an early endosome in plant cells.

Additionally, RabA4b, an *Arabidopsis* homolog of the Rab11 family of mammalian small Rab-GTPase was shown to partially localize to the TGN compartment in growing tips of root hairs (Preuss *et al*., 2004a and 2006). Rab11 proteins in mammals are known to mediate recycling processes from early endosomal compartments to the PM by acting on recycling endosomes (Ullrich *et al*., 1996; Mohrmann *et al*., 1999). Interestingly, only the minor part of RabA4b seems to be associated with the TGN, while the major part labels a yet unidentified compartment (Preuss *et al*., 2004a). These data provided the first indication for the existence of an additional early endosomal compartment, resembling the recycling endosome in mammals.

### 1.4.4 Multivesicular body as late endosomal compartment

In plants, similarly to other eukaryotic organisms, vacuolar/lysosomal protein degradation as well as the delivery of vacuolar proteins is mediated through a compartment
described as either prevacuolar compartment (PVC) or multivesicular body (MVB). Moreover, proteins of the secretory pathway as well as proteins delivered to protein storage vacuoles (PSV) apparently have to pass through such a compartment in the cell (for review see Mo et al. 2006). MVBs could be defined as round-shaped electron-transparent vesicular compartments enclosed by a limiting outer membrane and containing small internal electron dense microvesicles (Tse et al. 2004; Hause et al. 2006; Lam et al. 2007). In addition to the role of MVBs in the secretory/vacuolar pathway, it is known for a long time that organelles possessing multivesicular structures are also involved in endocytotic processes in plants. Already in the 1980s and the early 1990s uptake studies using electron dense tracers indicated their presence within MVBs (Hillmer et al. 1986 and 1988; Tanchak and Fowke 1987; Galway et al. 1993). During the last years, several molecular markers were developed and used to describe late endosomal/secretory compartments. Markers like vacuolar sorting receptors (VSRs) (Li et al. 2002; Tse et al. 2004), the Arabidopsis syntaxin SYP21/PEP12p (da Silva Conceicao et al. 1997; Uemura et al. 2004), as well as small Rab-GTPases (Sohn et al. 2003; Kotzer et al. 2004; Lee et al. 2004) contributed significantly to the study of MVB/PVC functions. The use of these markers in combination with vital endocytotic marker dyes made it also possible to show that there are cross talks between the secretory/vacuolar and the endocytotic pathway on the level of MVB/PVCs (for review see Müller et al. 2007b; Robinson et al. 2008). Further, there is growing evidence for the existence of bidirectional trafficking also allowing retrograde transport from MVBs to the TGN via a cytosolic protein complex named retromer (Oliviusson et al. 2006; Shimada et al. 2006; Jaillais et al. 2006, 2007 and 2008).

1.5 Interactions between MAPKs and cytoskeleton in plants

Like in animals and yeast, the cytoskeleton in plants is involved in the establishment of cell polarity and regulated vesicular trafficking in endo- and exocytosis (reviewed by Šamaj et al. 2006; Boutte et al. 2007; Yang 2008) as well as in cell division, morphogenesis and in the general maintenance of intracellular motility (reviewed by Pollard and Borisy 2003; Panteris 2008; Wasteneys and Ambrose 2008). A precise regulation and high dynamic of actin filaments (AF) and microtubules (MT) are fundamental requirements in order to accomplish all these functions.

Importantly, the behaviour of AF and MT is mainly influenced by specific binding proteins called microtubule associated proteins (MAPs) and actin binding proteins (ABPs)
(reviewed by Hamada 2007; Higaki et al. 2007). From animal cells it is well known that MAPKs are involved in the regulation of the cytoskeleton either by direct association with cytoskeletal elements or by phosphorylation and regulation of cytoskeleton-binding proteins (for example Hai and Gu 2006; Pullikuth and Catling 2007; Gerthoffer 2008).

Over the recent years evidence has grown about crosstalk between MAPKs and MAPs/ABPs also in plants. Interestingly, MAPKs not only regulate the behaviour of these proteins by phosphorylation but seem to be regulated in turn by the cytoskeleton including the binding proteins. For example, it was shown that pharmacological inhibition of SIMK activity in *Medicago sativa* by the synthetic drug UO126 not only affects the arrangement of actin filaments and inhibits vesicle trafficking and polar growth of root hairs, but vice versa, the disturbance of actin dynamics by latrunculin B activates SIMK (Šamaj et al. 2002). Since actin cytoskeleton dynamic is regulated through actin binding proteins and the modulation of the G-actin pool, it is interesting to note the obvious connection between MAPKs and ABPs. For instance, it was found in tobacco by Limmongkon et al. (2004) that two tobacco MAPKs, namely p45\textsuperscript{Nid4} and SIPK, were able to phosphorylate profilin 2.

Apparently, SIMK not only interacts with the actin cytoskeleton but also with microtubules. It has been localized to the phragmoplast after salt stress (Baluška et al. 2000) and after stabilization of microtubules by taxol (Šamaj et al. 2004b). Other MAPKs were also shown to localize to the phragmoplast (Calderini et al. 1998; Bögre et al. 1999). This suggests that MAPKs connect the incoming stress signal with the microtubule system. One group of molecular players, which modulate MT-dynamics are the members of the MAP65 family. In *Arabidopsis*, it has been shown by *in vitro* phosphorylation assays that MAP65-1, which is associated with the cortical MT-system but is also involved in cytokinesis, is negatively regulated by MPK4 and MPK6 (Smertenko et al. 2006).

Another example for a tight association of a MAPK with the cytoskeleton is the tobacco NPK1, a MAP3K that is known to be essential for cell plate formation (Nishihama et al. 2001). During cytokinesis, NPK1 interacts with NACK1, a kinesin-like protein that is necessary to transport NPK1 to the equatorial region of the phragmoplast (Nishihama et al. 2002). Interestingly, the NACK1 binding domain of NPK1 contains a nuclear localization signal and it has been shown that NPK1 resides in the nucleus during interphase (Ishikawa et al. 2002). This was a first indication of spatial and temporal organization of distinct MAPK subpopulations in plants.
1.6 Aims

This thesis work aims to characterize the subcellular organization and function of three MAPKs in *Arabidopsis thaliana* roots, namely MPK3, MPK4 and MPK6, with the main focus on MPK6. The combined use of biochemical methods together with *in-vivo* and *in-vitro* localization techniques, such as the application of recombinant GFP-reporters and immunofluorescence co-labelling, should provide insight into the potential organization and function of MAPK subpopulations on specific organelles or the cytoskeleton. To this end, a set of specific antibodies against organellar marker proteins, cytoskeletal components and MAPKs are used. Where applicable, selective activation of MAPKs by well established elicitors and stimuli have been employed to examine the specific role of these subpopulations. Moreover, using Brefeldin A as an inhibitor of membrane trafficking, this work should test, whether one or more of these MAPKs are integrated in or regulated by the endocytotic / secretory pathway. These experiments will be supplemented by phenotypically characterizing a *mpk6* knockout mutant line and further studies on the cellular level. Finally, this work is aims at characterizing the cell type specific changes of the actin- and microtubule-cytoskeleton in course of heat shock, a type of physical stress, which is also known to activate MPK6. Live cell imaging of *Arabidopsis* lines transformed with GFP-fusion reporter constructs, are used in order to reveal the cell type specific behaviour of these cytoskeletal components.

*Arabidopsis thaliana* is the best characterized experimental model system in plant biology. It has advantages such as the fully sequenced genome, availability of mutant lines, reliable transformation methods, established whole mount immuno-localization protocols and commercially and non commercially available antibodies against MAPKs as well as a set of organellar marker proteins.

Experimental work of this thesis is focused on the root of *Arabidopsis* seedlings representing an important organ for water and nutrition uptake and for plant – environment interactions. Moreover, roots are chosen, because of some practical advantages. They allow to use protocols for whole-mount immunofluorescence labelling and, because of their small size, they are suitable for microscopy without further embedding and sectioning. Further, they allow easy application of chemical probes such as vital dyes and drugs and the microscopic observation of effects caused by these probes.
2. MATERIAL AND METHODS

Plant growth conditions
If not stated otherwise, sterile *A. thaliana* seedlings of the Columbia 0 (Col-0) wildtype or the *mpk6-2* mutant line (SALK_073907) were grown on vertically oriented Phytagel plates containing ½ Murashige and Skoog (MS) medium (pH 5.7) under a 16-h-light/8-h-dark cycle at 22°C.

Seed sterilization
Seeds were sterilized for 15 min using 12% sodium hypochlorite solution in 0.1% v/v Triton X100. After successive washing with sterile double-destilled (MilliQ) water, seeds were dried on filter paper and stored at 4°C.

Extraction of proteins and isolation of cytosolic and microsomal fractions
For protein extraction, roots from 1-2 weeks old seedlings were homogenized in ice cold extraction buffer (30mM Tris at pH 8.3; 150mM NaCl; 10mM EDTA; 20% [V/V] Glycerol; 2mM DTT; 1mM PMSF; protease inhibitor cocktail (SIGMA-ALDRICH)) and subsequently filtered twice through Miracloth (CALBIOCHEM). To isolate cytosolic and microsomal fractions the resulting suspension was fractionated by the following centrifugation steps: (i) 15min at 10,000g resulting in a nuclear/waste sediment and a post-nuclear supernatant (PS); (ii) The PS was centrifuged for 60min at 100,000g (BECKMANN L8-70M ultracentrifuge) resulting in a microsomal pellet and a cytosolic supernatant. The pellet was resuspended in the extraction buffer.

Fractionation of microsomes
For the fractionation of microsomes *A. thaliana* (Columbia 0) seedlings were grown in liquid ¼ MS medium on a shaker for 2 weeks (16-h-light/8-h-dark at 22°C) and subsequently prepared as described above. The microsomal fraction was resuspended in extraction buffer and layered over a 30 to 55% continuous sucrose gradient prepared in centrifugation buffer (10mM Tris at pH 7.6; 30/55% [w/v] sucrose; 5mM EDTA; 2mM DTT; 1mM PMSF; protease inhibitor cocktail (SIGMA-ALDRICH)) and centrifuged using a SW40 swinging bucket rotor at 100,000g at 4°C for 18 h (BECKMANN L8-70M ultracentrifuge). Up to
twenty 0.5ml-fractions were collected from the top of the gradient. Sucrose concentration in the fractions was determined by refractometry.

**Immunoblot analysis**

Protein extract samples were precipitated by methanol and chloroform according to Wessel and Flügge (1984). First a MetOH:Sample:Chloroform (4:4:1 ) mixture was prepared in an Eppendorf tube and vortexed. After a centrifugation step (10min, 13,000rpm) the upper phase of the resulting three-phased sample was discarded. Another volume of MetOH was added and the sample was centrifuged a second time (10min, 13,000rpm). The resulting pellet was dried completely and resuspended in 1x SDS-PAGE sample buffer. SDS- PAGE was performed on a Minigel setup (MINI-Protean II cell system, BIORAD) using the Laemmli system (Laemmli 1970). Identical protein concentrations were loaded in all quantitative analyses and all fractions from sucrose density gradients. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (PEQLAB) in a wet tank unit (BIORAD) at 100 V for 1,5 h using the transfer buffer according to Towbin et al. (1979). For immuno-detection of protein bands the membrane was blocked with 6% [w/v] bovine serum albumin (BSA) in Tris-buffer-saline (TBS, see buffer list below) for 1h, and subsequently incubated with a primary antibody diluted in TBS-T (TBS; 0,1% Tween 20) containing 1% [w/v] BSA at room temperature for 1,5h or at 4°C overnight. After washing in TBS-T the membrane was incubated with a secondary antibody diluted in TBS-T containing 1% [w/v] BSA at room temperature for 1,5h. Following at least six washing steps, proteins were detected by incubating the membrane in freshly prepared enhanced chemoluminescence (ECL) reagent for 2min. ECL reagent was prepared using the following solutions: 1ml of solution A (200 ml 0,1M Tris-HCl (pH 8,6); 50 mg Luminol (SIGMA-ALDRICH)), 100µl of solution B (11 mg para-Hydroxycoumarin acid (SIGMA-ALDRICH) in 10 ml DMSO) and 0.3µl H₂O₂ (37%). Luminescence was detected using Hyperfilm ECL (AMERSHAM) in a dark room.

**Antibodies used for SDS-PAGE**

The following primary antibodies were used: rabbit polyclonal anti-AtMPK3 (diluted 1:3500, SIGMA-ALDRICH); rabbit polyclonal anti-AtMPK4 (diluted 1:3500, SIGMA-ALDRICH); rabbit polyclonal anti-AtMPK6 (diluted 1:7500, SIGMA-ALDRICH), rabbit polyclonal anti-SCAMP1 (diluted 1:1000; provided by Liwen Jiang, University of Hong-Kong, China), rabbit polyclonal anti-phosphoenol pyruvate carboxylase (PEPC) (diluted 1:2000; ROCKLAND); mouse monoclonal anti-clathrin LC (diluted 1:1500; SIGMA-ALDRICH); rabbit polyclonal
anti-alpha-mannosidase1 (diluted 1:1000; provided by Sebastian Bednarek, Wisconsin University, USA); rabbit polyclonal anti-FLS2 (diluted 1:5000; provided by Silke Robatzek, MPI, Cologne, Germany) rabbit polyclonal anti-AtSec12 (diluted 1:1000; see Bar-Peled and Raikhel 1997); rabbit polyclonal anti-AtPEP12/SYP21 (diluted 1:1500; see Preuss et al. 2004); mouse monoclonal anti-actin (MabGPa) (diluted 1:1500; provided by Richard Meagher, University of Georgia, USA); mouse monoclonal anti-alpha-tubulin (diluted 1:500; SIGMA-ALDRICH) and rabbit polyclonal anti-PIP2;2 (diluted 1:2000; provided by Anton Schäffner, Helmholtz Zentrum München, Germany).

Secondary antibodies were anti-mouse and anti-rabbit IgGs conjugated with horse radish peroxidase (diluted 1:2000; CELL SIGNALLING).

Statistical distribution analysis of proteins in microsomal and cytosolic fractions
The average grey value intensity of the immunoreactive band of each fraction was measured using the ImageJ software (http://rsbweb.nih.gov/ij/). In order to prevent false-positive values, the background of the surrounding membrane was additionally measured and subtracted individually for each band. The sum of the resulting values of cytosolic and microsomal fractions was defined as 100%. The relative portion of each fraction was subsequently calculated. Each experiment was repeated 3-4 times and the final values presented as the average of these multiple experiments. The standard deviation represents the differences between the repeated experiments.

Phos-tag™ SDS-PAGE

Reverse transcriptase PCR analysis of Arabidopsis mpk6-2 mutant and wild-type line
For RT-PCR analysis frozen root tissue from two weeks old Col-0 and mpk6-2 mutant (SALK_073907) seedlings were homogenized in a 1,5ml Eppendorf tube and RNA was isolated using plantrna-OLS kit (OLS, Germany). RT-PCR analysis was performed with the help of the SuperScriptTMIII One-Step RT-PCR kit (INVITROGEN) using the following
specific primers: MPK6 forward 5' gc gga tcc atg gac ggt ggt tca agg 3' and reverse 5' gc act agt cta tgt ctg ata ttc tgg att g 3'.

The following run parameters were used for RT-PCR analysis:

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**GUS staining procedure**

Samples were processed according to Block and Debrouwer (1992) with 1 mM of 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-Gluc) in staining buffer consisting of 0,1 M NaH$_2$PO$_4$; 0,1M Na$_2$HPO$_4$; 10 mM EDTA; 2 mM FeK$_3$[CN]$_6$; 2mM FeK$_4$[CN]$_6$ x 3H$_2$O; 0.1% v/v Triton X100; pH 7.0 at 37°C for 2-18 hrs. To extract chlorophylls of aerial parts of the seedlings, samples were transferred to ethanol/acetic acid (1:1) with two or three changes. Specimen were examined with a stereo microscope (Leica MZFL III) equipped with a CCD camera. For image documentation a Diskus-program (Carl Hilgers, Königswinter, Germany) was used.

**Whole mount immunofluorescence labelling**

*A. thaliana* (Columbia 0) seedlings or the respective transgenic lines (all 4-6 days old) were fixed *in vacuo* using 1,5% formaldehyde and 0,5% glutaraldehyde in half strength microtubule stabilizing buffer (1/2 MTSB, see buffer list below) at pH 6,8. After successive washing (at least 4 times) with 1/2 MTSB followed by Phosphate buffered saline (PBS, see buffer list below), free aldehyde groups were reduced by 0,1% borohydrite in PBS. Cell walls were permeabilized by a mixture of cell wall digesting enzymes containing 2% [w/v] cellulase (CALBIOCHEM); 1% [w/v] pectinase, and 2% [w/v] driselase (SIGMA-ALDRICH) at 37°C.
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for 30 min. Subsequently, the samples were permeabilized by 10% [v/v] DMSO and 2% [v/v] Nonidet P40 in PBS for 1 h. For subsequent immunolabelling, samples were blocked using 1% [w/v] BSA in PBS for 1 h and incubated with primary antibody (diluted in PBS containing 1% BSA) at 4°C overnight. Samples were washed with PBS and incubated with fluorescently labelled secondary antibody (also diluted in PBS containing 1% BSA) for 3 h (1.5 h at 37°C and 1.5 h at room temperature). Finally, samples were mounted in one drop of mounting medium (0.1% [w/v] para-phenylenediamine prepared in 90% [v/v] glycerol in PBS). In the case that primary antibodies were raised in different host animals double labelling was performed using both primary antibodies simultaneously followed by secondary antibodies which were also applied simultaneously. If antibodies were raised in the same animal, immunolabelling was done successively and with increased washing periods between steps. In this case additional negative controls were performed using only one primary antibody and successive incubation with two fluorescent secondary antibodies.

Antibodies used for single and double labellings:
Rabbit polyclonal anti-AtMPK3 (diluted 1:350, SIGMA-ALDRICH); rabbit polyclonal anti-AtMPK4 (diluted 1:350, SIGMA-ALDRICH); rabbit polyclonal anti-AtMPK6 (diluted 1:750, SIGMA-ALDRICH); rabbit polyclonal anti-SCAMP1 (diluted 1:300, kindly provided by Liwen Jiang, University of Hong-Kong, China); mouse monoclonal anti-clathrin LC (diluted 1:300, SIGMA-ALDRICH); mouse monoclonal anti-Golgi 58k (diluted 1:350, SIGMA-ALDRICH); mouse monoclonal anti-GFP (diluted 1:500, ROCHE); rat monoclonal anti-tubulin (YOL1/34) (1:300-500, SEROTEC); plant specific mouse monoclonal anti-actin (1:100, SIGMA-ALDRICH, A0480)

Secondary antibodies were: AlexaFluor®488 and AlexaFluor®546, both goat anti-rabbit IgG (H+L) and goat anti-mouse IgG (H+L) (diluted 1:500, INVITROGEN). AlexaFluor®488 anti-rat IgG (1:500, INVITROGEN)

Transgenic Arabidopsis lines
For visualization of microtubules an Arabidopsis line expressing a fusion-construct of GFP with the microtubule-binding domain (MBD) of the animal MAP4-protein (Marc et al. 1998) was used. Control experiments were done with a line expressing a fusion-construct of YFP with TUA5, an isoform of Arabidopsis alpha-tubulin (Shaw et al. 2003). For visualization of the actin cytoskeleton another Arabidopsis line was used expressing a fusion-construct of
GFP with the actin-binding-domain 2 (ABD2) of the plant actin binding protein fimbrin (Voigt et al. 2005). For visualization of MPK6 a transgenic line was used expressing a 35S:GFP:MPK6 construct (generated by Martina Beck in our laboratory). For whole mount labelling of the endoplasmic reticulum, a transgenic line expressing a mGFP5-ER construct was used (kindly provided by Jim Haseloff, University of Cambridge, UK).

**Confocal microscopy**

For heat stress experiments:
The cytoskeleton in living *A. thaliana* roots was observed with a LEICA TCS 4D confocal laser scanning microscope (LEICA, Germany), equipped with an argon/krypton laser, using a 40x oil immersion objective. GFP fluorescence of chimeric MT and actin constructs (see above) was imaged using the 488nm excitation line of the laser and a 515nm long pass or 530nm band pass emission filter. Time-lapse series were generated from single optical sections of cells or tissues, which were captured at defined time intervals. Serial confocal optical sections were taken for Z-stack projections at different step sizes. The projection of image stacks and further image processing was done with the Scion Image software package (Scion Corporation) and Adobe Photoshop, respectively (Adobe Systems Inc.).

For all other studies:
Microscopical analysis was performed using an Olympus FV1000 upright confocal laser scanning microscope. All images were acquired with 4x/0.16 (dry), 10x/0.4 (dry) and 60x/1.35 (oil immersion) UPlanSApo objectives (Olympus). GFP as well as Alexa488-conjugated antibodies were excited at 488nm and detected between 505-530nm. Alexa546-conjugated antibody was excited at 543nm and detected at 550-650nm. Fluorescent vital dyes FM4-64 and AM4-65 were excited at 515nm or 488nm and detected at 650-750nm. To avoid bleed-through in double labelling experiments, all images were captured using line-sequential scanning mode. Post-processing of images was done with the aid of Olympus software FV1000 (Ver.1.7a), Image J 1.38x, Photoshop 6.0/CS, Microsoft Powerpoint and Open office applications.

**Heat treatment and recovery**

Slides with mounted plant seedlings were placed on a float in a water bath with temperatures set between 35°C and 42°C for 5 min up to 6 hours. Recovery took place at room temperature (20°C) for up to 2 days.
**Inhibitor and stress treatments for biochemical analysis**

In order to prevent unspecific stress reactions, plants grown on vertically oriented plates were layered horizontally and slightly covered with liquid ¼ strength Murashige & Skoog (¼MS) medium, prior to any treatment, for several hours. Brefeldin A (BFA, 50µM; 1.5h), H₂O₂ (10mM; 10min) and bacterial flagellin elicitor peptide, flg22 (10µM; 10-15min) were diluted in ¼ MS medium and carefully applied by exchanging liquids. Controls were identically treated by exchanging liquid medium.

**Phenotype analysis of the mpk6-2 mutant**

Mutant seedlings were grown as described above. As a control, Col-0 seedlings were always grown in parallel on the same phytagel plate. Root and hypocotyl length of wild type versus mutant seedlings were measured after 5 days using the stereo microscope Leica MZFL III. Lateral roots were counted on 8 days old seedlings. Root length was only measured in those plants, which developed a visible root.

**Dyes, inhibitors and elicitors**

The following dyes, inhibitors and elicitors were used:

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<td>SIGMA-ALDRICH</td>
<td>20µM / 0.5-2h</td>
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<tr>
<td>Flg22</td>
<td>Provided by Silke Robatzek, MPI, Cologne, Germany</td>
<td>10µM / 10-15min</td>
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<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
<td>SIGMA-ALDRICH</td>
<td>10mM / 10min</td>
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Frequently used buffers and solutions

½ MS medium
MS basal salt mixture (without vitamins) 2.3 g
sucrose 10 g
add Aqua dest. 1 l
pH 6, autoclaved
for solid medium incl. 4 g/l Phytagel

PBS
0.14 M NaCl 8.0 g
2.7 mM KCl 0.2 g
6.5 mM Na₂HPO₄ x H₂O 1.15 g
1.5 mM KH₂PO₄ 0.2 g
add Aqua dest. 1 l
pH 7.3

MTSB (1x)
50 mM PIPES 15.1 g (PIPES free acid MW = 302.4)
5 mM MgSO₄ x 7H₂O 1.23 g
5 mM EGTA 10 ml of a 0.5 M stock solution
add Aqua dest. 1 l
pH 6.9 (with KOH)

Mounting Medium
p-Phenylenediamine 100 mg
Glycerol 90 %
PBS 10 %
pH 8.0; adjusted with carbonat/bicarbonate or Tris

Solutions for SDS-PAGE and Western-Blotting

Resolving gels (10-15 %)
Acrylamide/Bis (30%/2,67) 3.3 - 5.0 ml
**Aqua dest.**          2.4 - 4.1 ml  
1,5 mM Tris-HCl, pH 8,8     2.5 ml  
10 % SDS                  100 µl  
10 % ammonium persulfate  50 µl  
TEMED                     5 µl

**Stacking gel (4 %)**  
Acrylamide/Bis (30%/2,67)   1,3 ml  
Aqua dest.                     6,1 ml  
0,5 mM Tris-HCl, pH 6,8  2,5 ml  
10 % SDS                        100 µl  
10 % ammonium persulfate    50 µl  
TEMED                         5 µl

**4 x sample buffer**  
(62,5mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol)  
Aqua dest.               3,0 ml  
0,5M Tris-HCl, pH 6,8   1,0 ml  
glycerol                1,6 ml  
10 % SDS               1,6 ml  
β-mercaptoethanol      0,4 ml  
0,5% (w/v) bromphenol blue 0,4 ml

**SDS-running buffer (10x)**  
250 mM Tris         30 g  
1,92 M Glycine         144 g  
10 % SDS                10 g  
add Aqua dest.          1 l

**Transfer buffer (1x)**  
20 mM Tris          9,68 g  
150 mM glycine        45,05 g  
10% methanol            400 ml  
add Aqua dest.          4 l
MATERIAL AND METHODS

TBS-buffer (10x)
100 mM Tris-HCl (pH 7,4)  12,1 g
1,5 mM NaCl     87,6 g
Add. A. dest.        1 l

TBS-T buffer
0,1 % Tween 20    500 µl
TBS buffer       500 ml

Solutions for molecular biology methods

Sample buffer for agarose gel electrophoresis
Brome phenol blue     0,25 %
Xylene Cyanole FF     0,25 %
Ficoll 400            15 %
in Aqua dest. (MilliQ)

TAE (Tris acetate EDTA) buffer (50x)
Tris      242 g
0,5 M EDTA, pH 8,0    100 ml
Acetic acid 100 %   57,1 ml
add Aqua dest. (MilliQ)     1 l

TBE (Tris Borate EDTA) buffer (10x)
Tris          53 g
Boric acid   27,5 g
0,5 M EDTA, pH 8,0    20 ml
add Aqua dest. (MilliQ)     1 l
3. RESULTS

3.1 Specificity of antibodies against *Arabidopsis* MPK3, MPK4 and MPK6

In order to characterize the antibodies used for the detection of MPK3, MPK4 and MPK6, immunoblots of protein extracts from *Arabidopsis* roots were performed. Each antibody detected its specific MAPK at the predicted molecular weight of 43kDa for MPK3, 41kDa for MPK4 and 47kDa for MPK6 (Fig 4 A). However, the antibody against MPK4 but not MPK3 and MPK6 detected an additional band at ~55kDa. This additional band most likely represents a cytosolic protein, because it solely appeared within the cytosolic but not within the microsomal fraction (Fig 4 B).

Taken together, each antibody detected an individual protein band of predicted molecular size for the respective MAPK without cross-reacting with the other two MAPKs.

![Figure 4. Characterization of MAPK-specific antibodies.](image)

**(A)** Immunoblot detection of MPK3 (43kDa), MPK4 (41kDa) and MPK6 (47kDa) using crude protein extracts from roots of one week old *Arabidopsis* seedlings. Note that each antibody detects a specific band at the predicted molecular size. **(B)** Immunoblot detection of MPK4 on isolated cytosolic and microsomal fractions prepared by differential centrifugation using a postnuclear extract of root proteins. Note that the additional 55kDa band only appears within the cytosolic fraction.

3.2 MPK3, MPK4 and MPK6 associate with microsomal and cytosolic fractions

To analyze the subcellular distribution of MPK3, MPK4 and MPK6, a quantitative immunoblot evaluation of cytosolic and microsomal protein fractions was performed. Previous to the measurement, cross-contamination of isolated fractions was excluded in both fractions by western blot analysis using marker antibodies against: (i) cytosolic phosphoenol pyruvate carboxylase (PEPC) and (ii) against PEP12, a transmembrane protein known to reside at late endosomes and pre-vacuolar compartments (PVC) (da Silva Conceição et al. [2000]).
1997; Tse et al. 2004) (Fig 5 A). Although these results suggested relative purity of the
fractions, both fractions were further tested by microscopic analysis. For this purpose the
fluorescent lipophilic marker FM4-64 was directly applied on isolated fractions as a marker
for microsomal membranes (Fig 5 B). FM4-64 stained only the microsomal but not the
cytosolic fraction.

Subsequent immunoblotting experiments using antibodies against MPK3, MPK4 and
MPK6 revealed high abundance of all three MAPKs in both cytosolic and microsomal
fractions (Fig 6 A). Quantification of the intensity of immunoreactive protein bands from four
independent experiments (Fig 6 B-C) revealed 55.1% of the total MPK3 signal to be
associated with the microsomal and 44.9% with the cytosolic fraction. This suggests a major
microsomal association of MPK3 with a difference of 10.2 % between both fractions. In
contrast, MPK4 and MPK6 showed slightly different distributions. The respective average
microsomal association was 40.5% for MPK4 and 44.8% for MPK6. This represents 19%
difference between these two fractions for MPK4 and 10.4% for MPK6, suggesting a small
majority of cytosolic localization. Taken together, all three MAPKs can be characterized as
partially associated with the microsomal fraction.

Figure 5. Purity test for cytosolic and microsomal fractions. (A) Immunoblot
detection of the cytosolic protein PEPC and the transmembrane protein PEP12 on
cytosolic and microsomal protein fractions prepared by differential centrifugation of a
post-nuclear root extract. Note that there is almost no cross-contamination. (B)
Microscopical analysis of cytosolic and microsomal fractions directly labeled with
FM4-64 (5µg/ml for 10min). White spots represent labelled microsomal structures.
Note that there is no labelling within the cytosolic fraction.
3.3 MPK3, MPK4 and MPK6 co-localize with PM, clathrin-, TGN- and Golgi-markers in isolated subcellular fractions

The association of all tested MAPKs with the microsomal fraction raised the question as to the identity and composition of microsomal membrane compartments. To address this, microsomal extracts of 2-week-old plants were sub-fractioned by density gradient centrifugation on a continuous sucrose gradient (30-55% sucrose). Total proteins of the different fractions were prepared for immunoblotting and probed with MAPK antibodies and with antibodies specific for different organellar marker proteins. Due to the fact that fractionation of microsomal extracts only yields relatively small amounts of protein per fraction, two gradients were prepared (Fig 7 A and B). Results obtained from the first gradient showed that all three MAPKs noticeably co-fractionated with the clathrin light chain recognized by monoclonal antibody specifically binding to a 28-29 kDa protein band...
corresponding to one of three *Arabidopsis* CLCs, which were shown to functionally interact with clathrin heavy chain (CHC) (Scheele and Holstein 2002) (Fig 7 A). MPK3, MPK4 and MPK6 also co-fractionated with secretory carrier membrane protein1 (SCAMP1), representing a reliable marker for the trans-Golgi network (TGN), a tubular-vesicular secretory/endocytotic compartment partially coated by clathrin (Lam *et al.* 2007a). Further, all three MAPKs co-fractionated with two bands (63.5kDa and 66kDa) that were detected by an antibody against α-1,2-mannosidase I, a commonly used Golgi marker (for example Preuss *et al.* 2004a). On the other hand, MAPKs did not co-fractionate with SEC12, an ER-resident protein (Bar-Peled and Raikhel, 1997) or with PEP12, a marker of prevacuolar compartments (PVC).

Data from the second gradient shown in Fig 7 B revealed that the tested MAPKs co-localized with plasma membrane-intrinsic protein2;2 (PIP2;2), used as PM marker. Overall, there was only a negligible contamination with PEPC within the first fractions (30-40% sucrose) representing cytosolic protein. Because distribution of all three MAPKs was almost identical in all experiments, MPK6 was used as a representative MAPK in the second gradient.

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**Fig 7 A**

<table>
<thead>
<tr>
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<tr>
<td>MPK3 (42)</td>
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<tr>
<td>Clathrin LC (28)</td>
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<tr>
<td>Scamp1a (37)</td>
</tr>
<tr>
<td>α-Mannosidase (63.5/66)</td>
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<td>Sec12 (43)</td>
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<td>Pep12 (32.5/34.5/36)</td>
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**Fig 7 B**

<table>
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</tr>
<tr>
<td>PIP2;2 (30)</td>
</tr>
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<td>PEPC (110)</td>
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</tbody>
</table>
### RESULTS

3.4 MPK3, MPK4 and MPK6 localize to distinct spots/patches in the cytosol, at the cell cortex and in the nucleus of root cells

To further investigate the localization of all three MAPKs at the subcellular level, whole-mount immunolabellings on fixed *Arabidopsis* roots were performed using MAPK antibodies. To exclude unspecific labelling, control experiments were done using solely the secondary antibody. No signal was detected in such controls (Fig 8).

Microscopical analysis of several root tips from at least three independent experiments and for each MAPK revealed multiple localizations (Fig 9). Besides a diffuse cytosolic background, a multitude of spot-like and patch-like structures were labelled by all MAPK antibodies. Nuclear localization was found to be present in all cell types but showing some variability from cell to cell. Moreover, all MAPKs showed pronounced labelling at the PM (Fig 9 A, D and G; overviews).

Nevertheless, some clear differences among the MAPKs could be described. Thus, MPK3 showed the strongest microsomal labelling supporting the results from quantitative immunoblotting experiments. In meristematic cells, MPK3 labelling occurred all-over the surface of the PM (Fig 9 B, arrows) and was highly abundant within cytosolic spot-like and rod-shaped microsomal structures in post-meristematic elongating cells (Fig 9 C, arrowheads). Labelling of the PM did not change significantly within the different cell types. In contrast, both MPK4 and MPK6 generally labelled round shaped spot- and/or patch-like structures (Fig 9 E-F and H-I). Interestingly, dense spots often appeared along the PM in meristematic and post-meristematic epidermal cells (Fig 9 E and H, arrows), while both MPK4 and MPK6 seemed to be distributed randomly within cytosolic spots and patches in elongating cells (Fig 9 F and I, arrowheads). Both MAPKs sometimes lost clear association with the PM in elongating cells.
Taken together, these localization experiments strongly support the results from immunoblotting experiments (chapter 3.2) showing a clear association of MPK3, MPK4 and MPK6 with the microsomal fractions and microsomal subcellular structures in intact roots.

**Figure 8. Secondary antibody control.** Overview of a whole-mount labelled root-tip of a 6 days old *Arabidopsis* seedling. Labelling was done with the secondary antibody only in order to exclude detection of an unspecific signal caused by this fluorescent antibody. The figure shows a DIC image on the left and the corresponding immunolabelling on the right side. Note that there is no unspecific labelling. DIC= differential interphase contrast.

**Figure 9. Whole-mount immunofluorescent localization of MPK3, MPK4 and MPK6.** (A, D, G) Overview of fixed, antibody-labelled root tips of 6 days old *Arabidopsis* seedlings. All pictures show labelling of meristematic and post-meristematic elongating cells using specific MAPK antibodies. Note that there is a multitude of spot-like structures at cell peripheries and in the cytosol. (B, E, H) Detail of the distribution of MAPKs in meristematic cells. Arrows indicate punctuated labelling of the PM, which was found for all three MAPKs. (C, F, I) Detail showing typical distribution of MAPKs in post-meristematic elongating cells. Arrowheads indicate spot-like structures that often appeared within the cytosol of these cells. Note that the labelling of the PM is weaker in cells labelled with antibodies against MPK4 and MPK6 but not in cells labelled with MPK3.
3.5 **Immunofluorescence double-labelling studies**

In order to reveal the identity of the microsomal structures labelled by MAPK antibodies, whole-mount co-localization studies were performed using rabbit polyclonal MAPK antibodies in combination with the specific mouse monoclonal clathrin light chain (CLC) antibody, which was previously used for immunoblotting (chapter 3.3). Because this work mainly focusses on the characterization of MPK6, further double-labelling with marker antibodies against Golgi and TGN compartments were done only for this MAPK.

To avoid unspecific labelling and detection of unspecific signals, all experiments included negative controls with the two secondary antibodies alone (Fig 10) followed by microscopic analysis using the sequential scanning mode in a confocal laser scanning microscope (Olympus FV1000).

![Figure 10. Secondary antibody control. Overview of a whole-mount labelled root-tip of a 6 days old *Arabidopsis* seedling. Labelling was done with a combination of red and green fluorescent secondary antibodies in order to exclude detection of an unspecific signal. The figure shows green and red channel and the corresponding DIC image on the right. Note that there is no fluorescence signal detected.](image)

3.5.1 **MPK3 and MPK4 co-localize with clathrin at the PM**

Further supporting the above immunolabelling data, MPK3 was found to co-localize with CLC all along the PM (Fig 11 A-C, arrows). Interestingly, only the MPK3-positive spot-like but not the rod-shaped cytosolic microsomal structures partially associated with clathrin-labelled organelles (Fig 11 A-F, white arrowheads). In some cases, instead of a clear co-localization, MPK3-positive spots appeared only at specific domains of bigger compartments labelled by the CLC antibody (Fig 11 A-F, red arrowheads).

In contrast, MPK4 did not co-localize with CLC along the whole PM surface but rather within distinct spot-like domains (Fig 11 G-I, arrows). Similar to MPK3, cytosolic spots often associated with CLC-positive compartments (Fig 11 G-L, white arrowheads). As
in the case of MPK3, MPK4-positive spots were also sometimes overlapping with domains of CLC positive organelles (Fig 11 G-L, red arrowheads).

In summary, these experiments suggest an association of MPK3 and MPK4 with the PM and within specific domains of clathrin-labelled intracellular compartments.

### Figure 11. Whole-mount co-immunolabelling of clathrin light chain (CLC) and MPK3 or MPK4, respectively. (A-C and G-I) Single mid-sections of postmeristematic elongating root epidermal cells, showing typical distribution of CLC and MPK3 or MPK4, respectively. (D-F and J-L) Single cortical-sections directly underneath the PM. Arrows indicate co-localization of both proteins at the PM. Arrowheads indicate partial co-localization at spot like structures within the cytosol. Bigger red arrowheads indicate such MPK3- or MPK4-positive spot-like structures that partially co-localize with domains of bigger CLC positive compartments.

#### 3.5.2 MPK6 co-localizes with clathrin at the PM and at the TGN

Two different approaches were used for co-localization of MPK6 with clathrin-coated subcellular structures. First, double co-immunolabelling was performed on wild-type seedlings according to the above method used for MPK3 and MPK4. Second, transgenic *Arabidopsis* seedlings stably expressing a CLC:GFP construct (using the At2g40060 CLC sequence, which is coding for the 28,8 kDa light chain protein) (Konopka *et al.* 2008) were used to visualize clathrin:GFP in combination with MPK6. In this case, seedlings were co-labelled with rabbit MPK6 antibody and with a monoclonal mouse GFP antibody (Fig 12 A-I).

Microscopic analysis revealed that MPK6 co-localized *in-situ* with both native CLC (Fig 12 A-F) as well as with the CLC:GFP fusion protein (Fig 12 G-I). As in the case of MPK4, this co-localization occurred mainly in spot-like structures/domains directly at the PM.
(Fig 12 A-I, arrows) or within the cytosol (Fig 12 A-I, arrowheads). In comparison to the in-situ CLC labelling, CLC:GFP fusion protein strongly labelled spot-like cytosolic organelles that often clearly co-localized with MPK6 (Fig 12 G-I, arrowheads).

To further test, whether these spot-like structures represent the partially clathrin-coated TGN, antibodies against the TGN localized secretory carrier membrane protein1 (SCAMP1) (Lam et al. 2007a) were used for co-localization with MPK6. Double labelling of MPK6 and SCAMP1 clearly revealed their co-localization at the TGN (Fig 13 J-O, arrowheads). Because MPK6 and SCAMP1 were both raised in rabbit, specific negative controls were performed in order to exclude unspecific labelling (Fig 13).

In order to localize Golgi-stacks a specific commercial mouse monoclonal antibody against Golgi resident 58k protein was used (Li and Yan 2000; Baluska et al. 2002). As shown in Fig 12 P-U, epidermal cells contained widely dispersed Golgi-stacks within the cytosol. In analogy to the TGN, which is known to be closely attached to the Golgi-stacks in some cases (reviewed by Staehelin and Kang 2008), MPK6-positive spots showed no direct co-localization but were occasionally found in association with Golgi-stacks. This further supports the conclusion of MPK6 being localized at the TGN.

In summary, these experiments suggest an association of MPK6 with clathrin at the PM and at the TGN.
RESULTS

Figure 12. Co-localization of MPK6 with clathrin, TGN and Golgi in *Arabidopsis* root epidermal cells using whole-mount immunofluorescence labelling.  


(D-F) Magnification of boxed area in C.  

(G-I) Co-localization of CLC:GFP and MPK6 in transgenic root epidermal cells expressing a 35S::CLC:GFP construct. Arrows indicate co-labelling at the PM, arrowheads indicate co-localization on cytosolic microsomal spots. Note that MPK6 massively co-localizes with clathrin at spot-like structures along the PM and within the cytosol.  

(J-L) Co-localization of MPK6 and TGN marker SCAMP1.  

(M-O) Magnification of boxed area in L. Note that both proteins almost completely co-localize on spot-like structures within epidermal cells. Arrowheads indicate some of the spots that are co-localizing.  

(P-R) Co-localization of MPK6 and Golgi marker 58k.  

(S-U) Magnification of boxed area in R. Arrowheads indicate spots that are tightly associated with each other. Arrows indicate spots that are clearly unconnected. Note that there is partially a tight association but no direct co-localization of spots labelled by both antibodies. Images A-I show single midplane sections of the cells in order to better illustrate PM localization, while images J-U show single cortical section of the cells in order to illustrate cytosolic, spot-like localization. Bars are 5µm.
RESULTS

3.6 GFP:MPK6 co-localizes with the vital endocytotic tracer FM4-64

To further characterize the identity of MPK6-positive subcellular compartments, a transgenic *Arabidopsis* line stably expressing a 35S::GFP:MPK6 construct (developed by Martina Beck in our lab) was used for in-vivo and in-situ microscopic co-immunolabelling studies. Additionally, these transgenic plants were incubated with the endocytotic tracer FM4-64. FM4-64 is a red fluorescent amphiphilic styryl dye that, if applied exogenously, incorporates into the PM. Following the endocytotic pathway, this marker sequentially labels early endosomes, late endosomes and vacuolar membranes (tonoplast) in plants (Bolte et al. 2004). Therefore, it is commonly used to determine, if a protein is associated with the subcellular endocytotic network (for review see Müller et al. 2007b).

Immunoblotting analysis of microsomal and cytosolic protein fractions from these transgenic plants using antibodies against MPK6 and GFP revealed that the GFP:MPK6 (74kDa) fusion protein was clearly present in both fractions (Fig 14 A). This suggests that the GFP-tag does not disturb the microsomal association of MPK6. Moreover, the fact that the MPK6 antibody but not the GFP antibody specifically detects two bands representing the fusion protein and native MPK6 (47kDa), again demonstrates the antibody specificity.

In-vivo microscopic analysis showed that GFP:MPK6 co-localizes with FM4-64 at the PM and on spot-like endosomes within root epidermal cells (Fig 14 B-D). Further, time-lapse imaging provided evidence that these spots are moving along selective tracks throughout the cell (Fig 14 E-J). Therefore, these experiments strongly support the biochemical fractionation and co-immunolabelling data presented in chapters 3.2 - 3.5.

In contrast to growing epidermal root cells, which mainly showed spot-like structures embedded in an overall diffuse cytoplasmic fluorescence of GFP:MPK6, fully grown epidermal cells often showed labelling of a network like structure strongly resembling the ER-
network of such cells (Ridge et al. 1999) (Fig 15). Bright fluorescent spots that co-localized with FM4-64 labelled endosomes appeared within this network (Fig 15, arrows). To further test, whether MPK6 localizes to the ER, transgenic seedlings stably expressing a mGFP5-ER construct (kindly provided by Jim Haseloff, University of Cambridge, UK) were co-immunolabelled with a mouse GFP antibody and with the antibody against MPK6. mGFP5-ER is a fusion protein containing an N-terminal signal peptide derived from the Arabidopsis vacuolar basic chitinase and the C-terminal amino acid sequence HDEL. HDEL is a tetrapeptide sequence that acts as an ER retention signal and therefore specifically labels ER structures.

Microscopical analysis of fully grown root epidermal cells showed that MPK6 did not localize to the ER network (Fig 16) but several MPK6-positive spots were clearly embedded in gaps within this network (Fig 17, arrows). These spots probably represent those bright fluorescent spots, which co-localized with FM4-64 (Fig 15).

Taken together, these experiments supported the biochemical results, excluding an association of MPK6 with the ER and it provides further evidence that native MPK6 localizes to endosomal structures.
RESULTS

Figure 14. In vivo localization of GFP:MPK6 in transgenic Arabidopsis roots. (A) Distribution analysis of GFP:MPK6 (74kDa) in cytosolic and microsomal fractions using MPK6- and GFP- specific antibodies. Note that the GFP:MPK6 fusion protein is abundant in both fractions. (B-D) Co-localization of GFP:MPK6 and FM4-64 on endosomal organelles (white arrows) and at the PM (arrowheads) in root epidermal cells. (E-J) Time series showing co-labelled mobile GFP:MPK6 and FM4-64 positive endosomes (arrows). Time is indicated at the bottom of each image. Bars represent 10µm.

Figure 15. In vivo localization of GFP:MPK6 in fully grown root epidermal cells of transgenic Arabidopsis roots. (A) Localization of GFP:MPK6 within an ER-like network in fully grown root epidermal cells. Arrows indicate bright fluorescent spots within the network. (B) Labelling of endosomes (arrows) by the endocytotic marker FM4-64. (C) Merged image of A and B. Note that FM-labelled endosomes co-localize with the bright fluorescent spots visualized by the GFP:MPK6 fusion protein.

Figure 16. Whole-mount co-immunolabelling of mGFP5-ER and MPK6 in fully grown root epidermal cells of transgenic Arabidopsis roots. (A; E) Labelling of the ER network by a GFP-specific antibody detecting mGFP5-ER. (B; D) Labelling of MPK6 using MPK6-specific antibody. (C; F) Merged images. Arrows indicate MPK6 positive spots and their localization with respect to the ER network. Note that almost all MPK6 labelled spots are located in gaps within this ER network.
3.7 Oxidative stress causes phosphorylation of cytosolic but not microsomal MAPKs

In order to test the phosphorylation status of microsomal and cytosolic MPK3, MPK4 and MPK6 under oxidative stress conditions, 6-8 days old seedling roots were treated with 10mM H$_2$O$_2$ for 10 minutes (Noriega et al. 2009) and compared to control plants by immunoblot analysis using Phos-tag$^\text{TM}$ acrylamide gels. Phos-tag$^\text{TM}$ is a phosphate binding tag that is embedded in the acrylamide network of the SDS-gels. By loosely binding phosphorylated proteins, this tag is able to slow down their movement during electrophoresis. This causes the appearance of multiple bands with each band representing a specific phosphorylation status of the detected protein (see also Material and Methods).

Application of H$_2$O$_2$, which is thought to act as a general upstream activator of MAPKs (reviewed by Colcombet and Hirt 2008) affected the phosphorylation status of all three MAPKs (Fig 17). In untreated plants, the MPK3 antibody detected multiple phosphorylated bands of different intensity and size in both cytosolic and microsomal fractions. Interestingly, the intensity of some bands in the microsomal fraction significantly decreased, while those in the cytosolic fraction became significantly stronger in H$_2$O$_2$ treated plants.

A similar result was found also for MPK4 and MPK6. Phosphorylated bands were clearly detected within the cytosolic but not the microsomal fraction after H$_2$O$_2$ application for both MAPKs. Taken together, these experiments indicate that H$_2$O$_2$-induced activation of all three MAPKs mainly occurs in the cytosol.
3.8 Treatment with Brefeldin A (BFA) alters MAPK distribution but does not affect MAPK activity

Brefeldin A (BFA) is a fungal toxin that is extensively used as a general inhibitor of secretion and membrane recycling, which causes the accumulation and aggregation of early endocytotic TGN vesicles in Arabidopsis (for detailed overview see chapter 1.4.2). Because proteins residing on organelles of the early endocytotic pathway mostly show some sensitivity to BFA (see below) it can be used as an indicator to identify the specific localization of those proteins.

Immunoblotting experiments on isolated cytosolic and microsomal fractions of control and BFA-treated roots (50µM for 1.5h) of 6-8 days old seedlings were performed to test, whether MPK3, MPK4 and MPK6 distribution is affected by this pharmacological disturbance of membrane recycling. BFA treatment caused a significant increase in all MAPKs within the cytosolic fraction accompanied by a comparable decrease in the microsomal fraction (Fig 18 A). Distribution analysis combined from three independent experiments revealed an average amount of 61.3 % (MPK3), 64.4 % (MPK4) and 68.7 % (MPK6) of cytosolic MAPK protein in treated roots (Fig 18 B and C). According to the previous measurements done on non-treated plants (see Fig 6), this values represent a cytosolic shift of 32.8 % for MPK3, 9.8 % for MPK4 and 27 % for MPK6 (Fig 18 B).

In summary, all three MAPKs seem to partially loose their association with microsomes upon BFA treatment. To further check, whether the observed reaction is accompanied by activation the phosphorylation status of microsomal and cytosolic MAPKs in BFA-treated versus control plants was compared by immunoblot analysis using Phos-tag™ Acrylamide gels. No significant additional phosphorylated bands could be detected upon BFA treatment (Fig 19).
Figure 18. Distribution analysis of MPK3, MPK4 and MPK6 in BFA treated roots. (A) Immunoblot analysis of the distribution of cytosolic (C) and microsomal (M) MAPKs in control versus BFA treated (50µM/1.5h) roots of 6-8 days old seedlings. (B) Average cytosolic and microsomal distribution of all three MAPKs after BFA treatment, calculated from 3 independent experiments. Also shown are the values for the cytosolic shift for each MAPK, calculated by direct comparison with values from untreated plants shown in Figure 6. (C) Graphical view of the average MAPK distribution after BFA treatment. (D) Graph showing changes in the distribution of all MAPKs after BFA in comparison to untreated cells.
3.9 Microsomal MPK6 participates in flagellin signalling

3.9.1 Microsomal and cytosolic MPK6 becomes phosphorylated in flg22-treated plants

Until now, flagellin induced internalization of FLS2 is the only experimentally proven example of receptor-mediated endocytosis (RME) in plant cells (see also chapter 1.3). To investigate, whether microsomal MPK6 is specifically involved in the flagellin-triggered stress response, *Arabidopsis* seedlings were treated with the 22-amino-acids long flagellin peptide flg22, which is known to activate MPK6 (Nühse *et al.*, 2000). When the phosphorylation status of both cytosolic and microsomal MPK6 fractions was tested upon flg22 treatment, two phosphorylated bands appeared in the cytosolic fraction and a weaker single band of phosphorylated MPK6 in the microsomal fraction (Fig 20), suggesting that differently phosphorylated MPK6 subpopulations are associated with different cellular compartments.

Because H₂O₂ was shown to activate cytosolic but not microsomal MPK6 (see Fig 17) this experiment provided a first indication that stimulus-specific signalling of MPK6 is coupled with its spatial cellular organization.
3.9.2  FLS2 redistributes to early endosomal fractions after flagellin treatment

In *Arabidopsis* leaves it was shown previously that a FLS2:GFP fusion protein localizes to the PM, but becomes rapidly internalized to yet unidentified endocytotic organelles after flagellin triggered activation (Robatzek *et al.* 2006). In order to test, whether FLS2 and MPK6 associate with the same endocytotic organelle, plant cells were treated with the elicitor epitope flg22. Microsomal extracts from flg22-treated plants were fractionated in sucrose density gradients and FLS2 distribution was determined in treated versus control plants and this was compared with previously described localization of MAPKs and organellar markers. For this purpose, a polyclonal FLS2 antibody was used (kindly provided by Silke Robatzek, MPI, cologne, Germany). As shown in Fig 21 A, the FLS2 antibody specifically detected one band at the predicted size of around 170kDa (Chinchilla *et al.* 2007).

Untreated plants showed the strongest localization within fractions of 35.5-38.5 % sucrose. A second, small maximum was additionally found in the 49.5 % sucrose fraction (Fig 21 B and D). In contrast, flg22-treated plants showed an extended distribution including the fractions between 39-43 % sucrose (Fig 21 C-D). Compared with previous results (see Fig 7), untreated plants showed an FLS2 distribution corresponding with the localization of the late endosomal marker PEP12. Moreover, the much smaller maximum at 49.5 % sucrose corresponds with one of the two CLC maxima. In contrast, flg22 treated plants showed an additional distribution that also included fractions typically accumulating MAPKs and early endosomal compartments, indicating that activated FLS2 partially relocates to the same organelles, MAPKs are associated with.
3.9.3 FLS2-GFP localizes to the PM and co-localizes with FM4-64 in Arabidopsis roots

To further characterize the localization of FLS2 in roots, a transgenic Arabidopsis line stably expressing FLS2-GFP under the control of its native promoter (kindly provided by Silke Robatzek, MPI, Cologne, Germany) were microscopically analyzed. In order to test, whether FLS2 is associated with the membrane compartments of the endocytotic pathway, transgenic plants were co-labelled with FM4-64 and additionally treated with BFA (50µM/1.5h). As shown in Fig 22 A, FLS2-GFP clearly localized to the PM and in cytosolic spots and co-localized with the endocytotic marker FM4-64 at the PM (Fig 22 A-C, arrows) but not within endosomal compartments (Fig 22 A-C, arrowheads).

Interestingly, when treated with BFA FLS2-GFP accumulated within BFA compartments and partially co-localized with FM4-64 on smaller cytosolic spots (Fig 22 D-F). However, some of the FLS2-GFP labelled spots clearly did not co-localize with FM4-64.
3.10 Cytoskeletal association of MPK6

Growing evidence suggests that MAPK activity is also involved in the regulation of cytoskeletal arrangement and dynamics in plants (Šamaj et al. 2002 and 2004). Interestingly, several studies described MAPK regulation of tubulin- and actin-binding proteins such as kinesins (Ishikawa et al. 2002; Nishihama et al. 2001 and 2002), MAP65 proteins (Smertenko et al. 2006) or profilins (Limmongkon et al. 2004).

In order to check, whether MPK6 is directly associated with cytoskeletal elements in *Arabidopsis* roots whole-mount co-localization studies were performed on 5-6 days old seedlings using the rabbit MPK6 antibody in combination with a specific rat derived antibody against α-tubulin or with a mouse derived antibody against plant actin, respectively.

3.10.1 MPK6 co-localizes with mitotic and cortical MTs

Microscopic analysis revealed that the α-tubulin antibody clearly labelled all known microtubular structures, namely cytokinetic microtubules (MTs) (Fig 23 B, E, H and K), subcortical MTs (Fig 23 N) as well as a dense, well defined cortical array directly underneath of the PM (Fig 23 U). MPK6 was found to associate with mitotic microtubules including the
preprophase band (PPB) and the phragmoplast but not or only weakly with the mitotic spindle (Fig 23 A-L, arrows). Further analysis revealed that MPK6 also localizes within spot-like structures at the level of the maturing cell plate (Fig 23 G-I). Moreover, MPK6 co-localized within spot like structures in the subcortical area of elongating postmeristematic cells (Fig 23 M-O, arrowheads). In contrast, non dividing meristematic cells did not show cytosolic spots but revealed close association of MPK6 with the PM (Fig 23 P-R, arrowheads).

MPK6 also co-localized with MTs of the cortical array. In most cases this localization appeared in a spot like manner though less clearly defined because of a higher diffuse cytosolic background (Fig 23 S-V). However, in some cases MPK6 clearly labelled the complete cortical MT array (Fig 23 W-Y).

Figure 23. Whole-mount co-immunolabeling of MPK6 and α-tubulin in root epidermal cells. (A-C) Co-localization of MPK6 and α-tubulin at the preprophase band (arrows). (D-F) Cortical section showing co-localization at the phragmoplast (arrows). (G-I) Subcortical section of the cell from D-F showing localization of MPK6 at the phragmoplast (arrows) and in spots at the level of the mature (arrowheads) and growing cell plate (right hand arrows). (J-L) Metaphase cell showing only very weak localization of MPK6 to the spindle (arrow). (M-R) Co-localization of MPK6 and α-tubulin in the subcortical area of postmeristematic cells (M-O) and non-dividing, meristematic cells (P-R). Arrowheads in M-O indicate co-localization with spot-like structures. Arrowheads in P-R point to the close association of MPK6 and α-tubulin at the PM. (S-V) Co-localization of MPK6 and cortical MTs in postmeristematic elongating cells. Lines in V are drawn to highlight some MTs that are obviously associated with MPK6. Note that co-localization is hard to distinguish from the cytotolic background. (W-Y) Single labelling of MPK6 at the cortical array of postmeristematic elongating cells. Note, that sometimes MPK6 nicely labelles the cortical array in these cells. Bars represent 10µm.
3.10.2 MPK6 does not co-localize with the actin cytoskeleton

In contrast to the well-defined microtubule network with its dense cortical array and only a few subcortical bundles or single MTs, which connect the nucleus with the PM, actin filaments are organized as a complex and dense network becoming more or less longitudinally oriented in postmeristematic elongating cells. This network runs through the center of the cell (Fig 24 E and H), through the cortical area directly underneath the PM (Fig 24 K) and is also present in the subcortical area underneath of the cortical MT array (Fig 24 N). Co-labelling of MPK6 with actin filaments revealed only partial and weak association in the different subcellular areas of both non dividing meristematic and postmeristematic elongating epidermal cells (Fig 24 D-O). Within early stage phragmoplasts actin mostly resided in the central part which probably contains the young cell plate while MPK6 localized to the whole phragmoplast except the mid-zone. Nevertheless, in a small area MPK6 overlapped with actin in the phragmoplast (Fig 24 A-C). Moreover, because MPK6 was found to localize within spot-like structures at the level of the maturing cell plate (Fig 23 J-L) it can not be excluded that there is also a partial co-localization.

**Figure 24. Whole-mount co-immunolabeling of MPK6 and actin in root epidermal cells.** (A-C) Co-labelling of MPK6 and actin in a cytokinetic cell at the stage of cell-plate formation. Note that the entire phragmoplast is clearly labelled by MPK6 (arrow) while actin mainly labels the mid-zone representing the area of cell-plate maturation (arrowhead). (D-I) Co-labelling in the subcortical area in the central parts of meristematic cells. Note that there is only weak co-localization of actin and MPK6 within the observed cell types and subcellular areas. (J-L) Co-labelling of MPK6 and actin at the cortical array directly underneath the PM. (M-O) Co-labelling at the early subcortical area directly underneath the area, which contains the cortical microtubules. Bars represent 5µm in A-I and 10µm in J-O.
3.11 Treatment with BFA alters arrangement of actin and subcellular distribution of microtubules

3.11.1 α-tubulin and MAPK behave similarly in BFA-treated cells

Microsomal and cytosolic fractions were prepared from roots of control and BFA-treated 5-6 days old Arabidopsis seedlings in order to test the effects of BFA on the plant cytoskeleton. Subsequent immunoblotting of these fractions using antibodies against actin and α-tubulin revealed that tubulin but not actin followed the behavior of MAPKs by showing a clear shift towards the cytosolic fraction (Fig 25).

![Figure 25. Cytosolic and microsomal distribution of α-tubulin and actin in control versus BFA-treated roots.](image)

3.11.2 BFA causes rearrangement of actin but not of MT and MPK6

To test whether there is a significant and visible change in the arrangement and/or distribution of the cytoskeletal elements and of MPK6, whole-mount immunolocalization and in-vivo localization experiments on control and BFA-treated plants were performed.

For co-visualization of MTs and MPK6 BFA treated Arabidopsis seedlings were co-immunolabelled with antibodies against MPK6 and α-tubulin. However, except a minor reduction of the number of MPK6 and α-tubulin labelled spots that could be observed sometimes, no significant visible changes were found on the arrangement or distribution of MPK6, the cortical MT array, the subcortical MTs or the cytokinetic structures like the phragmoplast (Fig 26).

To study the actin cytoskeleton, a transgenic line was used stably expressing a GFP:ABD2 fusion protein under the control of the constitutive 35S promoter (Voigt et al. 2005). ABD2 (actin binding domain2) is one of the two binding domains of the actin bundling protein fimbrin 1 and known to specifically visualize the actin cytoskeleton in Arabidopsis (for example Sheahan et al. 2004; Wang et al. 2004; Voigt et al. 2005). This transgenic line
was chosen because it allowed dynamic in vivo visualization of the actin cytoskeleton upon BFA treatment. In this case, observations were focused on elongating cells because they also allowed the tracking of BFA induced compartments.

Untreated cells showed a fine, dense and highly dynamic network of actin filaments and bundles within root epidermal cells (Fig 27 A). Embedded within this network, a prominent actin cage around the nucleus was visible in each cell (Fig 27 A, arrows), consistent with our previously published results (Voigt et al. 2005). After BFA treatment, especially the fine actin network strongly disintegrated and only a few thick bundled filaments as well as the prominent actin cage around the nucleus remained (Fig 27 B-D, arrows). Interestingly, actin accumulated within another type of cage that was observed around the BFA compartments (Fig 27 B-D, arrowheads). This was evident when plants were additionally pre-treated with the red fluorescent endocytotic marker FM4-64, which labels early endosomal TGN that is known to accumulate in BFA compartments in Arabidopsis root cells (for example Jaillais et al. 2006). FM4-64 clearly labelled the core of BFA compartments surrounded by newly formed actin cages (Fig 27 E-G, arrowheads). It remained unclear, whether the changes in the structure of the filamentous actin network were the consequence of a non-regulated disintegration of the dense filaments or whether the BFA-induced disturbance in vesicular trafficking causes a regulated modulation of the arrangement of the actin network. A non-regulated disintegration most likely would be accompanied by a breakdown of the ability of actin filaments to build motility tracks throughout the cells. Time series were performed to analyse the ability of BFA-treated cells to maintain actin-dependent subcellular movements. The newly formed BFA induced compartments were highly mobile and moved along actin bundles throughout the cell with a velocity up to 2.2µm per second as shown in Fig 27 H-M. This velocity is within the typical range described for vesicular structures in plant cells (for example Voigt et al. 2005).
Figure 26. Whole-mount co-immunolabelling of MPK6 and α-tubulin in root epidermal cells treated with BFA. (A-C) Co-labelling of MPK6 and cortical MTs in the area directly underneath of PM. (D-F) Co-labelling of MPK6 and α-tubulin in the subcortical area. (G-I) Co-labelling of MPK6 and MTs in the phragmoplast. Note that, except a minor increase in diffuse MPK6 labeling, there is no significant visible difference between BFA treated cells and the control cells showed in Figure 23. Bar represents 10µm.
RESULTS

3.12 MPK6 gene expression pattern in Arabidopsis roots

In order to analyze the expression pattern of MPK6 in root tissues in greater detail, wild type Arabidopsis seedlings stably transformed with a MPK6prom::GUS construct (generated by Martina Beck in our laboratory) were examined. As shown in Fig 28 A-B most roots showed the strongest MPK6 promoter activity in the early apical root meristem and lateral root cap as well as in the post-meristematic, transition zone of primary roots. The transition zone (also called distal elongation zone) is defined as the region of isotropic growth directly following the meristem (for review see Verbelen et al. 2006). On the other hand, the MPK6 promoter was less active in fully elongated differentiated cells of the root. This result indicates a role of MPK6 in meristematic and in young post-meristematic root tissues.

Figure 28. GUS staining of the transgenic PromMPK6::GUS root. (A) Overview of a root; (B) Detail on a tip of a primary root. Arrows indicate the approximate position of the stem cell niche at the root tip and transition zone following the root meristem. Bars represent 100µm.

3.13 mpk6-2 knockout mutant shows disturbed post-embryonic root growth

Previous studies on mpk6-2 and mpk6-3 knock out mutant lines described a weak phenotype such as reduction of fertility and a decrease in the size of siliques and internodes between flowers (Bush and Krysan, 2007). These authors also described an aberrant embryo development in the mutant lines, sometimes causing embryos to burst out of their seed coat.
RESULTS

during development. However, nothing was reported about the early post-embryonic development of seedling roots so far.

Because this thesis is mainly focused on the analysis of MAPKs, and especially of MPK6 in roots, further analysis was focused on the root phenotype of the mpk6-2 mutant line (SALK_073907).

To prove that this mutant was a homozygous knock-out, RT-PCR and immunoblotting experiments were performed. As shown in Fig 29, both mRNA and MPK6 protein were detected only within the wild type but not in the mpk6-2 mutant. This result showed that the mutant line used was homozygous. Additionally, the immunoblotting experiment further demonstrated the specificity of the MPK6 antibody, which was used in previous experiments described in this thesis.

To perform phenotypic studies, wild-type Col-0 and mpk6-2 knock-out mutants (in the Col-0 background) were grown on ½ MS phytagel plates for 5 days and compared with each other. Interestingly, some mpk6-2 mutant seedlings showed serious defects in the initial growth of roots but not in the green tissue. Direct comparison with wild-type seedlings revealed only a minor difference in the development of hypocotyls and cotyledons but mpk6-2 mutants sometimes almost completely lacked roots or roots were significantly shorter, especially during early post-embryonic development (3-8 days after germination) (Fig 30, A-C). Altogether, five experiments were performed comprising at least 350 seedlings for each Col-0 control and mutant plants. Depending on the experiment, the number of mutant seedlings that completely lacked root development was in the range between 16% and 44%. In contrast, no single wild-type seedling showed such an aberrant development. Interestingly, mutant seedlings with this phenotype did not permanently lack root development but most of these plants rather showed a significant delay in root growth. If those plants were measured again after 8 days, seedlings started to develop short roots (compare Fig 30 C with D).

Quantitative analysis of the average size of 5 days old seedlings revealed an average decrease of 34.44% of hypocotyl length and an average decrease of about 50.99 % in the length of primary roots in the mpk6-2 mutant line as compared to the Col-0 wild-type (Fig 30 H and I). Thus, there is likely a general growth inhibition during the first days of post-embryonic development after germination. Moreover, a noticeable difference in the development of lateral roots was found in mpk6-2 mutant plants after 8 days of growth. While there was almost no difference in the average number of developed lateral roots, mutant plants showed a significant increase in the formation of adventitious roots (Fig 30 E-G, arrows). Quantitative analysis revealed that while the average number of lateral roots per plant
(including adventitious roots) was only slightly decreased in the *mpk6-2* line the average portion of adventitious roots increased from 20.45% to 44.26% in the mutant compared to the Col-0 wild type (Fig 30 J).

**Figure 29.** RT-PCR and immunoblot analysis on wild type Col-0 and *mpk6-2* mutant line. (A) RT-PCR analysis using isolated RNA from roots and MPK6 specific primers. Note that mRNA for MPK6 is only detected in the wild-type (B). Immunoblot of crude extract from roots using the MPK6 antibody. Note that protein is only detected in the wild-type (Col-0) but not in the mutant line.

<table>
<thead>
<tr>
<th>Hypocotyl Length</th>
<th>Root Length</th>
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<tbody>
<tr>
<td>Col-0: 1353 μm, 8163 μm</td>
<td>Col-0: 2891 μm, 2880 μm</td>
</tr>
<tr>
<td><em>mpk6-2</em>: 887 μm, 4001 μm</td>
<td><em>mpk6-2</em>: 273 μm, 2688 μm</td>
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<tr>
<th></th>
<th>Col-0</th>
<th><em>mpk6-2</em></th>
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<tbody>
<tr>
<td>Total nr. of plants</td>
<td>68</td>
<td>90</td>
</tr>
<tr>
<td>Total nr. of lateral roots</td>
<td>220</td>
<td>244</td>
</tr>
<tr>
<td>Average nr. of lateral roots/plant</td>
<td>3.23 ± 1.77</td>
<td>2.71 ± 1.41</td>
</tr>
<tr>
<td>Total nr. of adventitious roots</td>
<td>45</td>
<td>108</td>
</tr>
<tr>
<td>Quotient of adventitious roots</td>
<td>20.45%</td>
<td>44.26%</td>
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RESULTS

3.14  mpk6-2 mutant shows irregular cell divisions and disturbed cell division planes

*Arabidopsis* root tips have a simple and invariable architecture with distinct and highly ordered cell files originating from non-differentiated meristematic cells (Fig 31 A) called initials. All initials are in contact with four central cells called the stem cells or quiescent center (QC) (Fig 31 A; C-D). Together, QC cells and initials are called the stem cell niche. In early post-embryonic root development the QC is characterized by strict mitotical inactivity and it was shown that QC cells directly control division and differentiation of the surrounding meristematic initials (for review see Doerner 1998; van den Berg *et al.* 1998; Jiang and Feldmann 2005). The stem cell niche is followed by meristematic tissue of around 200µm in length which is followed by the so called transition (distal elongation) zone (for review about nomenclature and partitioning of zones see Verbelen *et al.* 2006), a zone of very slow cell elongation where postmitotic cells undergo several physiological changes in order to prepare for rapid elongation and differentiation (Verbelen *et al.* 2006).

In order to further characterize the *mpk6-2* root phenotype on the cellular level 5-6 days old seedlings were stained with the red fluorescent membrane marker FM4-64 and microscopically analyzed. Examination of the root tip revealed disturbances in the cell division planes as well as additional irregular cell divisions within the quiescent center (QC) and in the transition zone (Fig 31). The extent of this phenotype was directly correlating with the degree of disturbance in root development and elongation. Seedlings with extensively reduced root growth showed a strong disturbance in the alignment of cells and the control of cell size and division in the area of the stem cell niche (Fig 31 E-G).
It is known that mitotic and cortical MTs regulate cell division, cell growth and morphology (for review see Müller et al. 2009; Wasteneys and Ambrose 2009). Because the previous results (chapter 3.10) revealed a direct association of MPK6 with cytokinetic and cortical MTs (see Fig 23) it is likely that the inhibited growth and the irregular cell divisions in the mpk6-2 mutant resulted from deficient organization of MTs. In order to test this hypothesis, whole-mount immunolabellings were performed on mutant seedlings using the α-tubulin specific antibody. Microscopic analysis of epidermal and cortex cells of the root tip showed clusters of irregularly dividing cells in the transition zone in strongly shortened mpk6-2 mutant roots (Fig 32 A-D). Careful examination revealed no cytokinetic defect, because all cells were able to build and finalize a normal cell plate. However, within these clusters and partially also in single cells of the surrounding tissue a multitude of cells showed redundant cell divisions with abnormal division planes caused by disoriented alignments of PPBs, mitotic spindles and phragmoplasts (Fig 32 E-G). In summary, these experiments revealed that the mpk6-2 knockout mutant is disturbed in the control of cell division planes, specifically in the stem cell niche and in the transition zone. These abnormalities are mainly
due to the appearance of redundant/abnormal mitotic activity and disturbances in the cell division plane caused by an aberrant alignment of mitotic microtubules in mutant root cells.

3.15 Inhibition of MAPK activity affects distribution of cortical MTs

In order to test, whether the general inhibition of MAPK activity affects the distribution and alignment of cortical MTs, transgenic seedlings stably expressing a GFP:MBD fusion protein under the control of the constitutive 35S promoter were treated with PD98059. MBD (microtubule binding domain) is part of the microtubule binding protein MAP4 (microtubule-associated protein 4) from mammals and it is known to visualize MTs in Arabidopsis (Marc et al. 1998; Müller et al. 2007a). PD98059 is a cell permeable highly specific inhibitor of MAPKKs and therefore represents a useful tool to generally inhibit downstream MAPK activity (for example Alessi et al. 1995, Dudley et al. 1995).

In untreated elongating root epidermal cells, cortical MTs showed their typical uniformly oriented transversal orientation (Fig 33 A and B). In contrast, when treated with PD98059 those cells showed bundling of cortical MTs (Fig 33 C and D, arrows). Interestingly, this bundling did not affect the ability of cells to rearrange the overall MT array towards a transversal alignment in young, elongating cells (Fig 33 E-F).