THESE DE DOCTORAT DE L'UNIVERSITE D'EVRY

Ecole Doctorale « Génome Aux Organismes »

Spécialité Biologie Cellulaire

**Pour l'obtention du grade de**

**Docteur ès Sciences de l'Université d'Evry Val d'Essonne**

**Présenté par**

**Souha BERRIRI**

# Identification of constitutively active forms of *Arabidopsis* MAP Kinases brings more evidence on MPK4 function in plant immunity

*Identification de mutants constitutivement actifs de MAP Kinases d'Arabidopsis. Démonstration de leur intérêt à travers l'étude de la fonction de MPK4 dans les réponses aux pathogènes*

***Soutenue le 10 Janvier 2012, devant le jury composé de :***

| | |
|---|---|
| Pr. Thorsten Nürnberger, Professeur à l'Université de Tübingen | Rapporteur |
| Dr. Sylvain Merlot, Chargé de Recherches au CNRS | Rapporteur |
| Dr Martine Thomas, Maitre de conférences à Paris Sud11 | Examinateur |
| Pr. Bénédicte Sturbois, Professeur à l'Université d'Evry | Examinateur |
| Dr. Jean Colcombet, Chargé de Recherches INRA, Evry | Encadrant |
| Pr. Heribert Hirt, Directeur de Recherches INRA, Evry | Directeur de Thèse |

// Résumé heading
# *Résumé*

La phosphorylation/déphosphorylation des protéines est un mécanisme de signalisation intracellulaire commun. Parmi les kinases végétales, les Mitogen-Activated Protein Kinases (MAPKs) sont impliquées dans de nombreux processus biologiques importants, comme la réponse aux stress biotiques et abiotiques, le développement et la dynamique du cytosquelette. Chez *Arabidopsis thaliana* et ce malgré de nombreux efforts, les fonctions des kinases impliquées dans les cascades MAPK restent peu inconnues. L'activation des kinases en utilisant des mutations mimant la phosphorylation des sites normalement phosphorylés est une approche qui a fait ses preuves dans le cas de MAP2Ks et a largement contribué à élucider leurs fonctions. Cette stratégie s'est révélée impossible dans le cas des MAPKs, puisque les résidus à muter restent encore à identifier.

Pour contourner ce problème, nous avons adapté un crible basé sur la complémentation fonctionnelle d'un mutant MAPK de levure avec des formes aléatoirement mutées de MPK6 d'*Arabidopsis* dans le but d'identifier des mutants présentant une activité constitutive. Nous en avons identifiés plusieurs et avons montré que ces formes constitutivement actives (CA) de MPK6 sont actives sans phosphorylation par les MAP2Ks. Par ailleurs, les mutations des résidus équivalents dans d'autres MAPKs les rendent également hyperactives, ce qui indique que cette stratégie peut être utilisée comme approche générale pour activer les MAPKs afin d'en comprendre les fonctions. L'étude des interactions protéine-protéine et l'analyse des profils de phosphorylation indiquent que les MAPKs CA conservent leur spécificité envers leurs substrats et interacteurs.

Comme preuve de concept, nous avons généré des formes actives du MPK4. La MPK4 CA exprimée sous son propre promoteur a parfaitement complémenté le mutant *mpk4*. La caractérisation des lignées exprimant MPK4 CA confirme le rôle négatif de cette kinase dans les réponses de défense aux pathogènes des plantes que ce soit dans la PTI (PAMP Triggered Immunity) ou dans la ETI (Effector Triggered Immunity).

Globalement, ce travail permettra de fournir des informations directes sur les cibles des MAPKs et devrait contribuer à la compréhension globale de la transduction du signal chez les plantes.

# *Summary*


Protein phosphorylations and dephosphorylations are common events occurring during intracellular signaling processes. Among plant kinases, Mitogen-Activated Protein Kinases (MAPKs) are involved in signaling of many important biological processes, including biotic and abiotic stresses, development and cytoskeleton organization. Despite an abundant literature on MAPKs, the exact roles and direct targets of many *Arabidopsis thaliana* MAPKs are not clear yet. The activation of kinases using phospho-mimicking mutations of the phosphorylated residues was a successful approach in the case of MAP2Ks, helping to elucidate their functions. This strategy failed in the case of MAPKs since the necessary residues to mutate remain unclear.

To bypass this problem, we adapted a screen based on the functional complementation of a MAPK yeast mutant with randomly mutated *Arabidopsis* MPK6 in order to identify the ones mutants showing constitutive activity. We identified several clones and showed that these constitutively active (CA) of MPK6 candidates are indeed active without phosphorylation by MAP2Ks. Interestingly, mutations of the equivalent residues in other MAPKs triggered constitutive activity as well, indicating that this strategy may be used as a general approach to activate MAPKs and identify their functions. Interaction and phosphorylation assays indicated that CA MAPKs retain their substrate and interactor specificity.

As proof-of-concept, we generated active versions of MPK4. CA MPK4 expressed under its own promoter successfully complements *mpk4* mutant plants. Characterization of CA MPK4 lines further confirmed the negative role of MPK4 in plant pathogen defense responses and its implication in both PTI (PAMP Triggered Immunity) and ETI (Effector Triggered Immunity).

Overall, the work will help to provide direct information on all MAPK targets and should be an important contribution to the overall understanding of signal transduction in plants.

# *Abbreviations*

AD - Activation Domain

BD – Binding Domain

CA – Constitutively Active

Dpi – Days Post Inoculation

ETI - Effector Triggered Immunity

ETS - Effector Triggered Suseptibility

HR - Hypersensitive Response

KO – Knock out

MAPK - Mitogen-Activated Protein Kinases

MAP2K – MAPK Kinase

MAP3K – MAP2K Kinase

MAP4K – MAP3K Kinase

MEK - MAPK/ERK1 Kinase

MEKK - MEK Kinase

MKK – MAPK Kinase

MKP – MAPK phosphatase

PR – Pathogen Related

*Pst – Pseudomonas syringae* pathovar tomato

PTI – PAMP Triggered Immunity

ROS – Reactive Oxygen Species

SA - salicylic acid

SAR - Systemic Acquired Resistance

T3SS - Type III Secretion System

WT – Wild Type

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# *Chapter I*
# *Introduction*

# *Chapter I -* ***Introduction***

Plants are able to sense environmental conditions in order to adapt and maintain their metabolism, growth and development. They possess for that a complex system of long distance perception and cell signaling networks leading to appropriate adaptive responses. The signal transduction from receptors to the cellular responses usually brings into play phosphorylation/dephosphorylation events catalyzed by protein kinases and phosphatases. Around 30% of the plant proteins have potential phosphorylation sites and *Arabidopsis thaliana* genome encodes for more than 1000 kinases and 100 phosphatases. Interestingly, the number of genes encoding for kinases represent 4% of total *Arabidopsis* genes (Wang *et al.,* 2003) compared with 1,7% in human and 2% in yeast (Hunter & Plowman 1997; Manning *et al.,* 2002). Different types of kinases have been identified in plants and classified into distinct groups. One of the largest and most important categories is the Mitogen Activated Protein Kinases (MAPKs also called MPKs). MAPKs are present in both cytoplasm and nucleus compartments and are involved in different signal transduction pathways. Genetic studies coupled with biochemical approaches have shown that MAPKs are not only involved in biotic and abiotic stress signaling, but also in other processes such as hormonal signaling and development.

MAPKs were first discovered by Sturgill and Ray in 1986 in animal cells as kinases associated to cyto squeleton. They were for this reason firstly named Microtubule Associated Protein-2 Kinase (MAP-2 Kinase). These kinases were later found to be related to a set of proteins that are phosphorylated at tyrosine residue in response to mitogens (agents which induce mitoses) and were then renamed as Mitogen-Activated Protein Kinases (MAPKs) (Rossomando *et al.,* 1989). In 1992, MEK (MAPK/ERK1 Kinase) proteins were discovered as activator of ERK1 in mouse (Crews & Erikson 1992). Seven years later, the first plant MAPK, MsERK1 in alfalfa (Duerr *et al.,* 1993) and D5 kinase in pea (Stafstrom *et al.,* 1993), were discovered. In the same year, MAPKs were cloned from *Arabidopsis thaliana* (Mizoguchi *et al.,* 1994) and tobacco (Wilson *et al.,* 1993). Additionally, a first clue on their functions *in planta* was provided by an important genetic work which assigned a function to the Raf-like MAP3K CTR1 in ethylene signaling (Kieber *et al.,* 1993).

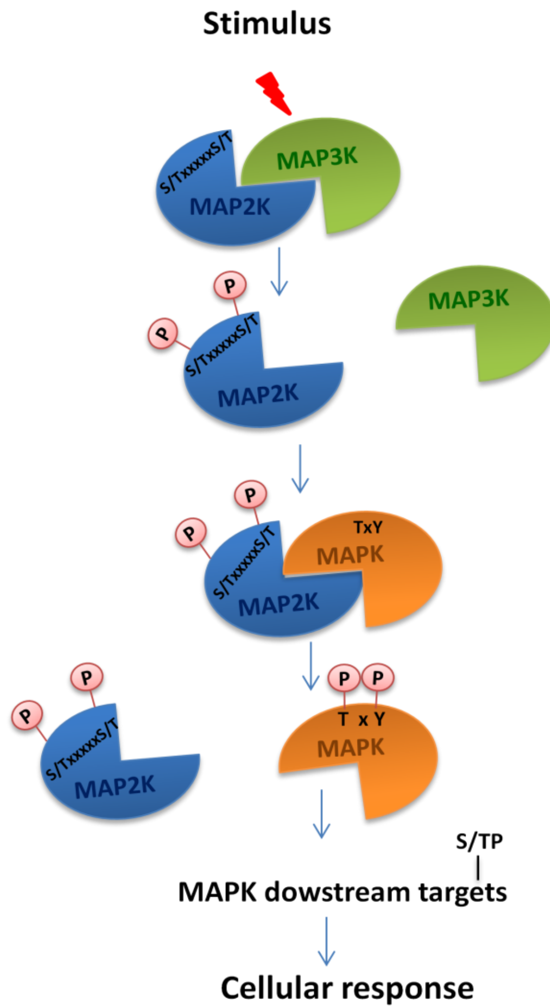



**Figure I.1: Signaling through MAPK cascades:** An appropriate signal, external stress signal or internal developmental signal, is perceived by a receptor and transmitted by phosphorylation events, optionally via additional interactors, to a MAP3K. The activated MAP3K phosphorylates and thereby activates an MAP2K, the active MAP2K phosphorylates its cognate MAPK. The MAPK phosphorylates downstream targets like transcription factors or other kinases, leading to the required cellular response.

# *A. MAPK cascades are conserved signaling modules in eukaryotes*

A typical MAPK module is constituted of 3 kinases able to activate each other by sequential phosphorylation (Figure I.1): a MAP2K Kinase (MAP3K) activates a MAPK Kinase (MAP2K) which in turn activates a MAPK. This module is turned on after perception of different environmental cues (abiotic stresses and pathogen) via a plasma membrane receptor. The MAP3Ks are serine/threonine kinases which phosphorylates MAP2Ks at a conserved **S/T-$X_{3-5}$-S/T** motif. An activated MAP2K which is a dual specificity kinase phosphorylates the threonine and the tyrosine residues of the MAPK conserved **T-X-Y** motif (Chang and Karin 2001). MAPKs phosphorylate proteins, mostly transcription factors, metabolic enzymes or transporters in order to modulate directly gene expression and cell homeostasis in response to extracellular stimuli. The deactivation of MAPK cascades is mediated by tyrosine and serine/threonine-specific phosphatases (Luan 2003). In animal and yeast systems, the formation and integrity of a specific MAPK cascades can be mediated by scaffold proteins or shared docking domains and adaptor or anchoring proteins (developed in chapter C.II.) (Whitmarsh & Davis 1998; Bardwell 2006).

### *1. MAPK and related kinase properties*

Kinases putatively belonging to MAPK modules are found in all eukaryote cells. They were very well characterized in mammal systems in which at least 7 sub-groups of distinct MAPKs exist (Cargnello & Roux 2011). *Saccharomyces cerevisiae* genome encodes for only 6 MAPK modules. Mammals and yeast kinases are models for plant studies and the signaling mechanistic seems to be roughly conserved between kingdoms. In this chapter, I will describe MAPK cascade important components in plant signaling, some examples being picked from mammal or yeast studies due to their better characterization in these systems compared to plant.

The sequences of putative MAPK components from several plant species are available from genome sequencing projects. *Arabidopsis thaliana* genome annotation allowed the identification of approximately 110 genes coding for putative MAPK pathway components: 20 MAPKs, 10 MAP2Ks and more than 80 MAP3Ks (Mapk_Groups 2002). Homologues of these

*Arabisopsis thaliana* kinases have been identified in other species, in which the family sizes are more or less identical (Mapk_Groups 2002).

a. *MAP kinase*

Plants MAPKs, also called MPKs, are rather globular proteins. They are activated by phosphorylation by MAP2K on their threonine and tyrosine residues of their characterized TDY or TEY motif located in the activation loops. Their activation motif is similar to the animal ERK kinases. However, no MAPK with a TGY motif similar to the animal p38 and yeast Hog1, or a TPY similar to JNK MAPKs was found in plants (Mapk_Groups 2002). Thus, all plant MAPK genes described so far belong to a single group, the so-called Extracellular signal-Regulated Kinase (ERK) subfamily. In mammals, members of this subfamily are mainly responsible for the transduction of mitogenic signals but, in plants, ERK like kinases seem to have evolved in such a way as to be able to transmit a broader range of stimuli (Ligterink & Hirt 2001). Based on sequence alignment analysis, *A. thaliana* MAPKs have been organized into four groups (A–D; (Mapk_Groups 2002)). TEY MAPKs constitute the groups A, B, and C, whereas group D contains TDY MAPKs. The most studied *A. thaliana* MAPKs, MPK3 and MPK6 belong to the Group A. Orthologs of these kinases in tobacco, alfalfa, rice, and poplar are involved in the same signaling processes including developmental and environmental stress perception (Kiegerl *et al.,* 2000; Zhang & Klessig 2001; Seo *et al.,* 2007). The third most studied MAPK in *A. thaliana*, MPK4, belongs to group B and has been reported to be implicated in mostly pathogen defense as well as in abiotic stress responses (Petersen *et al.,* 2000; Droillard *et al.,* 2004; Teige *et al.,* 2004; Andreasson *et al.,* 2005; Brodersen *et al.,* 2006; Qiu, Fiil *et al.,* 2008). It was also recently reported to function in cytokinesis and in the microtubule organization (Beck *et al.,* 2010; Kosetsu *et al.,* 2010; Beck *et al.,* 2011; Zeng *et al.,* 2011). The 8 MAPKs of the Group D have also a long typical C-terminal which is suspected to act as an auto-inhibitory domain. Little is known about this family. Approximately the same number of gene was predicted in poplar, 21, close to the twenty known from *A. thaliana*, while rice appears to have only fifteen MAPKs with more splicing variants. Microarray data showed remarkable disparity in their expression at the tissue and organ levels suggesting that such variants are the products of recent genomic

duplications (Suarez-Rodriguez *et al.,* 2010), and that plant MAPKs undergo rapid evolution and a significant level of sub-functionalization (Hamel *et al.,* 2006).

b. *MAPK kinase*

MAP2Ks also known as MEKs and MKKs constitute homogenous family of well defined kinases. *Arabidopsis* genome encodes for ten putative MKKs that fall into four groups (A-D), which are described in *A. thaliana* and rice (Mapk_Groups 2002; Hamel *et al.,* 2006). The N-terminal extension of plant MKKs shows a putative MAPK docking sites (detailed in chapter C.II.2) [K/R][K/R][K/R]×(1-5)[L/I]×[L/I] similar to that found in animal MAP2Ks (Bardwell 2006).

Group A includes MKK1 and MKK2 that act upstream of MPK4 (Ichimura *et al.,* 1998). MKK2 is also involved in abiotic stress like cold and salinity, and both MKK1 and MKK2 are important players in defense responses (Teige *et al.,* 2004; Meszaros *et al.,* 2006; Qiu, Zhou *et al.,* 2008). MKK6 that belongs to the same group was proposed to be the activator of MPK13 (Melikant *et al.,* 2004). Group B formed by MKK3, is distinguished by an unusual structural feature consisting of a nuclear transfer factor (NTF) domain (Hamel *et al.,* 2006). An NTF domain was also found in its tobacco orthologue NPK2. NTF domains prototypically function to enhance the nuclear import of cargo proteins (Quimby *et al.,* 2000), indicating that plant MAP2Ks with NTF domains are involved in cytoplasmic-nuclear trafficking. In addition, MKK3 participates in cascades that are elicited by pathogens and are dependent on jasmonic acid (JA) signaling (Doczi *et al.,* 2007; Takahashi *et al.,* 2007). MKK4 and MKK5 which form the group C are involved in abiotic (Kovtun *et al.,* 2000; Ren *et al.,* 2002) and biotic (Asai *et al.,* 2002) stress responses (PAMPs and oxidative stress) but also in stomatal and ovule development using the same downstream components MPK3 and MPK6 (Wang *et al.,* 2007; Wang *et al.,* 2008). MAP2K of group D, with MKKs 7–10 are not really characterized. MKK9 was reported to work upstream of MPK3 and MPK6 in ethylene signaling (Yoo *et al.,* 2008).

In general, phylogenetic studies showed that all plant phyla appear to use a more limited number of MKKs compared to MAP3K and MAPK, suggesting that cross talk between various signal-transduction pathways might be concentrated at this level in plant MAPK cascades (Suarez-Rodriguez *et al.,* 2010).

c. *MAPKK kinase*

Also called MAP3K or MEKKs, count for about 60 members (Mapk_Groups 2002). Relationship analysis based on the amino acid sequences of the protein kinase catalytic domain shows that *Arabidopsis* MAP3Ks fall into two main classes: MEKKs like subfamily such as the MEKK1 from mammals and STE11/BCK1 from yeast and RAF-like similar to the mammalian RAF1 (Mapk_Groups 2002).

MEKK-like kinases have a conserved catalytic domain. *A. thaliana* has 21 members in this group. Most *A. thaliana, Brassica*, and tobacco MEKK-like proteins seem to participate in canonical MAP kinase cascades that activate downstream MAP2Ks. MEKK1 was the first identified functional MAP3K in plants (Ichimura *et al.,* 1998), and it was found to regulate MKK2 after salt and cold stress and positioned upstream of MKK1/MKK2 in pathogen defence signalling (Gao *et al.,* 2008; Qiu, Zhou *et al.,* 2008). The MAP3K YODA was published to be involved in embryo development and later to regulate stomatal patterning (Lukowitz *et al.*, 2004) upstream of the MKK4/5-MPK3/6 cascade (Wang *et al.*, 2007). Also ANP1, ANP2 and ANP3, regulators of cell division (Krysan, Young *et al.,* 2002) belongs to the MEKK-like group of MAP3Ks. ANP1 can phosphorylate MPK3 and MPK6 in transient expression assays in protoplasts after $H_2O_2$ treatment, and the ANP1 ortholog NPK1 influences abiotic stress tolerance in *Nicotiana tabacum* (Kovtun *et al.*, 2000). In contrast, within this group, MEKK-like members called MAP3Kε1 and ε2 have not been proved to act in MAPK cascades and are similar to CDC (cell division control) proteins of fission yeast (Champion *et al.,* 2004).

The function of the 48 *Arabidopsis* members of Raf-like kinases is predominantly unknown. Two of the best-studied Raf kinases in *A. thaliana* were identified in genetic screens and are known as CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) and ENHANCED DISEASE RESISTANCE1 (EDR1). They participate in ethylene-mediated signaling and defense responses respectively (Kieber *et al.,* 1993; Frye & Innes 1998; Frye *et al.,* 2001; Huang *et al.,* 2003) and have not been clearly involved in any canonical MAPK cascades. Recent studies suggested that CTR1 is rather a negative regulator of MAPK pathway (Yoo *et al.,* 2008). Previous phylogenetic analyses of plant kinase families have discussed their duplication and divergence from animal and yeast ancestral genes (Champion *et al.,* 2004).

d. *Other players: MAP4K Kinases and phosphatases*

Additionally, *Arabidopsis thaliana* genome encodes for 10 kinases of the MAP3K Kinase (MAP4K) family whose functions are unknown.

MAPK cascades are also negatively regulated by phosphatase activities (Bartels *et al.,* 2010). The only ones being identified so far, act at the MAPK level, although it could be expected to find, among the 110 phosphatases of *Arabidopsis*, some able to target MAP2Ks and MAP3Ks. Dephosphorylation of the threonine and/or tyrosine residue within the activation motif inactivates the MAPKs. The most well known are MAPK Phosphatase 1 (MKP1) and MKP2 which belong to the dual specificity (Ser/Thr and Tyr) phosphatases (DSP) family and target MPK3 and MPK6 during abiotic and biotic stresses (Lee & Ellis 2007; Lumbreras *et al.,* 2010; Anderson *et al.,* 2011; Besteiro *et al.,* 2011). Some PP2Cs, another group of serine/threonine phosphatases, also contain interaction domain for MAPKs and were shown to inactivate them. For example, AP2C1 target and inactivated MPK4 and MPK6 after stress perception and affect plant hormonal balance (Schweighofer *et al.,* 2007).

## ***2. Signal specificity and docking interactions in MAPK signaling***

The very different size of kinase families involved in MAPK cascades suggests functional overlapping. The points which will be developed in this sub-chapter are how protein kinases distinguish their correct substrate from putative targets containing similar target residues, and how different signals transmitted by the same components elicit distinct responses. Specificity in MAPK cascade transductions is achieved by multiple mechanisms:

a. *Consensus phosphorylation motif*

The fist level of substrate specificity arises from the consensus sequence of the phosphorylation site. The consensus phophorylation motifs for the MAPK cascade components allowing a MAP3K to recognize and phosphorylate correctly its downstream MAP2K, and the activated MAP2K to phosphorylate its cognate MAPK have been reported in the first part of this chapter. For MAP3K and MAP2K, the general specificity preferences are not really studied,

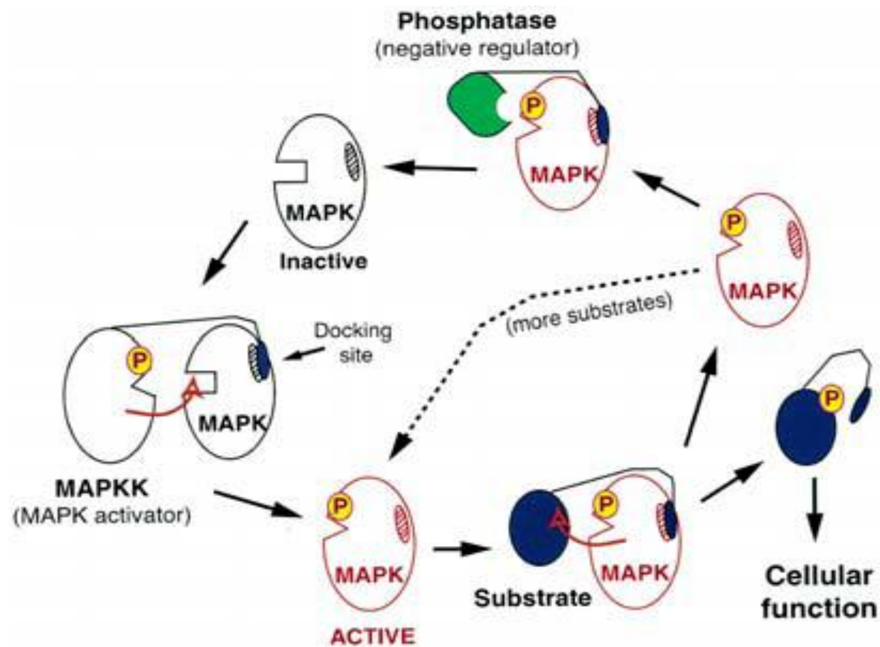



**Figure I.2: Docking mechanism in MAPK signaling.** The docking sequence of MAP2K is represented by a cluster of positively charged amino acids at the N terminal extension of the catalytic core (indicated by blue oval). This domain of the MAP2K binds to a docking pocket usually located at the back of the MAPK catalytic domain. Upon phosphorylation of the activation loop of MAPK and dissociation of the complex MAPKK-MAPK, the active MAPK can interact with and phosphorylate available substrates. MAPK uses the same docking pocket (hatched oval) to interact with docking sequences (blue oval) of substrates and phosphatases (which inactivate the MAPK) (Biondi & Nebreda, 2003).

probably because it is assumed that they only targets cognate kinases of the pathways and not other extra-pathway substrates. The preferences of MAPK were investigated in more detail as they contribute to the output specificity of the whole cascade.

MAPKs are proline-directed serine/threonine kinases. They minimally phosphorylate the serine or threonine in the dipeptide motif S/T-P. Although there is some preference for leucine at –1 and proline at –2 or –3 relative to the phospho-acceptor (Alvarez *et al.,* 1991; Songyang *et al.,* 1996). The minimal MAPK target site is simply S/T-P so-called P-site. However, the sequence S/T-P being found in approximately 80% of all proteins, other mechanisms are involved to dictate whether or not a particular protein is the substrate a particular MAPK (Bardwell 2006). It is also admitted that some potential phosphorylation sites might not be accessible due to structural conformational constraints (Ubersax & Ferrell 2007).

b. *Docking sites in MAP kinases*

The next level of substrate specificity often involves interaction between docking motifs on the substrate/interactor with specific interaction domains on the kinase. These additional binding motifs increase the affinity of the kinase for specific substrates and are often spatially separated from the kinase active site and the substrate phosphorylated residue (Ubersax & Ferrell 2007). This is a very well described mechanism for animal and yeast MAPKs (Figure I.2).

The best characterized MAPK-docking motifs found in MAPK interactors are the so-called D-motifs. In animal and yeast, the D-site consensus motif consists of a cluster of two or three basic residues, a short spacer, and a hydrophobic -X- hydrophobic sub-motif $\mathbf{(R/K)_{2\text{-}3}\text{-}(X)_{1\text{–}6}\text{-}\Phi\text{-}x\text{-}\Phi}$; where Φ denotes a hydrophobic residue such as Leucine, Isoleucine or Valine and X is any amino acid (Tanoue *et al.,* 2001; Remenyi *et al.,* 2005; Bardwell 2006). This motif is found in activators (MAP2Ks), negative regulators (phosphatases) and various substrates. They are usually located 50-100 residues away from the P-site on substrates (Ubersax & Ferrell 2007). Comparison of the MAPK docking interactions from yeast to human reveals a conserved mechanism of interaction. In MAPKs, a large surface, distinct of the kinase active site, is involved in the recognition of the interactor D-motifs. This site was then named common docking domain (CD domain). Crystal structure of ERK2 and p38alpha with peptides corresponding to D-motifs (Chang *et al.,* 2002; Liu *et al.,* 2006), showed that several part of the

kinase are involved in the interaction area to build this CD-domain. Mechanistically, the basic residues of the D-domain bind to a negatively charged area CD-site that is located just C-terminally of the kinase domain, and the hydrophobic residues bind to a hydrophobic Φ-x-Φ groove of the MAPK. Differences in the composition and spacing of residues in the docking site and the local preferences of the catalytic core for different amino acids around the P-site, work together to increase the overall selectivity of kinase–substrate interactions (Reményi *et al.*, 2005). MAPK docking occurs in all mammalian MAPK families (ERK, p38 and JNK) and crystal structures now exist for most of these MAPK docking complexes (Remenyi *et al.,* 2005; Bardwell 2006).

A second class of MAPK docking were found to be special for the ERK signaling, called DEF motif and is found in the MAPK interactors (including transcription factor, MAPK phosphatase and other regulators). The DEF domains have as consensus sequence FxFP which can bind to a separate MAPK surface and is located ten amino acids downstream of the phosphorylation site (Lee *et al.,* 2004; Vinciguerra *et al.,* 2004; Dimitri *et al.,* 2005). Hydrogen-exchange mass spectrometry (HXMS) data have located the DEF docking groove near the kinase active site. FxFP motif binding to ERK2 is coupled to the positioning of its activation loop. It has been demonstrated that the phosphorylated MAPK binds this docking motif better than the inactive kinase (Lee *et al.,* 2004).

c. *Scaffolds tether together the functional signaling compounds*

In addition to the direct interactions between protein kinases and their substrates, sometimes the two proteins interact through the intermediacy of adaptors or scaffolds, which act as organizing platforms that recruit both the kinase and the substrate to the same complex (Pawson & Scott 1997). Signaling scaffolds can be defined as proteins that join two or more signaling components of a pathway to each other. They are ubiquitous in eukaryotic signaling, and mammalian MAPK scaffolds have been recently reviewed (Morrison & Davis 2003). Scaffolds have been proposed to accelerate signaling reactions by binding multiple components of the same pathway. Tethering the correct components in close proximity with each other could allow the scaffolds to raise the local concentration of the bound signaling components, thereby

promoting signaling reactions. Many MAPK cascades are dependent on scaffolding proteins to maintain kinase specificity.

One of the most well characterized examples of scaffold in yeast is Ste11. The yeast MAP3K Ste11 (Sterile-11) is a component of three distinct MAPK cascades that are involved in three different biological processes: mating, invasive growth and high-osmolarity responses (Schwartz & Madhani 2004). The protein scaffold, Ste5 tether Ste11, Ste7 (MAP2K) and Fus3 (MAPK) together in pheromone signaling. The activation of the HOG MAPK module (Ste11-Pbs2-Hog1) however is dependent on the presence of the Sho1, Pbs2 co-scaffold (Saito 2010). The choice of scaffolds determines, at least in part, the process that Ste11 regulates and ensures that active Ste11 activates only one of the three pathways to prevent cross-talk with other MAPK cascades.

Scaffold proteins are not identified yet in plants but some MAP3Ks appears to bind directly MAPKs, suggesting that they have a scaffold function. The alfalfa oxidative stress activated MAP3K OMTK1 was shown to be able to interact in protoplast system with the MAPK MMK3 in response to $H_2O_2$ (Nakagami *et al.,* 2004). In *A. thaliana* it was published (Ichimura *et al.,* 1998) and confirmed in our laboratory (not published) that the MAP3K MEKK1 has scaffold role since it interacts not only with its cognate MAP2K MKK2 through its kinase domain but also with the downstream MAPK, MPK4, through its long N-terminal tail. Moreover at the opposite of MPK4, the kinase inactive version of MEKK1 rescued the *mekk1* phenotype indicating that MEKK1 has also a kinase-independent function (Suarez-Rodriguez *et al.,* 2007). As it will be developed further MEKK1 is also involved in another cascade with MPK3 and MPK6. Similar evidence was observed in the mammalian MAP3K MEKK1 which is able to interact with both JNK/SAPK and the ERK1/2 modules suggesting its role as scaffold for two separate MAPK cascades (Karandikar *et al.,* 2000).

## B. ***Plant responses regulated by MAP Kinases***

Kinases involved in MAPK modules count for more than 10% of the serine-threonine kinases found in higher plants. They transduce extracellular signals and modulate the activity of cellular enzymes. In animal, an active MAPK can regulate transcription factors, cytoskeleton dynamics and other kinases. MAPK pathway components can execute a wide variety of roles in plant cell signal transduction pathways such as osmoregulation, hormone signaling including auxin-induced cell proliferation or ethylene-responsive processes, cell wall biosynthesis, and cell growth and differentiation. However, there are only few complete tri-kinase cascades identified so far.

The role of these kinases in some of the important biological processes is described in the following sections. I choose to focus my introduction on functions of the MAPKs which are involved in stress perceptions. As the stress related kinases are also involved in other process, I will also present their stress unrelated functions.

### *1. The historical function of MAPKs: biotic stress signaling*

Plants, unlike mammals, lack mobile defender cells and somatic adaptive immune system. Instead, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites (Dangl & Jones 2001; Jones & Dangl 2006). MAPKs constitute a common way to sense biotic stresses, their activation being often observed upon pathogen treatment.

Two main progresses explain, in my view, the incredible increase of our knowledge on plant pathogen perceptions in the last years. The first one is the identification of small molecules from pathogens able to trigger alone numerous early plant responses to pathogens, allowing simplification of the experiments. The second one is the adoption of *Arabidopsis* as a plant model and the development of rich mutant libraries providing easy genetic tools for plant phyto-pathologists and biologists. This allowed the unambiguous identifications of the early signaling actors like MAPK cascades.

Plants respond to infection using a two-layered innate immune system. The first layer recognizes and responds to molecules common to many classes of microbes, including non-pathogens, commonly called Pathogen-Associated Molecular Patterns (PAMPs) or Microbe-

Associated Molecular Patterns (MAMPs). These molecules are perceived by transmembrane receptors, belonging to the Receptor Like Kinase (RLK) family and named Pattern Recognition Receptors (PRRs). This recognition activates non specific defense responses that include the reprogramming of host gene expression in a way to prevent pathogen progression and the production of antimicrobial compounds. This defense layer is referred to PAMP-Triggered Immunity (PTI). Examples of PAMPs are the *Pseudomonas* flagellin, the *Echerichia coli* Elongation factor Tu, lipopolysaccharides, and the fungal molecules chitin and ergosterol.

To circumvent PTI, pathogens deliver effector proteins into host cells by means of their type III secretion system (T3SS) in order to suppress the activities of PTI signaling components. This process is named Effector Trigerred Suseptibility (ETS) (Boller & Felix 2009). Plants have encountered this by evolving a second type of responses to pathogen virulence factors, either directly or through their effects on host targets: they develop mechanisms to detect microbial effectors via intracellular immune receptors, called resistance (R) proteins belonging NBS-LRR receptor family. This defense layer is referred to Effector Triggered Immunity (ETI) and triggers localized host cell death called the hypersensitive response (HR).

### a. *MAPK cascades are core signaling modules for PAMP Triggered Immunity*

PAMPs are highly active compounds produced by an invading pathogen and recognized by the plant through plasma-membrane receptors. They are defined as highly conserved pathogen surface-derived molecules (that usually have an essential function in microbial fitness or survival) which induce plant defense responses in both host and non-host plants (Nurnberger & Brunner 2002). One of the best studied PAMPs is flg22, a 22- amino acid active peptide from the *Pseudomonas* structural protein flagellin. Flg22 binds to the LRR-RLK receptor FLS2 (Flagellin-Sensing 2) (Zipfel *et al.,* 2004; Chinchilla *et al.,* 2006), which forms a complex with BAK1 (Bri1-associated receptor kinase1) to trigger MAPK signaling cascade (Chinchilla *et al.,* 2006; Heese *et al.,* 2007). Among the typical other known PAMPs, EF-Tu and Chitin are derived from the bacterial elongation factor Tu and from the fungal cell wall respectively and perceived by the Arabidopsis receptors EFR and CERK1 (Zipfel *et al.,* 2006; Miya *et al.,* 2007). As the first identified, flg22 is the PAMP for which we have the broader view of the signaling cascades. Even though PAMPs are perceived by specific receptors, they activate a common, and therefore

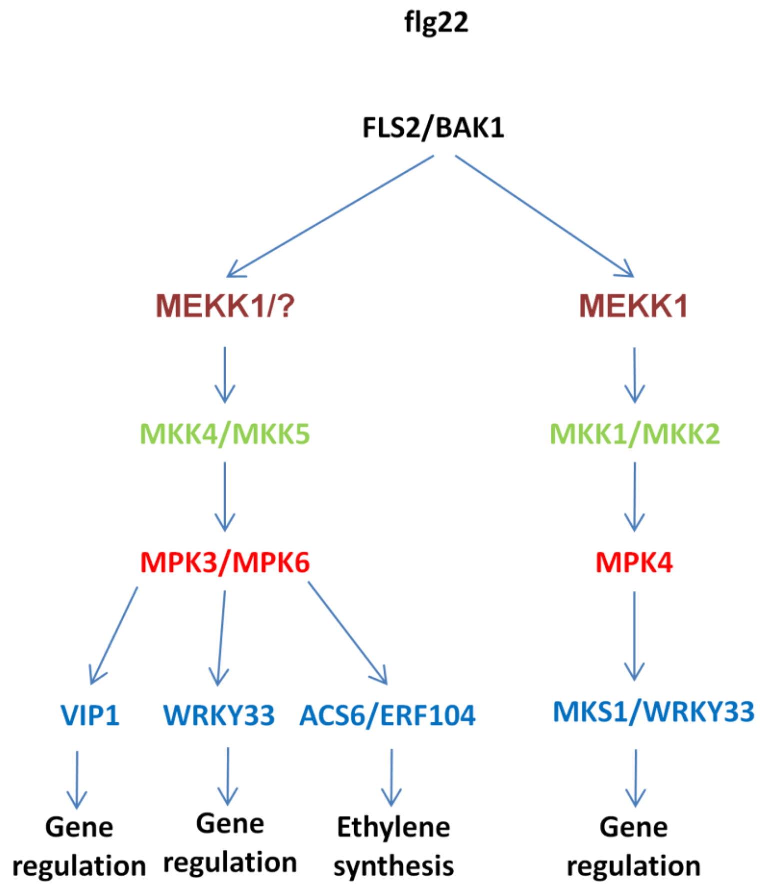



**Figure I.3: MAPK cascades in PAMP-triggered immunity.** These two models are supported by genetic and biochemical studies (Adapted from Colcombet *et al.* 2008).

not specific, set of signaling cascades leading to the activation of non specific stress responses. Among the signaling actors, a set of 2 or 3 (depending of the organism and the studies) MAPKs were shown to be activated and later on identified in the *Arabidopsis* as MPK3, MPK4 and MPK6. MAPK activation is considered as one of the earliest response in plants, within 1-5 min, after pathogen recognition (Frye *et al.,* 2001; Zhang & Klessig 2001; Cardinale *et al.,* 2002; Del Pozo *et al.,* 2004; Boller & Felix 2009). MAPKs cascades were largely proposed to be involved in PTI (Pitzschke, Schikora *et al.,* 2009).

*MKK4/MKK5-MPK3/MPK6 cascade positively regulates PAMP responses*

PAMPs activate two *A. thaliana* closely related MAPKs, MPK3 and MPK6 (Asai *et al.,* 2002; Wan *et al.,* 2004; Ranf *et al.,* 2011). As the constitutively active forms of MKK4 and MKK5 activate MPK3 and MPK6 in protoplasts, these four kinases were proposed to constitute a signaling module MKK4/5-MPK3/6 regulating positively defense responses (Asai *et al.,* 2002) (Figure I.3). This activation was initially shown to be triggered by MEKK1 but this result is now matter of debate now (Asai *et al.,* 2002; Ichimura *et al.,* 2006; Suarez-Rodriguez *et al.,* 2007). This MAPK cascade leads to the transcriptional activation of reponse genes such as *flg22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*) and transcription factors *WRKY22* and *WRKY29* (Asai *et al.,* 2002). Genetic studies of this cascade were made difficult by the fact that the MKK4/5-MPK3/6 seems to regulate important developmental processes.

The understanding of defense regulation via MAPKs is limited by the current knowledge of plant MAPK substrates; nevertheless information on the substrates of plant MAPKs is slowly emerging. Several *in vivo* substrates for MPK6 were identified even though not always directly linked to defense responses. The first identified is ACS6 (1-Aminocyclopropane-1-Carboxylate Synthase 6), important enzyme in the biosynthesis of stress hormone ethylene (Liu & Zhang 2004). Flg22-triggered ACS6 phosphorylation is necessary to stabilize the proteins. Two transcription factors are also phosphorylated by the MAPKs. ERF104 is a member of the ethylene responsive transcription factor, ERF104, which forms a complex with MPK6, this complex being disrupted by flg22 through ethylene signaling (Bethke, Unthan *et al.,* 2009). WRKY33 was also recently shown to be one of the MPK3/MPK6 targets and involved in the transcriptional regulation of camalexin biosynthesis genes (Mao *et al.,* 2011). A target proposed for MPK6 is NIA2, which nitrate reductase activity increases through its $H_2O_2$ induced

phosphorylation (Wang *et al.,* 2010). In the case of MPK3, VIP1 transcription factor is a direct substrate. Its phosphorylation induces its sub-cellular relocalization from cytoplasm to nucleus to activate stress gene (PR1) expression (Pitzschke, Djamei, Teige *et al.,* 2009). MPK3 and MPK6 were also proposed to target the poorly characterized protein PHOS32 which have been identified by high-throughput approaches using *Arabidopsis* protein microarray strategy (Merkouropoulos *et al.,* 2008). A network of MAPK targets is also proposed using the similar approach (functional protein microarray) (Popescu *et al.,* 2009). Functional characterization of these putative candidates remains to be done.

*MEKK1-MKK1/MKK2-MPK4 define a cascade with ambiguous functions*

Alternatively, PAMPs activate the MEKK1-MKK1/2-MPK4 cascade (Figure I.3). Evidence for this cascade comes initially from the interactions between these proteins in yeast and *in vitro* (Ichimura *et al.,* 1998; Gao *et al.,* 2008). In *mekk1* or *mkk1mkk2* mutant background, MPK4 is not activated anymore, indicating that they are important upstream activators. The loss-of-function *mekk1* and *mpk4* mutants and the *mkk1mkk2* double mutant have related phenotypes (Ichimura *et al.,* 2006; Nakagami *et al.,* 2006; Suarez-Rodriguez *et al.,* 2007; Qiu, Zhou *et al.,* 2008): They over-accumulate salicylic acid (SA) and Reactive Oxygen Species (ROS) and constitutively express pathogenesis-related (PR) genes. Therefore, loss of function mutants of this MAPK module produce paranoid dwarfed plants (Petersen *et al.,* 2000; Ichimura *et al.,* 2006; Suarez-Rodriguez *et al.,* 2007). Despite the crucial role of the MEKK1 in SA-mediated immune responses and its involvement in the regulation of reactive oxygen species (ROS) homeostasis (Nakagami *et al.,* 2006), MEKK1 may not interact with FLS2/BAK1 directly. Intermediates upstream of MAP3Ks that link membrane receptor to MAPK cascade need to be identified.

A single downstream target, of MPK4, MAP KINASE SUBSTRATE 1, has been identified (Andreasson *et al.,* 2005; Qiu, Fiil *et al.,* 2008). These studies have led to a model in which MPK4 plays the role of a negative regulator of PAMP response. The proposed model places MPK4 in a complex with its nuclear substrate MKS1 and the transcription factor WRKY33. Changes in MPK4 activity and phosphorylation of MKS1 on multiple sites (Caspersen *et al.,* 2007), somehow triggers the release of WRKY33 from nuclear complexes to activate the expression of target genes involved in defense responses such as the synthesis of the antibacterial

phytoalexin camalexin. Chromatin immunoprecipitation (ChIP) showed that, upon pathogen inoculation or flg22 treatment, WRKY33 binds to and activates the transcription of the *PHYTOALEXIN DEFICIENT3* (*PAD3*) promoter (Qiu, Fiil *et al.,* 2008). This mechanism does not follow the canonical mode of action of other ERK-like kinases, but it concurs with genetic evidence which also point to the negative role of MPK4 in defense responses.

b. *MAPKs are targets for pathogen effectors*

During evolution, plant pathogens developed strategies to circumvent PTI by injecting so-called effectors or virulence proteins into the plant cells. Effector proteins suppress signaling events (ETS) and thereby inhibit the plant defense mechanisms. Examples of effector targeting of MAPKs components exist in animals and in plants. Shigella type III effector OspF was shown to deactivate human MAPKs, and this by removing their phosphate groups from the phosphothreonine of the activation loop (Li *et al.,* 2007). This mechanism reminds the unique function of HopAI1 an effector conserved between plants and animals bacterial pathogens (Zhang *et al.*, 2007). MPK3 and MPK6 were specifically shown to be inactivated by HopAI1, leading to the suppression of PAMP-induced genes and callose deposition that is normally triggered to reinforce host cell walls (Zhang *et al.,* 2007). Another early response suppressed by HopAI1 is the accumulation of $H_2O_2$ as part of the oxidative burst. However it's still not known whether the phosphothreonine lyase activity of HopAI1 can also inactivate MPK4. The two effectors AvrPto and Avr PtoB were shown to be specific suppressor of early defense signaling (He *et al.,* 2006). They both block MPK3 and MPK6 activation in early PAMP signaling by suppressing an upstream component of MAP3K. Interestingly, MPK4, which belongs to a signaling cascade involved in negative regulation of defense responses, is phosphorylated by AvrB, a virulent factor having similarities to protein kinase (Cui *et al.,* 2010). Moreover AvrB virulence function appears to be correlated with its ability to induce JA signaling (through PDF1.2) in *Arabidopis*. Plants expressing AvrB in an RPM1 deficient background are more susceptible to *P. syringae* (Shang *et al.,* 2006). Many other *P. syringae* T3SS effectors including HopF2 (Wu *et al.,* 2011), HopZ1 (Macho *et al.,* 2010) were recently reported to target MAPK cascades to shut down PTI in plants (Shan *et al.,* 2007).

A clever bacterium system abusing host biological system and involving MAPKs components is the hitch-hiking mechanism used by *Agrobacterium tumefaciens*, the causative agent of crown gall blight. *Agrobacterium* invasion triggers the activation of MPK3, MPK4, and MPK6. An important player in shuttling the transfer DNA into the host nucleus is the bZIP transcription factor VirE1-Interacting Protein 1 (VIP1). The translocation is controlled by the MPK3-dependent phosphorylation of VIP1 (Djamei *et al.,* 2007). This mechanism also involves the up regulation of the PR1 gene, but it's still unknown whether this is a direct or indirect effect of VIP1. Many question are still open for this particular system, as how *Agrobacterium* weakens the innate immune responses because we saw with the study by Djamai *et al.* (2007) that *Agrobacterium* invasion results in the activation of several defense related MAPKs without eliciting a resistance to *Agrobacterium* transformation or colonization.

c. *Are MAPKs involved in ETI?*

In return, plants developed the Effector Triggered Immunity (ETI), also called 'gene-for-gene-resistance' reactions. Plant NBS-LRR (nucleotide binding site- leucine-rich-repeat) receptors act as resistance proteins (encoded by resistance- or *R*-genes). Upon specific recognition of pathogen effector proteins (or in this case also named avirulence (Avr) factors), a defence signaling cascade is triggered (Flor 1971). ETI was described as an accelerated and amplified PTI response which, exceeding a certain threshold, results in cell death known as the Hypersensitive Response (HR). But also ETI is not the last level in plant-pathogen interactions. Many pathogens already overcame ETI by a second level of ETS (Jones & Dangl 2006).

Examples based on studies on tobacco and tomato have shown the involvement of MAPK cascades in ETI and *R*-genes signaling. The activation of Salicylic acid–Induced Protein Kinase (SIPK) and Wound-Induced Protein Kinase (WIPK) by tobacco mosaic virus (TMV) and *Cladosporium fulvum avr9* (Cf-9) is gene-for-gene specific, suggesting their role(s) in disease resistance (Zhang *et al.,* 1998; Romeis *et al.,* 1999; Zhang *et al.,* 2000). The inhibition of SIPK and WIPK activation by staurosporine and K-252a suppresses the HR-like cell death in tobacco suspension cells treated with elicitin (Zhang *et al.,* 2000). These MAPKs were activated in tobacco cells that express the N- or Cf-9 resistance gene (Zhang *et al.,* 1998; Romeis *et al.,* 1999).

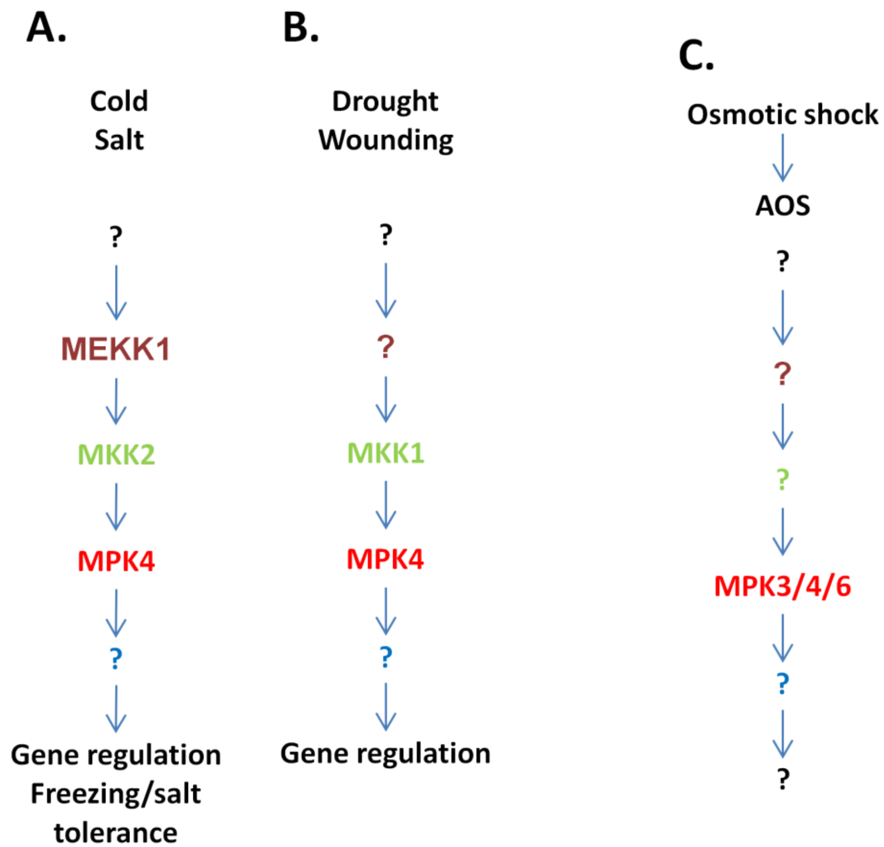



**Figure I.4: MAPK cascades triggered by different abiotic stresses: A.** Cold/salt stress induces the MEKK1–MKK2–MPK4 cascade; **B.** Drought and wounding induces MKK1–MPK4; **C.** Osmotic shock activates MPK3/4/6 via AOS production (Adapted from Colcombet *et al.* 2008).

Separate studies have also implicated the tomato MAP3K MAPKKKα in the Pto-mediated HR response. The silencing of MAP3Kα inhibits HR in resistant tomato plants, and the over-expression of this MAP3K promotes the development of HR lesions (Del Pozo *et al.,* 2004). In Tomato, Pedley and Martin identified two MAPKs LeMPK2 and LeMPK3 which are activated by agrobacterium-mediated expression of AvrPTO and AvrPToB in a Pto-specific way (Pedley & Martin 2004). Interestingly, over expression of MAP3Kα also activates LeMPK2 and LeMPK3. Additionally using VIGS (Virus Induced Gene Silencing), two MAPK modules, MEK1-NTF6 and MEK2-WIPK, were proposed to be involved downstream of the Pto resistance gene (Ekengren *et al.,* 2003). Thus far no evidence for direct protein interactions between MAPK modules and R-gene resistance process does exist due to the lack of identification of MAPK targets in the AvrPto-Pto mediated responses.

## *2. Abiotic stresses and MAPKs signaling*

Besides their role in pathogen signaling, MAPK cascades have an important role in the signaling of a wide range of abiotic stresses. Many studies on different plant species including *Arabidopsis* have shown their implication in signaling pathways activated by abiotic stresses such as cold, salt, wounding, heat, UV or osmotic shock. The best-studied MAPK cascade in abiotic stress signaling is the MEKK1-MKK2-MPK4/6 module, which is induced by salt and cold (Teige *et al.*, 2004) (Figure I.4A).

### a. *Water, Salt, Cold, and Osmotic Stress*

Involvement of *Arabidopsis* MAPK in abiotic stresses was suspected from studies showing that MEKK1 mRNA level increases in response to various stresses such as low-temperature, high salinity and mechanical stresses (Mizoguchi *et al.,* 1996). At this time, the kinase activation was not measured. It’s only with the study published by Ichimura and co-workers that two *Arabidopsis* MAPKs, MPK4 and MPK6, were shown to be activated by treatment with low temperature, low humidity, hyper-osmolarity, touch and wounding (Ichimura *et al.,* 2000). Droillard and co-workers confirmed the activation of MPK3, MPK4 and MPK6 in *Arabidopsis* suspension cells under hyperosmotic and hypoosmotic (mimicking the mechanical stress) shock

(Droillard *et al.,* 2002; Droillard *et al.,* 2004) (Figure I.4C). The identification of the other kinases of this stress pathways were realized using a combination of approaches. Directed yeast two hybrids indicated that MEKK1 interact with MKK2 and MPK4 (Ichimura *et al.,* 1998). These interactions were confirmed (Teige *et al.,* 2004): MKK2 interacts strongly with MPK4 and MPK6 and weakly with MPK5. This cascade was further established in a complementation assay of Hog1 pathway, which is involved in the osmotic stress signaling (Hohmann 2002). The double mutant *pbs2Δhog1Δ*, which lacks the MAP2K Pbs2 and its downstream MAPK Hog1 and therefore is hypersensitive to salt stress, was transformed with MKK2 and the *A. thaliana* MAPKs, MPK3, MPK4 and MPK6. In this assay only MKK2 together with MPK4 or MPK6 were able to complement the phenotype, showing that MKK2-MPK4/6 is a functional MAPK cascade (Teige *et al.,* 2004). Transient protoplast transformation of MPK4 or MPK6 alone or in combination with MKK2 resulted in a poor activation of the MAPKs, however co-expression with a constitutively active form of MEKK1 (MEKK1ΔN) together with MKK2 and MPK4 or MPK6 resulted in a stronger activation of both MAPKs. At least, *mkk2* mutant plants are hypersensitive to cold and salt stresses, likely due the loss of MPK4 and MPK6 activation under cold and salt stress conditions. Although MKK2 share 62% amino acid identity with MKK1, MKK2 respond more strongly to cold and salt stress whereas MKK1 activity was far more important after hydrogen peroxide ($H_2O_2$) and bacterial and fungal elicitation (flg22 and ß-laminarin) (Teige *et al.,* 2004). This data established a MAPK cascade formed by MEKK1-MKK2-MPK4/6 in response to cold and salt stress.

b. *Ozone and Reactive Oxygen Species (ROS)*

Redox homesotasis in plants needs a tight control by the antioxidant system (Pitzschke & Hirt 2009) since it's always subject to oxidative challenges both from internal, including potentially damaging "reactive oxygen species" (ROS) generated by high energy electron transfer systems in the chloroplasts, mitochondria, and peroxisomes in physiological processes such as control of stomatal aperture, and external sources by environmental stresses such as UV and ozone. It was very well established that ozone induces formation of ROS in plants, however ROS production can be also detected upon pathogen attack for instance (also described as oxidative burst) in this case ROS act as a signal transduction messenger (Pellinen *et al.,* 1999;

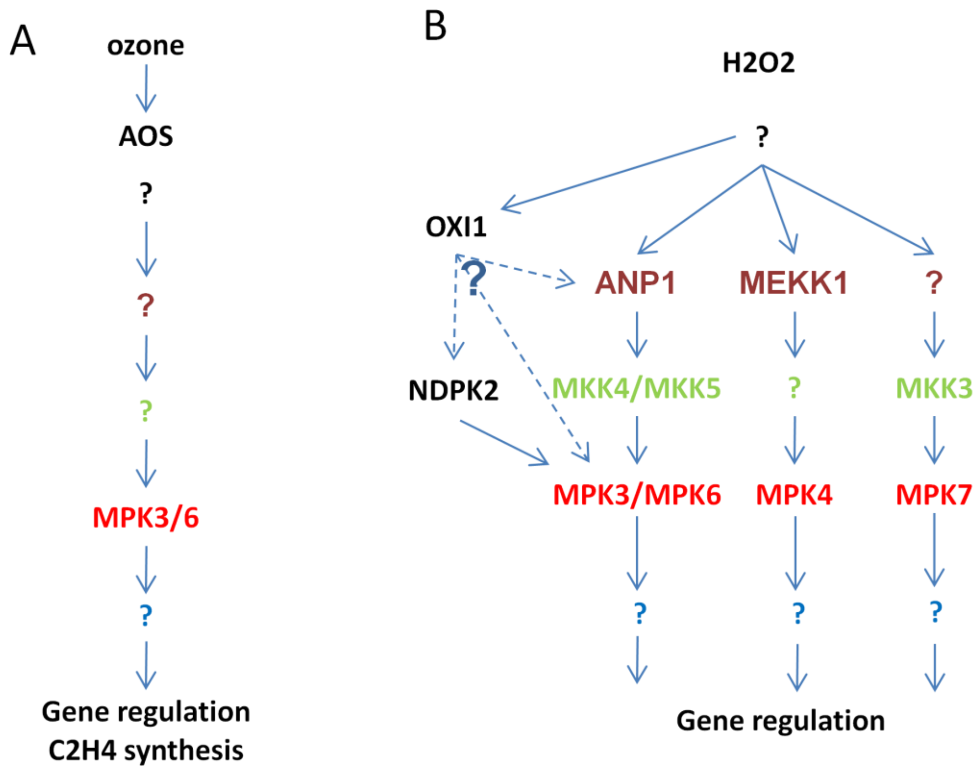



**Figure I.5: MAPK cascades activated in response to ozone and ROS.** Accumulation of ROS in the plant cell after abiotic stress perception like $O_3$ or pathogen attack activate MAPK cascades. $H_2O_2$ activates the three MAPK modules (ANP1–MKK4/5–MPK3/6, MEKK1–MPK4 and MKK3–MPK7) either directly or via the protein kinases NDPK2 (nucleoside diphosphate kinase 2) and OXI1 (Adapted from Colcombet *et al.* 2008).

Mittler 2002). To ensure a redox homeostasis, plants have evolved detection and intracellular signaling systems. Among them MAPK seems to play a central role in addition to alteration of the ET, SA, and JA balance leading to a PCD similar to HR. These similarities between responses to pathogens, $H_2O_2$ and $O_3$ allowed researchers to use O3 to manipulate the $H_2O_2$ content of plants (Colcombet & Hirt 2008).

The primary ROS activated MAPK in tobacco is SIPK and in a lesser extend WIPK (Kumar & Klessig 2000; Samuel *et al.,* 2000). In *A. thaliana*, MPK3 and MPK6 (SIPK orthologs) are activated by ozone treatments, and translocate to the nucleus (Ahlfors *et al.*, 2004) (Figure I.5A). *mpk3* and *mpk6* mutant plants appear to be hypersensitive to ozone as shown by O3-induced leaf damage. However, in each of these mutant backgrounds, the other MAPK remained activated longer (Miles *et al.,* 2005). MPK3 and MPK6 activation in response to ozone appears to be regulated by the MAPK phosphatase, MKP2, as the mutant MKP2 RNAi plants were more sensitive to O3 (Lee & Ellis 2007).

Catalase enzymes which have a role in $H_2O_2$ degradation are important to overcome oxidative stress. It was reported that ABA, drought, and salt stress-induced gene expression of CAT1 catalase is mediated by the MAP2K, AtMEKK1, and this by triggering $H_2O_2$ signal production (Figure I.5B). Using the MAPK kinase inhibitor, PD98059, Xing and co-workers showed that the kinase activity reduced considerably under ABA, drought, and salt which consequently reduced the stress-induced CAT1 expression (Xing *et al.,* 2007). This result demonstrate that catalase activity is linked to MAPK activation in response to abiotic stresses. And because MPK6 activity is lost in *mkk1* mutants after ABA treatment, MKK1-MPK6 could be the MAPK cascade which regulates $H_2O_2$ metabolism through CAT1, this is further confirmed by the altered response to ABA in *mkk1* and *mpk6* mutants. ROS signaling is also proposed to bring into play another MAPK pathway involving MEKK1-MKK1/2-MPK4. *mekk1* and *mpk4* have similar phenotypic alterations, they are severely dwarfed, accumulate high amounts of ROS and develop local lesions reminiscent of Programmed Cell Death (PCD) (Ichimura *et al.,* 2006; Nakagami *et al.,* 2006) and die at early developmental stages. Hydrogen peroxide can activate MEKK1, MPK3, MPK4 and MPK6 in protoplasts, though in the mutant background this activation is lost indicating that MEKK1 is specifically required for the activation of MPK4 by $H_2O_2$ (Nakagami *et al.*, 2006). Moreover transcriptome analysis of mutants in this pathway showed an important overlap between the genes regulated by MEKK1

and MPK4 (Pitzschke, Djamei, Bitton *et al.,* 2009). Particularly the expression of CAT2 encoding the major $H_2O_2$ scavenging enzyme is repressed in *mekk1* and in *mpk4* mutants. This down-regulation is consistent with the high level of $H_2O_2$ observed by DAB staining, a ROS-specific dye (Nakagami *et al.,* 2006). All together, these data point the important role of MEKK1-MPK4 in ROS metabolism.

Another MAPK involved in ROS signaling is the MPK7 from group C which was recently shown to be preferentially phosphorylated by MKK3 in response to $H_2O_2$ and that the module MKK3-MPK7 positively regulate the expression of PR1. *MKK3* and *MPK7* mRNAs accumulated upon bacterial infection, and global analysis of gene expression in the *mkk3* mutant supported the involvement of these kinases in pathogen responses functioning independently of PAMP induction (Figure I.5B). This module may be part of a secondary response promoted by the oxidative burst, which is not an exclusive phenomenon during pathogen attack. Moreover, other studies pointed the role of MKK3 in the control of JA-mediated developmental processes via the MAPK, MPK6 (Takahashi *et al.*, 2007).

### *3. MAPK regulates or are signaling pathways for plant hormones.*

Phytohormones have an essential role in plant physiology and particularly during plant pathogen interaction. They are involved in long distance pathogen signaling in one hand and in modulation of defense responses developed by the plant to escape infection. Moreover their role was highlighted since we discovered the ability of many pathogens to perturb hormonal homeostasis or signaling of the plant to their own benefit (Robert-Seilaniantz *et al.,* 2011).

#### a. *Salicylic and Jasmonic acid*

Salicylic acid (SA) and jasmonic acid (JA) together with ethylene (ET) play a pivotal role in plant immune signaling even though they have antagonistic function. Cross-talk between SA and JA/ethylene is very well studied (Koornneef & Pieterse 2008; Robert-Seilaniantz *et al.,* 2011). SA production and signaling is associated with resistance against biotrophic and hemibiotrophic pathogens such as *P. syringae* and *Hyaloperonospora*, while JA and ET are

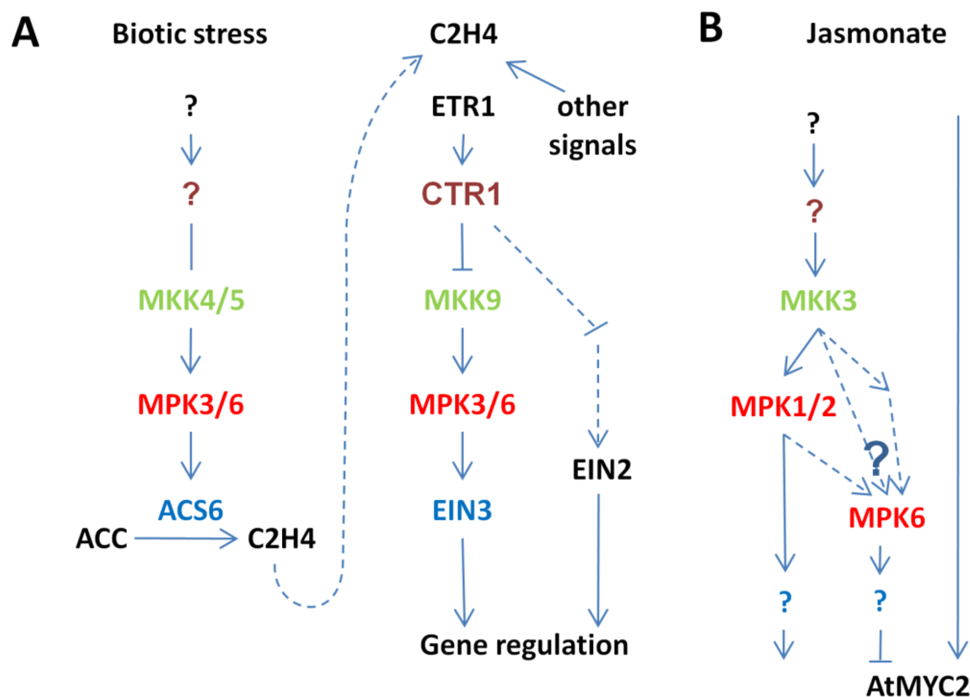



**Figure I.6: MAPK cascades activated in hormone signaling: A.** ET inhibits the ETR1 receptor and thereby alleviate the negative regulation by CTR1 of the downstream events, this derepression concerns EIN2 and the cascade MKK9–MPK6 module, which phosphorylates EIN3; **B.** JA regulates the MKK3–MPK1/2 module and, indirectly, MPK6. (Adapted from Colcombet *et al.* 2008).

mostly involved in signaling of defense responses against necrotrophic pathogens like *Altenaria* and *Botrytis* (Glazebrook 2005). JA has also a role in abiotic stresses such as ozone exposure, wounding and water deficit (Berger 2002; Devoto & Turner 2003). Using exogenous application of SA or expression of bacterial SA synthase trangenes (Verberne *et al.,* 2000), it was shown that SA accumulation induce Systemic Acquired Resistance (SAR) (defined as a long-lasting and broad spectrum disease resistance state in the plants) and Pathogenesis Related (PR) genes expression,

The role of MAPK in defense responses and therefore in hormone signaling specially SA and JA/ET is under investigation by several laboratories. A good example for the antagonistic regulation of immune response by SA and JA is the MEKK1-MKK1/2-MPK4 cascade . Mutants in these kinases accumulate SA and therefore constitutively express PR genes resulting in dwarfed plants that can be partially rescued by the expression of the bacterial salicylate hydroxylase NahG which converts SA into cathecol (Pearce & Humphrey 2001; Suarez-Rodriguez *et al.,* 2007). It was also proposed that MPK4 is the main modulator of JA- and ethylene responses through the regulation of downstream players of defense responses, PAD4 and EDS1, which enhance SA signaling and suppresses some JA/ET mediated responses like PDF1.2 expression (Brodersen *et al.,* 2006). The antagonistic effects of SA and JA signaling appear to be dose dependant. At low concentration, these hormones could have a synergic effect, however antagonistic effect was observed at higher concentrations (Koornneef & Pieterse 2008). In *mekk1* or *mpk4* mutants, the increased SA level results in a reduced expression of the JA-inducible marker gene PDF1.2 (Brodersen *et al.,* 2006; Ichimura *et al.,* 2006).

In Tobacco, the Wound-Induced Protein Kinase (WIPK) and the Salicylic-Induced Protein Kinase (SIPK) were shown to be important for the wound-dependent JA accumulation (Seo *et al.*, 2006). Indeed, RNAi lines or lines expressing MAPK phosphatases were shown to under-accumulate SA in response to wounding. Additionally, using protoplast system, SIPK, but not WIPK, was shown to be directly activated by SA and NO (Kumar & Klessig 2000).

In *Arabidopsis*, MAPKs were also proposed to directly belong to the hormonal signaling pathways. MPK6 is activated by JA signaling and this through the MAP2K, MKK3 (Takahashi *et al.,* 2007) (Figure I.6B). MKK3/MPK6 module is proposed to positively regulate JA responsive genes LOX2 and PDF1.2 and negatively regulate the expression of the bHLH transcription factor MYC2/JASMONATE INSENSITIVE1, an important player of JA pathway.

b. *Ethylene*

Ethylene is a key regulator of plant cell stress status, its role in plant defense was well established (Broekaert *et al.,* 2006; Van Loon *et al.,* 2006). It also plays important roles in developmental processes, such as fruit ripening, organ senescence, flower sex determination and skotomorphogenese. Genetic work in *Arabidopsis* showed that ethylene is perceived by 5 receptors of ETR1 family which possess an active kinase domain (Robert-Seilaniantz *et al.,* 2011). Ethylene receptors interact with the putative Raf-like MAP3K, CTR1 (Kieber *et al.,* 1993), which negatively regulate downstream components of ethylene response like EIN2 and EIN3. MAPK cascades are involved in both ethylene synthesis and ethylene response.

Two of the first MAPK substrates identified are ACS2 and ACS6 encoding an ACC synthases (Liu & Zhang 2004). In response to stress (flg22 or wounding), ACS6 is phosphorylated mainly by MPK6 and the phosphorylation stabilize the proteins, allowing the ACC biosyntheses and consequently an ethylene production (Liu & Zhang 2004; Joo *et al.,* 2008). The use of inducible lines expressing constitutively active MAP2K confirms that this MKK5 is involved in the production of ethylene triggering in this very particular lines cell death (Liu *et al.,* 2008).

On the other hand, application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) activates 2 MAPKs, MPK3 and 6 (Ouaked *et al.,* 2003; Yoo & Sheen 2008). The recent model proposed for ethylene signaling implies the cascade MKK9- MPK3/6 to works downstream of CTR1 (Yoo & Sheen 2008). In the presence of ethylene, CTR1 activity is blocked, and MKK9-MPK3/6 cascade is activated and phosphorylate EIN3 on T174 to promote its stability (Figure I.6A). In contrast, a lack of ethylene prompts CTR1 to block MKK9 activity, and then other MAPKs are free to phosphorylate EIN3 on its second phophorylation site T592 that promotes its degradation (Yoo & Sheen 2008). A work from Bethke and coworkers suggested a new mode of action of MPK6 via the release of the substrate Ethylene-Related Transcription Factors ERF104. In this work they emphasize a new function of MPK6 in ethylene signaling (Bethke, Unthan *et al.,* 2009).

In conclusion, ethylene signaling seems to be very complex as a single kinase MPK6, is needed for ethylene synthesis through ACS2/6 phosphorylation and playing also an important role in the downstream events after ethylene perception. Our limited knowledge on the role of

MAPKs in hormone signaling make it more difficult to understand plant defense responses since hormones are among the very important actors in the plant pathogen interaction.

c. *Abscisic acid (ABA) and Auxin*

These two hormones are grouped together because at some point they appear to share the same MAPK players in their signaling pathways.

Abscisic acid (ABA) plant hormone regulates various cellular processes, among them the seed dormancy and water loss control. ABA is an important transducer of environmental signals to protect plants mainly from abiotic stresses (Hirayama & Shinozaki 2007). It has been established that protein kinases and phosphatases plays a central role in regulation of ABA signaling network (Hirayama & Shinozaki 2007). The ABA signaling core mechanism is constituted of the PYR/PIL ABA receptors, PP2C phosphatases (such as ABI1, ABI2) and kinases belonging to the SnRK2 family (like the well characterized *Arabidopsis* OST1 ). In the absence of ABA, SnRK2s are inactivated by PP2C-phosphatase-mediated dephosphorylation. Upon ABA treatment, PYR/PIL ABA receptors bind phosphatases preventing the inactivation of SnRK2s which become consequently able to phosphorylate their targets. Additionally $Ca^{2+}$-dependent protein kinases (*CPK3*, *CPK6*, *CPK4*, *CPK11*) (Mori *et al.,* 2006; Zhu *et al.,* 2007), a protein phosphatase 2A regulatory subunit A (*RCN1*) (Kwak *et al.,* 2002), a receptor-like protein kinase (*RPK1*) (Osakabe *et al.,* 2005) and MAPKs act as positive regulators of guard cell ABA signaling (Jammes *et al.,* 2009). ABA activates 42–46 kDa kinases in *Arabidopsis* and Maize, which is consistent with average sizes of putative plant MAPKs (Lu *et al.,* 2002; Zhang *et al.,* 2006). An important player in ABA signaling, the Abscisic Acid Insensitive 5 (ABI5) transcription factor that have been found to be phosphorylated (Lopez-Molina *et al.,* 2001), MPK3 is suspected in this event (Lu *et al.,* 2002). Moreover *AtMPK3* gene, as well as the *ANP1* gene encoding the MAP3K of the stress-activated kinase cascade, are overexpressed in the *hyl1* mutant, an *Arabidopsis* ABA hypersensitive mutant. Increasing MPK3 expression by two times was enough to trigger hypersensitivity in post-germination and growth arrest by ABA (Lu *et al.,* 2002). However, it seems that the same MAPKs are also stimulated by many other signals. Both ABA and $H_2O_2$ tend to activate the same MAPKs and, thus, it is possible that ABA-induced

$H_2O_2$ production activates MAPK. The activation of MAPK also enhances the $H_2O_2$ production, forming a positive feedback loop (Zhang *et al.,* 2006).

Auxin is an important phytohormone for several aspects of plant development (Robert-Seilaniantz *et al.,* 2011). Connection between auxin effects and MAPK signaling was first observed in maize (Kovtun *et al.,* 1998). Using maize protoplast it was shown that the expression of the MAP3K, NPK1 represses GH3 and ER7 auxin responsive promoter and activate MAPK cascade. NPK1 ortholog from *Arabidopsis*, ANP1 is activated by $H_2O_2$ in protoplast. Moreover the active form of ANP1, ΔANP1 activated MPK3 and MPK6 which in turn suppressed promoter induction by auxin of auxin responsive promoters (Kovtun *et al.,* 2000). This seems to be consistent with previous data on maize.

Tobacco plants expressing constitutively active tobacco ANP1 ortholog NPK1 are more tolerant to multiple abiotic stress droughts, cold, and to abscisic acid hormone than wild type. $H_2O_2$ like ABA promotes expression of antioxidant genes *CAT1*, *cAPX*, and *GR1* and the total activities of the antioxidant enzymes catalase, ascorbate peroxidase (Zhang *et al.,* 2006). It seems that auxin role is not restricted to growth processes for plant it has also a stress-protective role by inducing an oxidative stress response through the MAP3K, ANP1.

As I mentioned before auxin and ABA signaling are connected. In a recent study, the *A. thaliana* MPK12 was found to be a direct substrate of the MKP INDOLE-3-BUTYRIC RESPONSE 5 (IBR5) (Lee *et al.,* 2009). In transgenic plants with reduced *MPK12* expression, the root growth is more sensitive to auxin. However *ibr5* mutants are less responsive to auxin and ABA, this phenotype was partially rescued by the silencing of MPK12 in *ibr5* plants but did not affect its ABA-related phenotype. Even though MPK12 colocalizes with IBR5 in the nucleus (Lee *et al.,* 2009), it seems that it has another function regarding ABA response. MPK12 and another MAPK from the group D were found in a cell type-specific functional genomic approach to be highly expressed in guard cells (Jammes *et al.,* 2009). These two kinases play a redundant role in Reactive Oxygen Species (ROS) signaling and in guard cell ABA signaling. Only the double mutant *mpk9/12* displays ABA-insensitive stomatal response, this phenotype being complemented by MPK12 (Jammes *et al.,* 2009). Another MAP2K from *Arabidopsis*, is proposed to be a negative regulator of polar auxin transport (PAT), as the auxin action defective mutant (fewer lateral roots and modified gravitropic responses) *bud1* over-accumulate MKK7 (Dai *et al.,* 2006).

All these examples showed that MAPK cascade appears to be an important hub for hormone signaling. It could be then worthwhile to put more effort on investigating their function in all circumstances regarding not only environmental but also developmental processes.

### *4. MAPK functions in plant development*

As with other eukaryotes, plant MAPK cascades play a central role in plant cell division and some MAP kinases are transiently induced during mitosis. Recent data shows the importance of stress induced MAPKs in very important developmental processes.

#### a. *MAPK NtNTF6/AtMPK4 defines a pathway involved in cell mitosis*

The Tobacco MAPK NTF6 was found to be specifically activated during late anaphase of mitosis and transiently localized at the phragmoplast (Calderini *et al.,* 1998). Using two-hybrid interaction analysis and pull-down experiments it was shown that tobacco MAP2K, NtMEK1 acts upstream of NTF6. The MAP3K of tobacco NPK1 (Nucleus- and Phragmoplast-localized protein Kinase 1; renamed from *Nicotiana* Protein Kinase 1) is involved in the regulation of cell-plate formation in plant cytokinesis (Nakashima *et al.,* 1998). The activity and the expression of NPK1 increases in the late M phase of the tobacco cell cycle, where it interacts with tobacco motor proteins, NACK1 (NPK1-activating kinesin 1) and NACK2. Both NPK1 and NACK1 localize at the center of the mitotic spindle and phragmoplast of dividing tobacco cells (Nishihama *et al.*, 2000). Expression of a kinase negative version of NPK1 interfered with the formation of cell plate and led to failed cytokinesis (Nishihama *et al.*, 2001). Recently substrates of NPK1 were identified and these include MAP2K, NQK1 (*Nicotiana* kinase next to NPK1), and the MAPK, NRK1 (*Nicotiana* kinase next to NQK1) (Takahashi *et al.,* 2004).

NPK1 is related to three *Arabidopsis* MAP3Ks, ANP1 (*Arabidopsis* NPK1 homolog), ANP2, and ANP3 (Nishihama *et al.,* 1997). Genetic studies using the three MAPK KO *anp1*, *anp2*, and *anp3* support a model in which the ANP family of MAP3Ks positively regulates cell division and growth (Krysan, Jester *et al.,* 2002). In recent studies, the cascade was completed: ANPs act upstream of the MAP2K MKK6 and the MAPK MPK4 in regulating cytokinesis (Kosetsu *et al.,* 2010; Takahashi *et al.,* 2011). Cytokinesis in plant cells is achieved by the

formation of the cell plate, it was striking to see that MPK4 and MKK6 both localize at the equatorial plane of the phragmoplast. Both *mpk4* and *mkk6* plants show a similar cell division defect (Kosetsu *et al.,* 2010). MKK6 also activates MPK4 in protoplasts. Moreover MPK4 activity was detected to be higher in organs that contain dividing cells (shoot and root apex, cotyledons) in *Arabidopsis* plants (Kosetsu *et al.,* 2010; Takahashi *et al.,* 2011).

Interestingly, independent studies propose ANP1/ANP2/ANP3-MKK6-MPK4 cascade in a larger context of cytoskeleton organization (Beck *et al.,* 2010; Beck *et al.,* 2011), explaining several aspects of mutant defects such as cell growth patterns, branching of root hairs and swelling of epidermal cells. Interestingly, these phenotypes are related to a decrease of the phosphorylation level of the Microtubule-Associated Protein 65 (MAP65) (Beck *et al.,* 2010).

What makes the story even more complex is the *mpk4* plants phenotype that appear to be also impaired in male meiosis cytokinesis (Zeng *et al.,* 2011). This phenotype was proposed to be due to the lack of callose deposition in pollen tetrads. and could be controlled by the same ANP3-MKK6-MPK4 cascade as the same defects were observed in the mutant plants (Zeng *et al.,* 2011). This observation could explain the decreased fertility of *mpk4*.

b. *MKK4/5-MPK3/6 cascade is involved developmental processes*

Another example that MAPK cascades functions in cell division and differentiation comes from the identification of *A. thaliana* MAP3K mutant *yoda* (MAP3K04) and its downstream MAPK components MKK4, MKK5, MPK3 and MPK6. *yoda* was identified in a genetic screen for mutants which have distinctive changes in the pattern of cell division (Lukowitz *et al.,* 2004). Then it appears that sterility, dwarfism with compact leaves of *yoda* mutant are associated to a compromised stomatal cell specification corresponding to a transgression of the 'one-cell spacing' rule: *yda* leaves present abnormal clustered stomata (Bergmann *et al.,* 2004). Evidence was provided subsequently using a reverse-genetic approach, that MKK4/MKK5 and MPK3/MPK6 are redundant downstream components of this MAPK signaling cascade, as *mkk4/mkk5* and *mpk3/mpk6* fail also to develop further than the cotyledon stage and show a typical *yoda*-like stomatal patterning at the epidermis (Wang *et al.,* 2007). bHLH transcription factor SPEECHLESS (SPCH) was proposed to be a target of MPK3 and MPK6 in the YODA cascade(s) (Lampard *et al.,* 2008). *spch* mutants do not develop stomata and is epistatic on *yoda*

(Macalister *et al.,* 2007). SPCH protein, at the opposite to two other bHLH involved in stomatal patterning, shows a MAPK phosphorylation domain containing 10 SP/TP sites. Phosphorylation on this transcription factor might be important to regulate its activity and stability, and to fine tune responses according to the phosphorylation site. This regulation could be ensured by MPK3 or MPK6. Positive regulation of stomatal differentiation may function downstream of this MAPK cascade and include other bHLH transcription factors, MUTE and FAMA which are related to SPCH (Ohashi-Ito & Bergmann 2006; Kanaoka *et al.,* 2008).

Interestingly the same signaling cascade (MKK4/MKK5-MPK3/6) functions in floral abscission (Cho *et al.,* 2008). Abscission is the natural process allowing plants to discard non functional or infected organs and promote dispersion of progeny. Plants silencing *MKK4/MKK5* or expressing a dominant negative form of MPK6 in *mpk3* background have abscission defects. Based on genetic interactions between the important player in abscission process, it was proposed that IDA (Inflorescence Deficient in Abscission), the receptor like protein kinase HAESA (HAE) and the HAESA-like 2 (HSL2) together with MPK3/6 cascade regulate in a sequential model floral organ abscission (Cho *et al.,* 2008).

### 5. Complexity of MAPK signaling

Several signaling pathways including abiotic and biotic stress response pathways, hormonal and developmental stimuli converge to the same MAPK cascades but triggers distinct responses. It looks like MAPKs are a hub of all modulating cellular functions. However, MAPK are not efficiently defined in separate parallel cascades, instead multiple interconnected MAPK pathways are required to transduce a signal, which involve a lot of overlaps and cross talks. Often the complexity is increased when the regulator of a MAPK is itself regulating several responses. MEKK1 for instance activates MPK3 and MPK6 in PTI processes through the two MAP2K MKK4 and MKK5 (Asai *et al.,* 2002) and at the same time MEKK1 functions in another cascade involving MKK1/2 and MPK4. This is also due to the disparity in size of the different families involved in the cascades. MAP3K family as 60–80 members and their putative MAP2K substrate, only 10… and the same thing for MAP2K and MAPK (20). This suggests a redundancy at the level of MAP2K for example, and the ability to be involved in different cascades. These expectations were observed experimentally.

In *Arabidopsis thaliana* two signaling pathways are very well characterized. The MEKK1-MKK1/2-MPK4 cascade is triggered by abiotic and biotic stresses. But in some recent reports it appears that MPK4 has also a role in developmental processes such as cytokinesis and microtubule organization but this time in association with the MAP3K ANP1/ANP2/ANP3 and the MAP2K MKK6. The combination of both function partially explain the fact that *mpk4* share phenotype traits with mutants of the two pathways. The second cascade involves MKK4/5-MPK3/6 and is proposed to work also in PTI and in responses to diverse abiotic stresses downstream of MEKK1. It also has a role in the stomatal patterning process but this time down-stream of the MAP3K YODA (Wang *et al.,* 2007). Yet it appears that flg22 is still able to activate MPK3 and MPK6 in the *mekk1* background, suggesting redundancy at the level of the MAP3K step in the MEKK1–MKK4/MKK5–MPK3/MPK6 signaling pathway.

A more comprehensive and exhaustive view of the MAPK cascades has being generated thanks to studies reported by Lee and co-workers and Popescu and co-workers. In the first study (Lee *et al.,* 2008), the authors used pair-wise yeast two-hybrid assays to test interactions between the 10 MAP2Ks and all 20 MAPKs. Popescu and co-workers made rather a phosphorylation assay with the same combinations and then used the working MAP2K-MAPK combination to screen for protein targets (Popescu *et al.,* 2009). Both studies confirmed the known interactions identified by genetic and *in vivo* studies, on the other hand it appears that no specific MAP2K is devoted for one MAPK. Numerous new interactions were proposed, but sometimes were not confirmed by both studies, showing techniques artifacts. Overall these studies provide new cascades that could be worth to check in vivo when the localization and expression data are in accordance with the probable interaction.

## C. Statement of approach

To find out the exact role of a protein in a specific biological process, scientists adopted different strategies. Genetic approaches were among the most successful. The use of knock out/down mutants or overexpressor lines allowed study the molecular and physiological alterations induced by the miss regulations or complete abolishment of the expression of a protein. After completing *Arabidopsis* genome sequencing, the creation of public libraries of KO plants using insertion mutagenesis accelerated research advances.

However quite often, the expression of a gene is tightly regulated. In this case, using over-expression lines could be subjected to down regulation. This approach is easy to apply to proteins which are not subjected to post-transcriptional or post-translational regulations. Moreover, gene for which the loss of function influence many phenotypic traits can often not be knocked out or give, when mutated, pleiotropic phenotypes which are usually difficult to interpret. A very promising approach allowing to better understand a protein function is to use gain of function (GOF) mutations. This allows monitoring the consequence of the activity on the phenotypic and molecular level. The kinases involved in signaling pathways are interesting candidates for such approaches.

### *1. Several tools to activate plant MAPKs*

In the case of MAPKs, increasing the protein level, if possible, does not necessarily result in elevated kinase activity (Hirt, *personal communication*). The activation of kinases that belongs to the MAPK families subsequent to a stimulus perception, depend on dual phosphorylation processes happening on conserved phosphorylation sites localize in the activation loop. Dual phosphorylation is the basis for the increase in MAPK activity (it can rich 1000 fold). This phosphorylation leads to structural changes and dynamic increases in specific activity (Canagarajah *et al.,* 1997; Prowse *et al.,* 2001). Studies on activation process of MAPK interested particularly the biochemists. Crystallization of the model mammalian MAPK ERK2 in active or inactive states has been reported (Canagarajah *et al.,* 1997). This work highlighted the role of the double phosphorylation occurring on the TEY motif and its relevance for the catalytic activity of the kinase and its specificity. This knowledge is likely transposable to plant MAPKs

since they all belong to the ERK like subclass of MAPKs (Mapk_Groups 2002) and all the amino acids important for the stabilization of phosphorylated kinase in a structurally active state are conserved in plant (Colcombet, *personal communication*). The main changes noticed concern the activation loop itself and another domain at the C- terminal extension (extending from $Pro^{309}$ to $Arg^{358}$) known as L16. Upon phosphorylation, the phospho-threonine (residue 183) of the TEY motif forms ionic contacts with the N-terminal domain which correspond to the L16 inducing the domain closure (Canagarajah *et al.,* 1997). The phosphorylation of the tyrosine serves to configure the ATP binding site, while phosphorylation of both residues is required to stabilize binding of the protein to the substrate (Prowse *et al.,* 2001). The kinetic of the phosphorylation is important as conformational changes happen accordingly in order to organize the catalytic residues in the active site. These modifications including new interaction between the phophorylation lip and the neighboring structures are important in promoting the substrate recognition specifically at the P+1 site (Canagarajah *et al.,* 1997). Knowing how complex is the regulation of MAPK phosphorylation, different strategies were employed in order to obtain active MAPKs. In the following chapter, I will present the different strategies developed to activate MAPKs and their limits.

a. *Stress activation of MAPKs pathways*

A classic way to activate MAPK is to apply extracellular stimuli known to activate the cascade. For plants, the activation of the three stress related kinases MPK3, MPK4 and MPK6 works perfectly by using the 22-amino acid fragment derived from the bacterial flagellin flg22. Once activated, the MAPK can be immuno-precipitated and used to phosphorylate specific substrates. The phosphorylation of VIP1 was demonstrated this way (Djamei *et al.,* 2007) and our laboratory used this strategy to test many putative new substrates identified in phospho-proteomic approaches (Hirt, *personal communication*). This strategy also allows studying downstream events controlled by MAPKs. Nevertheless, treatments often activate more than one MAPK and evoke many cellular responses difficult to sort out. Finally, his strategy is not suitable if the treatment or the conditions activating the kinase are unknown. As very few MAPK activities are detected in plants, activators of other MAPKs are still unknown.

b. *Activation of upstream steps of MAPK*

Many proteins which are activated by phosphorylation have been reported to be artificially modified into constitutively active forms when the phospho-acceptor amino acids at the active site are mutated to phospho-mimicking amino acids. Residues that mimic the phosphorylated state of the protein are usually acidic amino acids. In the case of MAP2Ks, the phosphorylation of the two adjacent Ser/Thr residues in the activation loop of MAP2K (**S/T-$X_{3-5}$-S/T**) is essential for its activation and in fact, mutation of these residues to acidic amino acids makes MAP2K constitutively active (CA) (Cowley *et al.,* 1994; Mansour *et al.,* 1994). This strategy was extensively and successfully used in protoplast expression system and allowed reconstitutions of the two last steps of some MAPK cascades (MKK1/MKK2-MPK4 (Teige *et al.,* 2004; Meszaros *et al.,* 2006), MKK4/5-MPK3/6 (Asai *et al.,* 2002), MKK3-MPK7 (Doczi *et al.,* 2007)). The CA MAP2Ks were also used to generate plant lines and identify downstream events (Ren *et al.,* 2002). Astonishingly to ensure a full activation of a MAP2K the double mutation of both phosphorylation sites is needed. The phospho-mimicking mutation strategy often works but our experience (and other's) suggests that each particular construct needs to be carefully characterized. Additionally, if serine and threonine have structural similarities with acidic amino acids (aspartate and glutamate respectively), this is not the case of the aromatic phosphorylable tyrosine (1.5 to 3 % of the identified cellular sites (Mithoe *et al.,* 2011). This may explain why a direct activation of MAPKs through the mutation of the TEY motif was never successful.

Kinases often have additional domain involved in inhibitory mechanisms. Several studies used active forms of plant MAP3K to activate the downstream kinases (MAP2K), among the first ones are the studies by Kovtun and co-workers who deciphered MAPK cascade involving NPK1 in response to auxin hormone (Kovtun *et al.,* 1998). Another successful example is the expression of the kinase domain of *Arabidopsis* MEKK1 to reveal the MAPK cascade working downstream of PAMP perception and abiotic stress (Asai *et al.,* 2002; Teige *et al.,* 2004). The activation of these MAP3K does not require mutation of the phosphorylation sites but rather truncation of the C terminal part of the protein. The expression of the kinase domain of the protein result an increased kinase activity compared to that observed after stimuli activation.

c. *Genetically triggered activation of MAPKs*

*Fusing constitutively active (CA) MAP2K*

A strategy to enforce activation of a given MAPK by a MAP2K is to constantly express them to an infinite proximity. This strategy consists in the case of MAPKs, to express a MAPK fused to its upstream activator MAP2K. The MAP2K-MAPK chimera results in the activation of the MAPK (Robinson *et al.,* 1998; Miyata *et al.,* 1999). For example Robinson and coworkers expressed the MEK1-ERK2 chimera, and showed that it was catalytically and biologically active because it was able to induce the activation of ERK2 substrates, the transcription factors Elk2 and AP-1. In another study (Zheng *et al.,* 1999), authors adopted this strategy to activate JNK1 MAPK through its fusion to JNKK2. Ateinza *et al*. (1999) validated the strategy for MEK-ERK1 chimera expressed in *S. cereviseae*. All these studies showed that the chimeras are biologically active. Nonetheless, a major drawback of this technique is that the MAP2Ks and MAPKs can lose their appropriate regulation and localization to have no specific activities and functions in the cell. MEK1 for instance has a nuclear export signal which has to be mutated when fused with ERK2 in order to ensure their expression directly in the nucleus in the vicinity of ERK2 substrates (Robinson *et al.,* 1998). Moreover ERK2 binds to its substrates and inhibitors (phosphatases) through the same domains (Tanoue *et al.,* 2000) and this could increase the competition for binding and reduces the binding with the substrate.

*Mutations triggering CA MAPKs*

Although the mechanisms of MAPK activation have been revealed, this knowledge could not be applied for the production of active forms of MAPKs because of the impossibility to mimic the pT-*X*-pY structure by mutagenesis (Robbins *et al.,* 1993). Many attempts to isolate active forms of MAPK, FUS3 in *S. cerevisiae* (Brill *et al*., 1994) or Rolled MAPK of *Drosophila Melanogaster* (Bott *et al.,* 1994) were performed in the previous decade, however they were not completely successful, as some mutations did not lead to a significant elevated basal kinase activity and therefore still require activation by MAP2Ks. A genetic screen in yeast to isolate active forms of the *S. cerevisiae* MAPK, Hog1 has been developed by Engelberg group (Bell *et al.,* 2001; Engelberg & Livnah 2006). This screen allowed the isolation of nine point mutations, most of them (6 mutations) are located in the conserved L16 domain, and activate the kinase independently of Pbs2 (the upstream MAP2K of Hog1) and without any induction (Bell *et al.,*

2001). The equivalent mutations introduced in other MAPK, like the human p38α (Bell *et al.,* 2001; Diskin *et al.,* 2004) or p38γ, β and δ, (Avitzour *et al.,* 2007) induce considerable increase in the kinase activity. In all these studies showed also that the constitutively active mutants preserve an authentic substrate specificity profile and sensitivity to inhibitors (Avitzour *et al.*, 2006; Diskin *et al.*, 2004).

Another smart approach developed by Emrick and coworkers (Emrick *et al.,* 2001) consisted to combine all the mutations described in previous studies (Brill *et al.,* 1994; Brunner *et al.,* 1994; Ikeda *et al.,* 1997) to create ten point-mutated ERK2 (Table I.1). It was reported that insertion of the single equivalent mutation in ERK2 resulted in just basal catalytic activity, similar to that of ERK2 WT. However, ERK2 proteins carrying combinations of some mutations manifested very high specific activity, reaching up to ~90-fold over that of wild-type non-activated ERK2. A combination of the three mutations L73P/S151D/D319N resulted in the highest activity recoded for ERK2. Surprisingly $ERK2^{L73P/S151D/D319N}$ showed less activity when tested *in vivo* compared to other mutation combination, its activity was lower than 1% of that of phosphorylated ERK2 (Emrick *et al.,* 2001).

**Table I.1: Mutations selected by Emrick and cowarkers (2001) to produce hyperactive ERK2.**

| Mutation found | Reference | MAPK |
|---|---|---|
| D48N, I161L, D227N, Y7H, I9K and C28Y | Brill *et al.* (1994) | FUS3 |
| L63P | Hall *et al.* (1996) | FUS3 |
| D334N | Brunner *et al.*, 1994 | FUS3 |
| K136Q | Ikeda *et al.* (1997) | ERK2 |
| S194D | Cowley *et al.*, (1994) | MKK1 |

### *2. Objectives of my PhD: identifying constitutively active form of MAPKs and use them to understand their functions in Arabidopsis*

Many aspect of MAPK function and mechanism had been revealed, however still the exact role of each MAPK in a given biological system is not fully understood as they are difficult to study independently of their signaling context. This is mainly due to complexity of signaling through MAPK cascade despite the fact that they are probably involved in almost all the biological responses of the cell (Suarez-Rodriguez *et al.,* 2010). A way to bypass this complexity is to activate one single MAPK at a time and follow the biochemical and physiological consequences of its activity, ideally in a time dependent manner. This approach was successful in the case of MAP2Ks, for which a combination of CA mutated forms with inducible promoters allowed the characterization of major downstream events of the cascades (Liu *et al.,* 2008). However, this approach is not possible yet concerning plant MAPKs as no simple way to activate them exists. When I started my work in Heribert Hirt's group, this development still needed to be initiated for plants MAPKs.

Constitutively activating mutations were identified for yeast and mammals MAPK but their transfer into plant MAPK was not successful. For example, in the laboratory, we introduced the mutations identified by Engelberg's group in *Arabidopsis* MPK4. When expressed in yeast, these putative CA forms failed to complement yeast mutants (*pbs2Δhog1Δ*) and did not show any increased kinase activity (Colcombet, *personnel communication*). Constructions of MAP2K-MAPK chimera, initiated in Heribert Hirt's group, were not functional neither (Kumar and Hirt, *personal communication*).

In this context, the aim of my study was to (1) find a strategy to **isolate constitutively active (CA) forms of a model MAPK** and (2) to demonstrate that **MAPK CA forms are useful to understand kinase function *in planta***.

As the strategy used by Engelberg was successful and knowing that MPK6 was functional in yeast (Teige *et al.,* 2004), we decided to adopt a similar approach. The chapter III describes the use of a genetic screen in yeast to isolate CA mutants of MPK6. In the chapter IV, I prove that mutations identified in the screen can activate other plant MAPKs from different group and in the chapter V that the specificity of interaction between the active MAPKs and known interactors, as well their substrate phosphorylation preferences are not altered. The last part (Chapter VI and VII) is devoted to the proof-of-concept that the mutations identified in our

screen can activate a plant MAPK *in vivo*. We choose to work on MPK4 whose cascade is very well described now, Heribert Hirt's laboratory being among the most involved in this characterization. We choose to generate stable transgenic lines in which *mpk4* mutant plants were complemented with active version of MPK4. To our knowledge the complementation failed with inactive kinase version (Petersen *et al.,* 2000). All the data concerning MPK4 demonstrated its essential and complex role in plant defense responses, yet recent studies demonstrated its role in developmental process. In this part of the project, we investigated first whether constitutively active version of MPK4 could complement *mpk4* mutant plants and whether the mutations could activate the kinase *in vivo* (chapter VI). Then, in the chapter VII, we studied the resistance of these lines towards pathogens in order to try to learn more about the exact role of MPK4 during plant pathogen interaction and more specifically its implication in PAMP triggered immunity (PTI) or effectors triggered immunity (ETI). Investigation of MPK4 function in development and particularly the microtubule organization using complemented CA-MPK4 lines is under studies.

# *Chapter II*
# *Material &Methods*

# *Chapter II - Material and methods*

## *A. Materiel*

### *1. Plant Material*

*Arabidopsis thaliana* ecotypes used in this study were Columbia-0 (Col-0) and Wassilewskija-0 (Ws-0). Mutant lines are listed in following table

**Table II.1: Mutants of *Arabidopsis* used in this study**

| Allele name | Ecotype | Mutagen | Reference/Source |
|---|---|---|---|
| *mpk4-2* (Salk_056245) | Col-0 | T-DNA | (Kosetsu *et al.,* 2010) |
| *mekk1-1* (Salk _052557) | Col-0 | T-DNA | (Nakagami *et al.,* 2006) |
| *eds1-2* | Col-0 (Ler-0)[a] | Fast neutron | (Bartsch *et al.,* 2006) |

[a]Ler eds1-2 allele introgressed into Col-0 genetic background, 8 backcrosses generation.

### *2. Pathogens*

*Arabidopsis* plants were challenged with different strains of the bacterial pathogen *Pseudomonas syringae*:

- *P syringae* pv. tomato (*Pst*) strain DC3000 harboring the empty broad host range vector Pvsp61 (Innes *et al.,* 1993)
- *avrRps4* strain*: P syringae* pv. tomato (*Pst*) strain DC3000 expressing the effector *P syringae* pv. pisi effector AvrRps4 (Hinsch & Staskawicz 1996).
- *HrcC* strain: mutant deficient in the type-III secretion system (Deng *et al.,* 1998).

*Arabidopsis* plants were also subjected to infection with the oomycete pathogen Emwa1 strain of *Hyaloperonospora parasitica* (*Hpa*). Emwa1 is an avirulent strain expressing the AvrRpp4 effector.

*Alternaria brassicicola:* White-shire, strain DSM 62008.

### *3. Escherichia coli strains*

Depending on the application different bacterial strains were used. Non recombined GateWay vectors were propagated in *Escherichia coli* DB3.1 strain (invitrogen). For Gateway recombined Donor and Destination vectors and for regular cloning protocol, we used the strain DH5α (invitrogen). For protein purification, we used the bacterial strains BL21 or the BL21 Rosetta$^{TM}$2 (Novagen, **http://www.emdbiosciences.com/html/NVG/home.html**). For plant cell transformation we used *Agrobacterium tumefasciens* strain C58C1 carrying the helper plasmid pSOUP (Hellens *et al.,* 2000).

### *4. Yeast*

Two *Saccharomyces cerevisiae* strains were used. For the genetic screen, osmosentive double mutant strain *pbs2Δhog1Δ was created in* the W303 ecotype (MATa/MATalpha, leu2-3,112 trp1-1, ura3-1 ade2-1 his3-11,15 PBS2::HIS3 HOG1::TRP) and is already described (Teige *et al.,* 2004). The yeast two hybrid strain was MaV103 (MATa; leu2-3,112; trp1-901; his3Δ200; ade2-101; cyh2R; can1R; gal4Δ; gal80Δ; GAL1::lacZ; HIS3UASGAL1::HIS3@LYS2; SPAL10::URA3).

### *5. Media*

All the used media were sterilized by 20 min autoclave at 120°C. All supplements (antibiotics, amino acids, plant hormones, chemicals) were sterile filtrated and added after autoclaving.

*Plant media:*

For *Arabidopsis* germination ½ MS medium was used: 0.22% MS powder (Murashige-Skoog salt mixture with B5 vitamins, Duchefa, Netherlands), 0.05% MES, 1% sucrose (if not stated differently); pH 5.8 adjusted with 1M KOH; for agar plates 0,8% plant agar was added (Duchefa, Netherlands) before autoclaving. For selection Antibiotics were added after autoclaving.

*Bacterial media:*

- Luria Bertani (LB) medium (for E. coli and *Agrobacterium*): 1% Bacto-tryptone, 0.5% NaCl, 0.5% Bacto-*yeast extrac*t, 1% NaCL; for solid medium 1% Bacto-agar was added before autoclaving
- NYG agar medium (for *Pseudomonas* strains): .5% Bacto-peptone, 0.3% yeast extract, 2% glycerol and 1.5% Bacto-agar.

*Fungus media:*

PDA medium: 20 % Potato infusion, 2 % Dextrose, 2 % Bacto-agar.

*Saccharomyces cerevisiae medium*

- YPD medium (complex medium): 1% yeast extract, 2% peptone, 2% dextrose, supplemented or not with 2% agar
- YNB medium (minimal medium): 0.17% yeast nitrogen base without amino acids, 2% dextrose, 0.5% of NH4SO4, 0.2% of Drop out mix (US Biological), pH 5.6 adjusted with 5M NaOH, . For solid medium, 2% of bacto agar was added before autoclaving.

## *6. Buffers and Solutions*

*Yeast transformation:*

- EDTA stock solution: 0.5 M EDTA in $H_2O$; pH 8.0 adjusted with NaOH.
- 10xTE: 100 mM Tris, 10 mM EDTA pH 8.0
- TE/LiAc mix: 1xTE, 100mM LiAc
- PEG TE/LiAc mix: 1x TE, 100mM LiAc, 40% PEG [MW3,350] (from autoclaved 50% PEG stock solution in $H_2O$)

*Yeast plasmid extraction:*

- Buffer A: 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA, 0.1% SDS
- TE buffer: 10 mM Tris-Cl, pH 7.5, 1 mM EDTA

*Isolation of plant genomic DNA*

EDM-buffer: 200mM Tris-HCl (pH 7,5), 250mM NaCl, 25mM EDTA, 0,5% SDS

*SDS-PAGE gel*

10 x SDS running buffer: 30.3g/l Tris base, 72,1g/l glycine, 144g/l, 10g SDS

*Laemmli buffer (SDS loading buffer)*

For 6x solution: 30% glycerol, 10% SDS, 0.6M DTT, 0.012% Bromophenol blue, 0.35M Tris Cl pH 6.8 or 0.28% SDS

*Western blotting*

- 10x Transfer Buffer (TB): 30.3g/l Tris base, 144g/l glycine
- TB for use: 10% 10xTB, 20% EtOH 96°
- 10x TBS: 87.66g/l $NaCl_2$, 121.1g/l Tris base, Ph 7.5
- TBS-T: 1xTBS + 0,1% Tween

*Coomassie staining*

- Staining solution: 0,1% Coomassie, Blue R-250, 40% ethanol, 10% acetic acid
- Distaining solution: 50ml ethanol, 75ml acetic acid, 850ml $H_2O$

*Purification of HIS-fusion proteins*

- Lysis buffer: 30 mM Tris pH 7.5, 300mM NaCl, 0.1% Triton X-100, 5mM imidazole, 2mg/ml lysosyme
- Washing buffer: 30 mM Tris pH 7.5, 300mM NaCl, 10mM imidazole
- Elution buffer: 30 mM Tris,pH 7.5, 300mM NaCl, 200mM imidazole, 20% glycerol
- Dialysais buffer : 30 mM Tris pH 7.5, 20% glycerol

*Crude protein extracts of A.thaliana plant material*

Laccus buffer: 25mM Tris-HCl pH 7,8, 10mM $MgCl_2$, 15mM EGTA, 75mM NaCl, 1mM DTT, 1mM NaF, 0,5mM $NaVO_3$, 15mM ß-glycerophosphate, 15mM 4-nitrophenylphosphate, 0,1% Tween-20, 0,5mM PMSF, 5µg/ml leupeptin, 5µg/ml aprotinine

*Phosphorylation assays*

- 1M DTT: in 0.01 M sodium actetate; pH 5.2

- SucI-buffer: 50mM Tris-HCl pH 7.5, 250mM NaCl, 5mM EGTA, 5mM EDTA, 0,1% Tween-20, 5mM NaF, 0.1% Nonidet P-40, 'complete, EDTA-free protease inhibitor cocktail' (Roche Diagnostics)
- Kinase buffer: 20mM HEPES pH 7,5 , 15mM $MgCl_2$, 5mm EGTA, 1mM DTT

*Trypan blue staining*

- Trypan blue stock solution: 10ml lactic acid, 10ml glycerol, 10ml phenol, 10mg trypan blue dissolved in 10ml $H_2O$. Before use 1volume this solution is diluted with 1volume of EtOH 100%
- Trypan clearing solution: 25g/10ml chloralhydrate in $H_2O$

*Flower deeping solution for Arabidosis thaliana transformation*

Sucrose solution 5% in $H_2O$, silvett L77 0,05%

## *7. Antibodies*

*Primary antibodies*

- Anti-AtMPK4: rabbit, polyclonal, antigen ELIYRETVKFNPQDSV (C-terminus) (Davids Biotechnologie, Regensburg, Germany); crude serum was used for pull down, affinity purified antibody for western blot: dilution 1:5000
- Anti-AtMPK6: rabbit, polyclonal, antigen FNPEYQQ (C-terminus) (Davids Biotechnologie, Regensburg, Germany); crude serum was used for pull down, affinity purified antibody for western blot: dilution 1:5000
- Anti-c-Myc (C3956, SIGMA): Polyclonal antibody from rabbit, antigen in carboxy terminal domain of human c-Myc; dilution for western blot: 1:5000

*Secondary antibodies*

Anti-rabbit IgG: Peroxydase conjugate from goat, polyclonal (SIGMA-ALDRICH, USA); dilution for western blot: 1:20000

### *8. Antibiotics*

Antibiotics were used at the following concentrations for 1000x stocks, ampicillin (amp): 50mg/ml in $H_2O$, chloramphenicol (CA): 34mg/ml in ethanol, gentamycin (gent): 10mg/ml in $H_2O$, hygromycin (hyg): 10mg/ml in $H_2O$, kanamycin (kan): 50mg/ml in $H_2O$, rifampicin (rif) : 34mg/ml in DMSO, spectinomycin (spec): 10mg/ml in $H_2O$ (100x stock).

### *9. Vectors:*

In our study we used these following vectors:

- **pDONR$^{TM}$207:** Donor Gateway® vector from Invitrogen, used to clone genes and generate entry clones
- **pDR195gtw:** Home-made destination Gateway® vector for protein expression in yeast under the control of PMA1 promoter (Oomen *et al*., 2009)
- **pDR195:** Cloning vector for expression of a protein in yeast under the control of PMA1 promoter
- **pDEST17:** Destination Gateway® vector from Invitrogen for IPTG-inducible expression of N-terminal hexahistidine (6xHis) protein (Invitrogen).
- **pDEST22:** Destination Gateway® vector from Invitrogen for constitutive expression in yeast of a protein fused to GAL4 Activation Domain (AD) under the control of ADH1 promoter (Invitrogen)
- **pDEST32 modified :** Destination Gateway® vector for constitutive expression in yeast of a protein fused to GAL4 Binding Domain (BD) under the control of ADH1 promoter. This vector was modified from Invitrogen to be kanamycin resistant.
- **Peri-HIS-MBP:** Destination Gateway® vector for IPTG-inducible expression of N-terminal Peri-His-MBP tagged protein in Coli (Nallamsetty *et al*., 2005).
- **pGREEN0229:** binary vector for expression of fusion protein.
- **pGEM®-T Easy:** vector to clone PCR products. Promega.

### *10. Oligo-nucleotides:*

For gateway cloning: Table II.2

| Clone name | Sequence of primer couple |
|---|---|
| pDNR207-MPK3-END/STOP | GGA GAT AGA ACC ATG AAC ACC GGC GGT GGC<br>TCC ACC TCC GGA TCM ACC GTA TGT TGG ATT GAG TGC T |
| pDNR207-MPK4-END/STOP | GGA GAT AGA ACC ATG TCG GCG GAG AGT TGT TTC<br>TCC ACC TCC GGA TCM CAC TGA GTC TTG AGG ATT GAA C |
| pDNR207-MPK6- | GGA GAT AGA ACC ATG GAC GGT GGT TCA GGT CA |

| END/STOP | TCC ACC TCC GGA TCM TTG CTG ATA TTC TGG ATT GAA AGC |
|---|---|
| pDNR207-MPK7-<br>END/STOP | GGA GAT AGA ACC ATG GCG ATG TTA GTT GAG CCA C<br>TCC ACC TCC GGA TCM GGC ATT TGA GAT TTC AGC TTC AG |
| pDNR207-MKK1-<br>END/STOP | GGA GAT AGA ACC ATG AAC AGA GGA AGC TTA TGC C<br>TCC ACC TCC GGA TCM CCA TTG CGA GAT GAA GGA GC |
| pDNR207-MKK4-<br>END/STOP | GGA GAT AGA ACC ATG AGA CCG ATT CAA TCG CCT C<br>TCC ACC TCC GGA TCM TGT GGT TGG AGA AGA AGA CGA G |
| pDNR207-<br>ERF104-END/STP | GGA GAT AGA ACC ATG GCA ACT AAA CAA GAA GCT<br>TCC ACC TCC GGA TCM AGT GAC GGA GAT AAC GGA AAA |
| pDNR207-VIP1-<br>END/STOP | GGA GAT AGA ACC ATG GAA GGA GGA GGA AGA GGA<br>TCC ACC TCC GGA TCM GCC TCT CTT GGT GAA ATC CAT |
| pDNR207-MKS1-<br>END/STOP | GGA GAT AGA ACC ATG GAT CCG TCG GAG TAT TTT<br>TCC ACC TCC GGA TCM ATC TTG ATC CCA AAT ATG ACT A |
| Δ*MPK16* | GGA GAT AGA ACC ATG CAG CCT GAT CAC CGC AA<br>TCC ACC TCC GGA TCM AGT TGG CTC TGA TCC ATC CAA G |

For point mutations: Table II.3

| Mutation | Sequence |
|---|---|
| $MPK3^{T119Y}$ | F: ATC CAT CAA CTC ACA AAC AAT ATA TAC<br>R: GTA TAT ATT GTT TGT GAG TTG ATG GAT |
| $MPK3^{D193G/E197A}$ | F: GAG AAT GGT TTT ATG ACT GCG TAT GT<br>R: ACA TAC GCA GTC ATA AAA CCA TTC TC |
| $MPK3^{T119C}$ | F: GTTTATATCTCTTGTGAATTAATGGAT<br>R: ATCCATTAATTCACAAGAGATATAAAC |
| $MPK4^{Y124C}$ | F: GTC CAT AAG CTC ACA AAC AAT GTA AAC<br>R: GTT TAC ATT GTT TGT GAG CTT ATG GAC |
| $MPK4^{D198G/E202A}$ | F: CGA GAC TGG CTT TAT GAC TGC ATA TGT TG<br>R: CAA CAT ATG CAG TCA TAA AGC CAG TCT CG |
| $MPK6^{Y144C}$ | F: GAT GTT TAC ATC GCG TAT GAG TTA ATG GAC AC<br>R: GTG TCC ATT AAC TCA TAC GCG ATG TAA ACA TC |
| $MPK6^{D218G}$ | F: TTC TGA GAG TGG TTT CAT GAC TGA A<br>R: TTC AGT CAT GAA ACC ACT CTC AGA A |
| $MPK6^{E222A}$ | F: TTC ATG ACT GCA TAT GTT GTC ACG AGA<br>R: TCT CGT GAC AAC ATA TGC AGT CAT GAA |
| $MPK6^{D218G/E222A}$ | F: GAG AGT GGT TTC ATG ACT GCA TAT GTT<br>R: AAC ATA TGC AGT CAT GAA ACC ACT CTC |
| $MPK7^{Y114C}$ | F: ATC CAT TAG CTC ACA AAC CAA ATA AAC<br>R: GTT TAT TTG GTT TGT GAG CTA ATG GAT |
| $MPK7^{Q188G/E192A}$ | F: GGT AAT GGA CAG TTC ATG ACT GCG TAT GTG GTT<br>R: AAC CAC ATA CGC AGT CAT GAA CTG TCC ATT ACC |
| $\Delta MPK16^{F106Y}$ | F: CAT TTA CGT GGT TTA CGA GCT TAT GGA AT<br>R: ATT CCA TAA GCT CGT AAA CCA CGT AAA TG |

For cloning MPK4-PC2 constructs: Table II.4

| Name | Sequence |
|---|---|
| K4_5p_F | GGT ACC GAC TTG TTT GTG AAT ATA GAG GAA ACA TG (Underline = Kpn1 site) |
| K4_5p_R | GGA TCC CAC TGA GTC TTG AGG ATT GAA CTT GAC (Underline = BamH1 site) |
| K4_3p_F | GGA TCC GAG AAA GAG AGA GAG ATA TAT ATC C (Underline = BamH1 site) |
| K4_3p_R | GCG GCC GCG ATA ATT AGT GGA TGT AAT TAG AGT TAA GAC (Underline = Not1 site) |
| PC2_F | AGA TCT CCA GCT TTC TTG TAC AAA GTG GTG ATC (Underline = Bgl2 site) |
| PC2_R | AGA TCT TCA CTT CTC GAA CTG AGG ATG AGA C (Underline = Bgl2 site) |

For genotyping of T-DNA insertion lines and transformed lines: Table II.5

| Primer's location | Sequence |
|---|---|
| mpk4-LB | TCA AAC AGG ATT TTC GCC TGC T |
| mpk4-RB | CTT GAA ATA TCT ACA GAG TTG GTG TG |
| MPK4-WT allele | GTG ACA ATG CAA GAA GAT ACG TTA GAC AGC |
| MPK4 terminator: transformed lines | GCGGCCGCGATAATTAGTGGATGTAATTAGAGTTAAGAC |
| mekk1-LB | GCT CTA TTT AGG TTT ATC GAA GTA ATC |
| mekk1-RB | AGA CCG ATA AAT CCG ATC CAT CTC TC |
| LB6316 (Salk tDNA) | TCA AAC AGG ATT TTC GCC TGC T |

### *11. Enzymes*

Restriction enzymes were purchased from New England Biolabs, and were supplied with the 10x reaction buffer which was used for restriction digestion following manufacturer's recommendations. Standard PCR reactions were performed using homemade *Taq* DNA polymerase or *Taq* DNA polymerase from New England Biolabs. High accuracy I-*proof* high fidelity DNA polymerase (BioRAD) was used for cloning and site directed mutagenesis. For random mutagenesis we used Mutazyme® II DNA polymerase from Stratagene. BP and LR cloning reaction were performed with BP and LR enzymes respectively from Invitrogen.

# B. Methods

## 1. DNA methods

### a. *DNA isolation*

*Isolation of genomic DNA from Arabidopsis*

Young leaf from 2-3 weeks old *Arabidopsis* plants was frozen in liquid nitrogen, grinded and the powder resuspended in 300µl EDM-buffer. After homogenization the samples were centrifuged at RT for 5min at max speed. The supernatant was transferred to a fresh tube and the DNA precipitated by addition of 1 Volume of isopropanol for 10min at room temperature, followed by a 10 min centrifugation at top speed and RT. The supernatant was removed and the pellet dried at 37°C for and then resolved in 40-100 µl $H_2O$ (overnight at 4°C). The genomic DNA was stored at -20°C.

*Quick Mini preparation of plasmid DNA from E. coli*

5-10ml overnight grown culture in LB with appropriate antibiotics were pelleted, the pellet either stored at -20°C until further use or extracted using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions.

*Plasmid rescue from yeast*

2ml overnight cultures in YNB medium with appropriate selection were pelleted at 5.000rpm for 1 min at room temperature. The pellet was resuspended in 200µl Buffer A and kept at on ice to then add 0.3g of glass beads and 200µl phenol, followed by vortexing for 15s and 1min centrifugation at 14.000rpm at room temperature (RT). A second phenol extraction was performed with the supernatant. To the last supernatant was added the same volume of $H_2O$ and 200µl of chlorophorme and vortexed for 15s before centrifugation at top speed for 1min at RT. Precipitation was done with 30µl of 3M NaAcetate and 800µl of 96% ethanol for 10min at -20°C, followed by 20min centrifugation at top speed and 4°C. The pelleted DNA was washed with 70% ethanol and air dried for 1h before resuspension in 50µl of TE pH8 buffer.

b. *Polymerase chain reaction (PCR)*

*Cloning, genotyping and colony PCR*

PCR reactions were carried out with Taq DNA polymerase (Biolabs) or homemade taq-polymerase for colony-PCR and genotyping, I-Proof (BioRAD) was used for cloning steps because of its higher accuracy in the synthesis.

*Cloning PCR*

| Component | Vol for 50 µl | Final Conc. |
|---|---|---|
| 5X iProof HF buffer | 10 µl | 1X |
| 100 mM dNTP mix | 0.1 µl | 100 µM each |
| Primer 1 (10µM) | 1 µl | 0.2 µM |
| Primer 2 (10µM) | 1 µl | 0.2 µM |
| DNA template | 1 µl | |
| Sterile $H_2O$ | 36.4 µl | |
| DNA polymerase | 0.5 µl | 0.25 U/µl |

PCR program:

**1 cycle** 94°C 30s
94°C 10s
**25-35 cycles** 53-55°C 30s
72°C depends on fragment length (1 min /kb)
**1 cycle** 72°C 8 mins

*Genotyping and colony PCR*

| Component | Vol for 20 µl | Final Conc. |
|---|---|---|
| 10X Taq buffer | 2 µl | 1X |
| 100 mM dNTP mix | 0.2 µl | 1 µM |
| Primer 1 (100µM) | 0.04 µl | 0.2 µM |
| Primer 2 (100µM) | 0.04 µl | 0.2 µM |
| Sterile $H_2O$ | 17.57 µl | |
| DNA polymerase | 0.15 µl | 0.25 U/µl |

PCR program:

**1 cycle** 94°C 30s
94°C 10s
**35 cycles** 55°C 30s
72°C 2min depends on fragment length (15-30s/kb)
**1 cycle** 72°C 7 mins

*Site directed mutagenesis*

Site directed mutagenesis was performed to exchange a specific amino acid residue (found in our screen, see primer sequence) in different MAPKs to turn the protein kinase active. This was achieved by two PCR reactions with *I-proof* DNA using 2 couples of primers: (5'primer-mutation primer and mutation primer-3') containing the desired mutation and the target gene as a plasmid template (pDONR-ORF). The PCR program was run as specified above. After agarose gel electrophoresis two DNA fragments were purified from gel slices using the Gel extraction kit (250) of Quiagen. A second run of PCR is done to reconstitute the ORF using universal primers containing the gateway flanking regions U5 for 5' and U3-GGA/TGA. Then the PCR product is purified by a size selective DNA precipitation method using 230µl of $PEGMgCl_2$, 1µl of glycogene azur and centrifugation at maximum speed for 15mins and RT. The pellet is then resuspended in 20µl of TE buffer.

Another method that was adopted to introduce mutations is to perform the site directed mutagenesis PCR reaction using only one PCR run using the couple of primer containing the desired mutation on the pDONR plasmids. After the PCR reaction, the DNA was digested with DpnI (Promega), which selectively degrades the methylated (non mutated) parental DNA. 17µl of the purified DNA were mixed with 2µl of buffer and 1 µl of DpnI (10U/µl) and incubated at 37°C for 1-1,5h. The entire digest was then transformed by heat shock into *E.Coli* without any additional purification steps before.

*Random mutagenesis*

Random mutations were introduced using GeneMorph® II Random Mutagenesis Kit (Stratagene) to introduce mutations in the MAPK gene according to the manufacturer's instructions.

*Restriction digest*

DNA digestion was ensured with restriction enzymes from Biolabs. 1-5µg of DNA was digested with 5U of restriction enzyme under appropriate buffer conditions at 37°C for at least 1,5h. Loading dye was added to the digest and the samples were run on an agarose gel.

c. *Agarose gel electrophoresis for DNA*

Agarose gels at 0,8-1% were prepared by heating the appropriate amount of agarose in TBE-buffer (10X solution: 108g Tris base, 55G Boric acid, 40ml EDTA pH8) until dissolved. After cooling to 50-60°C ethidium bromide was added to visualise DNA under UV-light. The Gene Ruler 1kb DNA ladder (Fermentas) was used as a size standard. The Gels were runned at approximately 5V/cm.

d. *Gateway cloning*

For creation of pENTR vectors, we used BP reaction and a two-step protocol kindly provided by Claire Lurin (http://www-urgv.versailles.inra.fr/atome/index.htm). For the destination vector (pDEST) for expression assays, we used the LR recombination reaction. Both reactions were made at 25°C over night.

| **BP Reaction mix:** | **Volume** | **LR Reaction mix:** | **Volume** |
|---|---|---|---|
| BP reaction buffer 5X | 1µl | LR reaction buffer 5X | 1µl |
| PEG purified PCR product | 2µl | pENTR (10 ng/µl) | 1µl |
| pDONR207 (200 ng/µl) | 0.25µl | pDEST (100ng/µl) | 1µl |
| TE pH7.5 | 1.35µl | TE pH7.5 | 1.6µl |
| BP clonase enzyme mix | 0.4µl | LR clonase enzyme mix | 0.4µl |
| **Total volume** | **5µl** | **Total volume** | **5µl** |

e. *E. coli transformation by heat shock*

Competent cells were prepared as described previously (Inoue *et al.*, 1990) and kept as -80C. 2-5µl of DNA were mixed with 100µl of heat shock competent cells already thawed on ice. The DNA-bacteria mixture is incubated for 20min in ice. Cells were then heat shocked for 1min30sec at 42°C and 10min on ice, then 900µl LB medium were added immediately and cells were incubated for 1h at 37°C. Cells were spread on LB-agar plates with respective antibiotics and transformants were grown overnight at 37°C.

f. *Agrobacterium C58C1 transformation by heat shock*

To prepare competent cells, an overnight culture grown in 8ml LB medium was inoculated from a single colony and left shaking over night at 30°C. Pre-culture was diluted in 200ml of fresh LB and grown for 4-5 hours. Cells were spun down 15min at 4000rpm and 4°C. The supernatant was then washed with a 10 mL solution of 10 mM Tris/HCl pH7.5 and spun down again. The cells were resuspended 5mL LB, alicotated and frozen in liquid nitrogen and conserved at -80C. To transform the cells, 200µl of competent *Agrobacterium* cells were mixed together with 5µl of plasmid DNA and kept on ice for 5-10 min, then frozen in liquid nitrogen for 5min. The mixture were incubated for 5min in a water bath at 37°C and then left for 2hours of gently shake incubation at 37°C after adding 800µl of LB medium.

g. *Cloning of MPK4 locus in pGREEN0229*

*MPK4* locus (2693 bp) and downstream (258 bp) of the termination codon was amplified from *Col0* genomic DNA using K4_3p_F/K4_3p_R and K4_5p_F/K4_5p_R couples of primers (Table II.4). PC2tag was amplified with PC2_F/PC2_R from pPC2 (kindly provided by H. Mireau). PCR fragments were cloned by T/A cloning in pGEMTeasy (Promega) and sequenced. Using appropriate restriction enzymes (Table II.4), MPK4 locus was reassembled in pGREEN0229. In a second step, the BglII DNA fragment containing the PC2 tag was cloned in the unique compatible BamH1 site to build pGREEN0229-MPK4L-PC2. The whole construct was then re-sequenced. Point mutations were realized as described above to generate pGREEN0229-MPK4$^{Y124C}$-PC2 and pGREEN0229-MPK4$^{D198G/E202A}$-PC2.

***2. RNA methods***

a. *RNA isolation from plant material using*

RNA extraction was performed on approximately 100mg of grinded plant material using RNeasy® Plant Mini Prep kit (50) (Quiagen) according to the manufacturer’s instructions.

b. *Deoxyribonuclease (DNAse) I treatment of RNA*

RNA was subjected to DNAse prior to synthetise cDNAs in order to differentiate between amplification from contaminating DNA in RNA isolation. 30µl extracted RNA was mixed with 5µl of 10xDNAseI reaction buffer and 5µl DNAse (Ambion) in a total volume of 50 µl and incubated at 37°C for 30min. The enzyme was inactivated by addition of 5µl of 25mM EDTA and incubation at 70°C for 10min.

c. *Reverse transcription (RT)*

A mix of 1µg of RNA and 1µl Oligo-(dT) (500µg/ml) primers were incubated at 65°C for 5min to denature the secondary RNA structure and chilled on ice for 2min to allow annealing of the primers to the RNA. A mix of 1 µl (20U/µl) of SUPERSCRIPT IIaa (Invitrogen), 4 µl of 5X First-Strand Buffer, 1 µl 10 mM dNTP mix, 1 µl RNaseOUT Recombinant Ribonuclease Inhibitor (40 U/µl) and 2 µl 0.1 M DTT were added. The samples were incubated for 50min at 42°C for cDNA synthesis in a total volume of 20µl. The reaction was inactivated by heating at 70°C for 15 min. cDNA was then subjected a template for amplification by PCR.

### *3. Proteins methods*

a. *Protein expression and purification*

WT and mutated proteins were expressed as 6xHIS tagged recombinant proteins in *Escherichia coli* cells (Rosetta strain). Cell cultures were grown in volumes of 0.1 to 0.3 liters at 37°C until they reached an A600 of 0.5 to 0.6. Protein expression was induced over-night at 25°C using 0.1mM Isopropyl β-D-1-thiogalactopyranoside. The cells were spin down by centrifugation and stored at -20°C. An aliquot of 200µl was pelleted in the same way and resuspended in Laemmli buffer to be run on an SDS-PAGE gel to verify the induction. For the purification, the frozen pellet was gently thawed on ice and resuspended in the 3 mL lysis buffer. Then the cells were lysed by sonication (6 times 10s with 10s of ice incubation in between), the lysate was centrifuged 10,000×g for 20 min at 4°C. The supernatant containing the soluble

protein was incubated for 30 min with 2 ml of cobalt resin (Thermo Scientific), equilibrated with lysis buffer, for binding. The matrix were washed 2 times with 1 mL washing buffer and eluted with from the columns with elution buffer. The different elution fractions were runned on an SDS-gel to check whether the protein was purified and the most concentrated protein fraction was then dialyzed overnight using dialysis cassette (Slide-A-Lyser®, Thermo Scientific) against dialysis buffer. Dialysis step was repeated the second day in a fresh buffer for 2 hours. After dialysis protein concentration was determined with Bradford assay. Most of the proteins were 90-95% pure as determined by SDS-PAGE.

b. *Total protein extracts of Arabidopsis thaliana plant material*

*Lacus extraction*

Grinded frozen plant materials (100-200mg) were resuspended in 200µl lacus buffer and left on ice before homogenizing by inverting the tubes for few seconds. The samples were afterwards centrifuged at 14000 rpm for 5min at 4°C. The supernatant was transferred to a new vial and the operation was repeated to have a clear lysate. Then protein concentration was measured by Bradford in order to adjust the protein concentration of all samples to 100µg for pull down experiments and 50µg for western blot analysis.

Lacus protein extraction was also carried for on yeast cells. For that purpose a 500 µl overnight yeast culture (started from a single colony) was diluted in 5ml appropriate YNB selective media and grown for 5-6 hours. Yeast cells were then spun down for 5mins at 6000 rpm and 4°C. The pellet was resuspended with 300µl of lacus buffer and then 0.3ml of glass beads were added to help grinding the cells by vortexing (10 x 20s maw speed votexing keeping the cells on ice in-between). 200µl of the supernatant cleared off beads and cell debrits was transferred in a new tube. Protein concentration was determined like cited before.

*Laemmli extraction*

Crude total protein extraction for SDS-PAGE gel analysis or western blot was realized using laemmli buffer extraction. 100mg of the frozen grinded samples were resuspended in adequate volume of laemmli (dependant on the material quantity and the laemmli concentration) and homogenized before centrifuging and retrieving the supernatant for further use. The samples were stored at -20°C for a monthes.

c. *Western blotting*

After running a protein extract on an SDA-PAGE gel, proteins were transferred on a PVDF membrane (Amersham Hybond$^{TM}$-P, GE Healthcare) according to Amersham and BioRAD recommendations. After transfer, the membrane was incubated in blocking solution (TBS-T with 5% milk powder or 3% of BSA) at room temperature for at least one hour. The first antibody was applied in a new blocking solution and incubated overnight at RT and very gentle shaking. Non-specifically bound antibody was removed by washing the 4 times the membrane with a TBS-T solution (4x10min). Then the secondary antibody was added for 1h at room temperature. After, the membrane was washed 4x10min with TBS-T solution. For detection the chemiluminescence of peroxidise product with the ECL Plus western blotting detection system reagent (GE Healthcare) was detected after 10min up to 1hour in a GeneGnome chemiluminsescence imaging system.

d. *In vitro phosphorylation assays*

*Phosphorylation assays after immunoprecipitation (IP)*

Protein Immunoprecipitation (IP) was performed from fresh protein extract in laccus as described above. 100µg of total protein extract were incubated with 30µl of a 50% slurry of Suc1-washed Protein A beads (Amersham) and 1µl antibody for 2h at 4°C. The beads were washed 3 times with SucI buffer, once with kinase buffer, and sucked dry with a syringe. The kinase assay reactions were performed by adding 16µl of reaction mix consisting of: 15 µl kinase buffer with 0,1µl 100mM DTT, 0,1µl 10mM ATP, 0,2µl $\gamma$-$^{32}$P-ATP (1µCi) and 1µl myelin basic protein (MBP, 10mg/ml) on the IPed proteins. The reactions were incubated for 30min at room temperature and stopped with 4x laemmli buffer and a 5min boiling. 10µl of the kinase reactions were used for SDS-PAGE on a 15% SDS-PAGE gel. Once run, the gel was stained with coomassie, destained and dried using a gel dryer (Biorad, model 583). After exposing the gel to a phosphoimager cassette (Amersham), the kinase activity was detected by autoradiography using a Storm detection system (Amersham, model 820).

*Phosphorylation assays with purified fusion proteins*

Phosphorylation assays with purified fusion proteins were performed in a total volume of 20µl with 14.6µl kinase buffer, 0,1µl 100mM DTT, 0,1µl 10mM ATP, 0,2µl γ-$^{32}$P-ATP (1µCi) and 1µl myelin basic protein (MBP, 10mg/ml). The reaction was incubated for 30min at room temperature and the samples treated like described above.

Phosphorylation assays in peptide chips were performed according to the protocols cited in (Turk *et al.*, 2007; Vlad *et al.*, 2008).

e. *Protein structure*

MPK6 structure was predicted from the crystallized structure of ERK2 (PDB-ID: 2erkA) using SWISS-MODEL (http://swissmodel.expasy.org) and visualized using SWISS-pdb viewer.

***4. Yeast methods***

a. *Yeast transformation*

Competent yeast cells were prepared fresh for each transformation. An overnight culture was diluted to $OD_{600}$=0.4 in fresh YPD media and grown up for 4 hours. Cells were spin down at 2500rpm for 5min at room temperature, resuspended in 40ml of TE buffer and spin down again. Cells was resuspended in 2ml LiAc/0.5xTE mix. The cells were then incubated at RT for 10min before use. For each transformation 1µg of plasmid DNA was mixed with 100µg of denaturated (after incubation at 95° for 5min) sheared salmon sperm DNA and 100µl of yeast suspension. 700µl of 40% PEG-3350/TE mix were added and mixed very well by vortexing. Cells were incubated for 30min at 30°C, followed by 7min heat shock at 42°C after adding 88µl of DMSO. To remove the PEG, the cells were spun down at 2500rpm for 10s and washed with 1ml of TE buffer then resuspended in 50-100µl of TE before plated on selective plates.

b. *Yeast screen for gain of function MAPK*

MPK6 ORFs were recombined in pDR195gtw to generate pDR195gtw-MPK6. The library of MPK6 mutants was produced using a PCR mutagenesis kit (Mutazyme® II DNA polymerase, Stratagene). A BamH1 linearised pDR195 plasmid carrying "autotrophy gene" together with purified PCR fragments of MPK6 were transformed into *hog1Δpbs2Δ* yeast competent cells. By homologous recombination, the plasmid is reconstructed in the yeast. The transformed cells using the protocol detail before, were plated on non selective medium for the first steps and then on appropriate selective media, SC-Uracil to only select yeast colonies that carry the reconstructed plasmid and grown at 30°C for 48h. To screen for putative auto-active MPK6 yeast colonies were replicated in liquid YPD media and then dropped on NaCl (400-500mM) supplemented solid medium and left to grow for at least 2 days at 30°C.

c. *Yeast two hybrid (Y2H) analysis for protein-protein interaction*

Genes of interest were recombined from Entry vectors to pDEST22 for GAL4-AD fusion or pDEST32 for GAL4-BD fusion. MAV203 yeast cells were co-transformed with 5µl of each plasmid DNA and transformed colonies selected on agar medium lacking tryptophan and leucine. Single colonies were cultured in 500 µL of the same selection liquid medium over night at 30°C. Cells were diluted 200 times in water and 5 uL droplets were spotted on agar plates containing selective medium lacking leucine, tryptophane and histidine and supplemented with 30, 65 or 100mM 3-AT (3-Amino-1,2,4-triazole). Growth was assayed during one week (30°C).

### 5. Plant methods

a. *Generation of stable transgenic lines by floral dip*

*Agrobacterium tumefaciens* transformed with pGREEN0229-MPK4L-PC2-like vectors was grown in 5mL LB and used to inoculate 250ml LB with antibiotic and grown for 5h. The bacteria were afterwards spun down (5000rpm, 15min, room temperature) and resuspended in 250ml freshly prepared 5% sucrose solution. 0.5% of Silwet L-77 (Lehle Seeds, USA) was

added, the solution filled into a beaker and a plant in early flowering stage was soaked in the bacterial solution for about 2min (Clough & Bent 1998). The plants were covered overnight in darkness to maintain high humidity. Dry seeds were harvested, and transformants were selected on soil after Basta spraying. These $T_1$ –transformants were proliferated, and the seeds of the T2-generation were screened for homozygous transformant lines by Mendelian segregation analysis.

b. *Arabidopsis seed sterilisation*

For all *in vitro* growth assays Arabidopsis seeds were sterilized. Seeds were placed in 1.5 eppendorf tube, supplemented by 1ml of 70%EtOH 0,05% Triton X-100 and left 15min in agitation . Seeds were washed in 1ml of 95% EtOH 2 times. EtOH was removed by pipetting and dried further for about 2h under the hood.

c. *Stress treatment for kinase assays*

Depending on the assay 2 weeks old seedlings were grown on ½ MS agar plates under sterile conditions and were transferred to liquid ½ MS medium and left at room temperature for at least 6-8 hour to equilibrate. This method was adopted to grow plantlets for growth inhibition assay experiments and to generate plant material for RNA extraction. For the other assays, seedlings were grown directly on liquid MS medium under the same conditions. flg22 treatment was done by removing the media carefully with a pipette and adding 4ml of fresh MS with 500nM of flg22 peptide.

d. *Lactophenol Trypan blue stainings*

Lactophenol trypan blue staining was used to visualize plant cell death after P. *syringae* infection as well as H. *parasitica* mycelium and necrotic plant tissue. For these assays 2-3 adult leaves or 3 weeks old plantlets, depending on the assay were collected in a 15ml tube, covered with lactophenol trypan blue solution and placed in boiling water bath for 2min. *Trypan* solution was then removed and replaced by chloral hydrate solution and incubated over night for

destaining. Cleared leaves were then left in 70% glycerol for at least 3hrs before mounting onto glass microscope slides with 70% glycerol and examined using light microscope.

e. *Pathogen assays*

*Pseudomonas syringae growth assay*

*P. syringae* pv. *tomato* virulent and avirulent DC3000 strains used (described in 2.1.2) were maintained by streaking onto selective media NYG agar plates and incubation at 28°C for 48hrs. *P. syringae* innoculations were performed on 4-5 weeks-old plant, raised under short day conditions, by spray inoculation with a bacterial suspension of 0,2 (circa 1x $10^7$ colony forming units(cfu)/ml) in 10mM $MgCl_2$ supplemented with 0.04% Siwet L-77. The plants were then covered with a cloche to ensure high humidity for 1 day and replaced in the growth chamber.

*In planta* bacterial titers were determined at two time points, two hours and day 3 after infection. For that purpose leaf discs from infected plants were inoculated in 1ml of 10mM $MgCl_2$/0.01% Silwet L-77 and left for 1h of shaking at 28°C to extract the bacteria (Tornero & Dangle, 2001). The resulting bacterial suspensions were serially diluted (Day0: 0- $10^{-1}$, $10^{-2}$/ Day3: $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$) and spots of 20µl per dilution were grown on appropriate selective NYG agar media at 28°C. After two days the colony forming units (cfu) were counted.

*Alternaria brassicicola infection*

The infection was performed according to the instruction of Elke Stein (IPAZ, Giessen). *Arbidopsis* seeds were germinated and grown for 5 weeks at 22°C with 150µmol/$m^2$/s light in 8/16h day/night photoperiod. About 30 leaves/replicate were detached and were placed in agar plates (with petioles in the agar to prevent leaves of moving). 5µl of *Alternaria* spore suspension ca. 5x$10^5$ spores/ml, spores washed of from a fully grown PDA-plate, kept at 4°C to increase sporulation. The infected leaves were kept in closed square petri dishes under high humidity (circa 100%). Visual evaluation of the *Alternaria* sensitivity was carried out at 7dpi.

*Hyaloperonospora parasitica infection*

The infection was performed according to the instruction of Marie Garmier (IBP, Orsay). H. *parasitica* isolates were maintained as mass conidiosporangia cultures on leaves of susceptible *Arabidopsis* ecotype over a 7 days cycle. H. *parasitica* inoculations were done on 2

weeks-old plants by spray innoculation with a conidiospore solution at $4x10^4$ spores/ml. Plant cell necrosis and development of H. *parasitica* hyphae in leaf tissue was monitored by lactophenol trypan blue staining 6 days after infection.

f. *Salicylic Acid hormone monitoring*

SA measurement was realized on spray inoculated plants as described previously (*Pseudomonas syringae* growth assay). About 300mg of plant material (leaves) were collected from 4 plants at least for each biological replicate. The extraction and mesurment of SA were performed in collaboration with Rozhon W. according to his protocol (Rozhon *et al.,* 2005). The only exception from the protocol described in the publication in the addition of 10µM of EDTA at the HPLC eluent.

# *Chapter III*

# *Identification of CA MPK6 mutants*

# ***Chapter III - Identification and characterization of Constitutively Active (CA) Arabidopsis MPK6 mutants***

In this chapter, I describe the identification of candidate mutations in Arabidopsis MPK6 which could trigger kinase constitutive activity. This identification was based on an adaptation of a functional genetic screen in the yeast *Saccharomyces cerevisiae* developed from Engelberg and co-workers (Bell *et al.,* 2001) (Figure III.1). In a second step, I bring direct evidences that the mutated MPK6 are constitutively active.

## ***A. Functional complementation screen of the yeast pbs2Δhog1Δ allows the identification of Constitutively Active (CA) forms of Arabidopsis MPK6***

### ***1. HOG1, a well characterized dispensable yeast MAPK pathway that provides a nice system to build a screen***

Yeast possesses three MAPKs in (Fus3, Kss1 and Mpk1/Slt2) which belong to the ERK subfamily and one MAPK (Hog1) which belongs to the SAPKs (Stress Activated Protein Kinase) (De Nadal *et al.,* 2002). One of the most well characterized yeast MAPK cascade is the Hog1 pathway which is activated after perception of extracellular hyper-osmolarity. This pathway consists of the MAPK module Ste11-Pbs2-Hog1 which is recruited on high external osmolarity or high salt concentration. The MAP2K Pbs2 activation by the MAP3K Ste11 depends on other protein kinase partners like Ste50, Ste20 and the Cdc42 GTPase (De Nadal *et al.,* 2002). However the most important player in the osmoadaptation response in yeast remains Pbs2 and Hog1 because Pbs2 is also directly activated by the transmembrane protein Sho1. Mutants of the HOG1 pathway could grow under normal conditions but are unable to develop under high osmotic conditions. Interestingly, the phenotype of *hog1*Δ cells (sensitivity to osmotic stress) could be rescued by MAPKs from various plants, animal, fungi and other yeasts (Popping *et al.,* 1996; Teige *et al.,* 2004). Our laboratory has previously shown that *Arabidopsis* MPK4 and MPK6 together with MKK2 rescue the salt sensitivity of the yeast *pbs2*Δ*hog1*Δ strain (Teige *et al.,* 2004). The fact that MPK6 or MPK4 without MKK2 does not restore the growth of

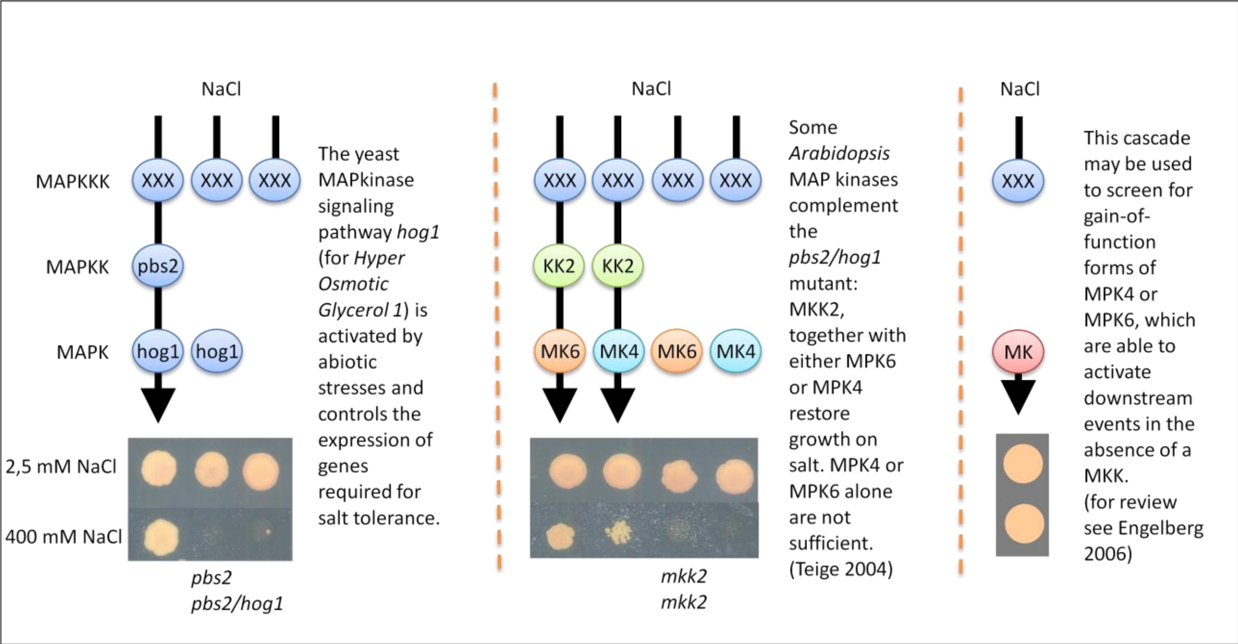
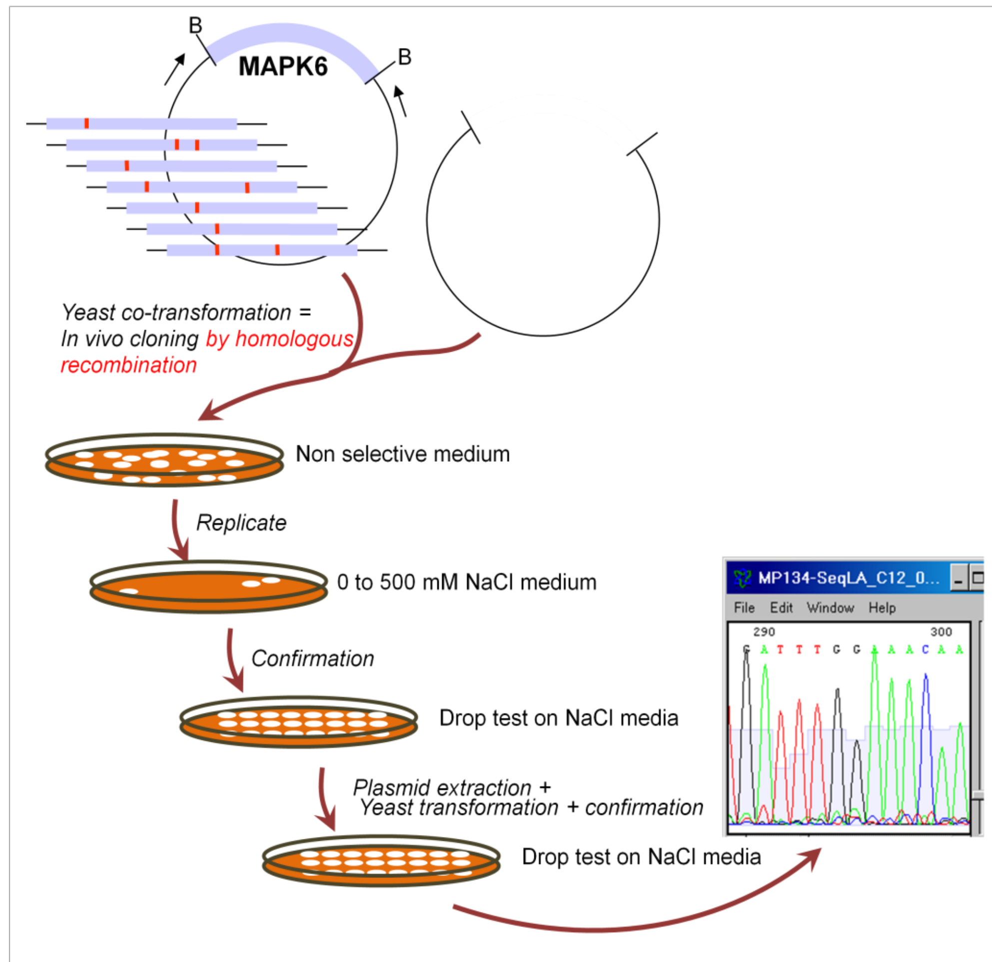



**Figure III.1: General scheme of the yeast *pbs2∆hog1∆* complementation and rationale of the genetic screen:** Arabidopsis MAP Kinases (MPK4, MPK6 and MKK2) can complement the yeast *pbs2-hog1* double-mutant.

**A**




**B**

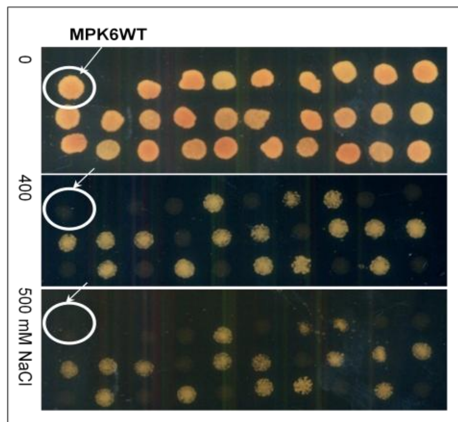



**Figure III.2: Identification of MPK6 candidates able to complement *pbs2Δhog1Δ* on salt. A.** Screening protocol for MPK6 candidates which complement *pbs2Δhog1Δ* on salt: *MPK6* mutated PCR fragment were cloned by *in vivo* recombination in the pDR195 BamH1 linearised vector. **B.** Some of *pbs2Δhog1Δ* yeast expressing *MPK6* CA candidate clone at the confirmation step are able to rescue the mutant growth phenotype on Salt (YPD media supplemented with 400mM NaCl).

*pbs2Δhog1Δ* on salt conditions, suggests that MPK6 or MPK4 could not be activated by salt stress without an upstream activator. In this study we took advantage of the yeast MAPK cascade that is highly similar to its mammalian or plant counterparts (Caffrey *et al.,* 1999).

### *2. The complementation screen allowed the identification of 3 Sets of CA candidate mutations*

We used a PCR strategy followed by *in vivo* recombination to generate a population of *pbs2Δhog1Δ* yeasts expressing a randomly mutated *MPK6* ORF (Figure III.2A and Materiel & Methods). As expected, when replicated on 400 mM NaCl, only very few colonies were able to grow: they putatively express an MPK6 protein mutated in residues controlling its activity (Figure III.2B). To confirm the link between the growth phenotype and the mutated MPK6 ORF, we re-extracted the plasmids and re-transformed them in *pbs2Δhog1Δ* double-mutant to double check the growth on salt medium. We finally selected 27 clones carrying constitutively active MPK6 candidates. Sequence analysis revealed between 2 and 10 mutations in the *MPK6* ORF corresponding to 1 to 6 amino acid residue changes (Table III.1). Except for one case, each clone was found to contain more than one single mutation in the coding sequence of *MPK6*.

The mutations Y144C, Y144F and R274H were identified, associated with other mutations, in 12, 9 and 4 clones respectively, indicating that they are important for the yeast complementation and suggesting that our screen is saturated for single mutants. Only two clones did not exhibit any of these three mutations meaning that their ability to complement the yeast growth defect on salt is linked to several combined important mutations.

### *3. The complementation of pbs2Δhog1Δ is linked to an increase of MPK6 activity*

To check whether the ability of the mutant candidates to rescue *pbs2Δhog1Δ* is a result of an increased kinase activity, we measured the kinase activity of MPK6 mutants expressed in *pbs2Δhog1Δ* yeast cells. We choose among the candidate clones able to complement *pbs2Δhog1Δ* under salt stress conditions, the ones that contain the main mutations Y144C, Y144F and R274H found in the screen. We also included a clone without any of these three mutations (clone #8). The MPK6 mutant proteins were immuno-precipitated from the yeast cells

**Table III.1: List of MPK6 mutations triggering amino acid exchanges able to complement *pbs2∆hog1∆* under salt stress conditions.**

| Clone number | Protein mutations |
|---|---|
| 1 | G43R;K112M;Y144F;S217G |
| 2 | Y144C;L387H;Q394L |
| 3 | Y144C |
| 4 | C78S;Y144C |
| 5 | D118N;Y144F;F219I |
| 6 | P68L;Y144C |
| 7 | P37L;Y144F;C360Y;H371Y |
| 8 | A9V;D218G;E222A |
| 9 | R109C;L133I;Y144F;F364L |
| 10 | F98L;R274H;P319H;F364I |
| 11 | S5P;A136P;Y144C |
| 12 | D103Y;Y144C |
| 13 | K112R;Y144C |
| 14 | H42Q;D240N;R274H;L276H;C360R |
| 15 | H116N;N237I;R274H |
| 16 | E34V;N138S;Y144F;V250L;F364L |
| 17 | K112T;Y144F |
| 18 | D268E;R274H;N294K;T361I |
| 19 | A97T;H116L;Y144C |
| 20 | E34V;D99A;Y144C;N349D;H371Q;I382S |
| 21 | Y144C;I299M;S309P |
| 22 | I69L;R267Q;D268N;E287K;H318N;F366L |
| 23 | N54I;Y144F;C360Y |
| 24 | A25T;I47V;I51M;I111V;Y144F;H165Y |
| 25 | F21Y;Y144F;K327R;P363S;F366I |
| 26 | N96T;Y144C;F366S |
| 27 | C78S;Y144C |

using anti-MPK6 specific antibody. They were then subjected to a kinase activity assay to test their ability to phosphorylate the Myelin Basic Protein (MBP), a common heterologous substrate for MAPKs. In these conditions, the activity of wild type (WT) MPK6 was barely detectable whereas all MPK6 mutants phosphorylated efficiently the MBP (Figure III.3). This experiment suggests that the *pbs2Δhog1Δ* growth rescue is linked to an increased MPK6 activity, as all the mutants forms tested have an important kinase activity compared to WT. It also shows that the mutations Y144C, Y144F, and R274H might trigger such increase.

## B. CA MPK6 candidates have an MAP2K-independent increased kinase activity

As I mentioned above, yeast cells have several MAPK cascades. The increased MPK6 activity of the clones found to be able to rescue *pbs2Δhog1Δ* growth on salt, might also be the consequence of MPK6 activation by yeast other endogenous MAP2Ks. If candidate mutations would improve the recognition of the MAPK by unrelated MAP2Ks, MPK6 mutants will not be really intrinsically active. To bring more evidences of this constitutive activity, we choose to express MPK6 candidates in *Escherichia coli* cells, a system lacking MAPK cascades and particularly MAP2Ks.

Sequence analysis of the clones able to complement the yeast mutant growth defect on salt allowed us to identify Y144 and R274 as important residues for the kinase activity: mutations of these residues were found in a majority of candidate clones (21/27 and 4/27 respectively) (Table III.1). One of the two remaining clones, clone #08, appeared to be also very promising: MPK6 showed three mutations, one at the ninth amino acid residue (A9V) in the N-terminal part which is a not conserved MAPK domain, and two mutations in the activation loop, D218G and E222A. Based on knowledge on MAPK activating mutations in yeast and mammals, no activating mutation occurred in the first amino acid residues of MAPK, I speculated then that the A9V mutation could not have an effect on the autoactivity of MPK6. For the further characterization steps, I used the double mutant MPK6 form carrying the D218G and E222A mutations.

Sequence alignment of *Arabidopsis* MAPKs showed that residues near the phosphorylation site of the kinase, D218 and E222, together with the tyrosine residue at the position 144 in

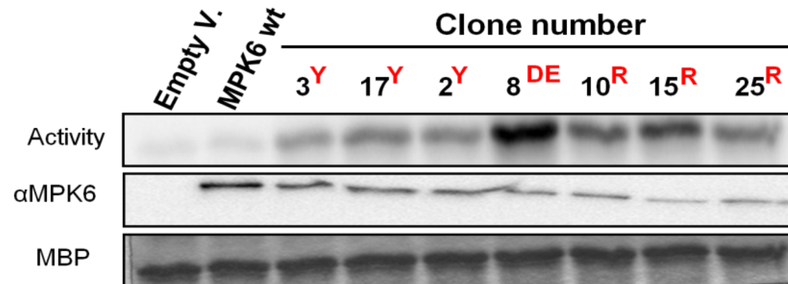



**Figure III.3: Kinase activity of some MPK6 CA candidate mutants expressed in yeast cells:** Kinase activity after immuno-precipitation from complemented *pbs2*Δ*hog1*Δ mutant yeast cells of MPK6 WT and of some putative gain of function clones. Clone's numbers refer to table 1. The main mutation in each clone in written with a red letter (Y: Y144C/F, DE: D218G/E22A, R: R274H.

```
                 144
MPK3      DVYISTELMDTDLHQIIRSNQSLSEEHCQYFLYQLLRGLKYIHSANIIHRDLKPSNLLLN 173
MPK6      DVYIAYELMDTDLHQIIRSNQALSEEHCQYFLYQILRGLKYIHSANVLHRDLKPSNLLLN 198
MPK10     DVYIVNELMEFDLYRTLKSDQELTKDHGMYFMYQILRGLKYIHSANVLHRDLKPSNLLLS 195

MPK4      DVYIVYELMDTDLHQIIRSNQPLTDDHCRFFLYQLLRGLKYVHSANVLHRDLKPSNLLLN 178
MPK11     DVHIVYELMDTDLHHIIRSNQPLTDDHSRFFLYQLLRGLKYVHSANVLHRDLKPSNLLLN 175
MPK12     DVYIVYELMDTDLQRILRSNQTLTSDQCRFLVYQLLRGLKYVHSANILHRDLRPSNVLLN 176
MPK5      DVYIVFELMDTDLHQIIRSNQSLNDDHCQYFLYQILRGLKYIHSANVLHRDLKPSNLLLN 178
MPK13     DVYIVYELMDTDLHQIIRSTQTLTDDHCQYFLYQILRGLKYIHSANVLHRDLKPSNLVLN 168

MPK1      DVYLVYELMDTDLHQIIKSSQVLSNDHCQYFLFQLLRGLKYIHSANILHRDLKPGNLLVN 167
MPK2      DVYLVYELMDTDLHQIIKSSQVLSNDHCQYFLFQLLRGLKYIHSANILHRDLKPGNLLVN 167
MPK7      DVYLVYELMDTDLHQIIKSSQSLSDDHCKYFLFQLLRGLKYLHSANILHRDLKPGNLLVN 167
MPK14     DVYLVYELMDSDLNQIIKSSQSLSDDHCKYFLFQLLRGLKYLHSANILHRDLKPGNLLVN 167

MPK8      DIYVVFELMESDLHQVIKANDDLTPEHYQFFLYQLLRGLKYVHAANVFHRDLKPKNILAN 239
MPK15     DVYVVFELMESDLHQVIKANDDLTPEHHQFFLYQLLRGLKYVHAANVFHRDLKPKNILAN 225
MPK9      DIYVVFELMESDLHQVIKANDDLTPEHYQFFLYQLLRGLKFIHTANVFHRDLKPKNILAN 158
MPK17     DIYVVFELMESDLHHVLKVNDDLTPQHHQFFLYQLLRGLKFMHSAHVFHRDLKPKNILAN 151
MPK16     DIYVVFELMESDLHQVIKANDDLTPEHYQFFLYQLLRGLKYIHTANVFHRDLKPKNILAN 160
MPK18     DIYVVFELMESDLHQVIKANDDLTREHHQFFLYQMLRALKFMHTANVYHRDLKPKNILAN 160
MPK19     DIYVVFELMESDLHQVIKANDDLTREHHQFFLYQMLRALKYMHTANVYHRDLKPKNILAN 148
MPK20     DIYVVFELMESDLHQVIKANDDLTREHYQFFLYQLLRALKYIHTANVYHRDLKPKNILAN 160
          *:::  ***: ** : ::  : *. ::  ::::*:**.**::*:*:: ****:* *:: .

                                     218 222
MPK3      ANCDLKICDFGLARPTS----ENDFMTEYVVTRWYRAPELLLNS-SDYTAAIDVWSVGCI 228
MPK6      ANCDLKICDFGLARVTS----ESDFMTEYVVTRWYRAPELLLNS-SDYTAAIDVWSVGCI 253
MPK10     TQCDLKICDFGLARATP----ESNLMTEYVVTRWYRAPELLLGS-SDYTAAIDVWSVGCI 250

MPK4      ANCDLKLGDFGLARTKS----ETDFMTEYVVTRWYRAPELLLNC-SEYTAAIDIWSVGCI 233
MPK11     ANCDLKIGDFGLARTKS----ETDFMTEYVVTRWYRAPELLLNC-SEYTAAIDIWSVGCI 230
MPK12     SKNELKIGDFGLARTTS----DTDFMTEYVVTRWYRAPELLLNC-SEYTAAIDIWSVGCI 231
MPK5      SNCDLKITDFGLARTTS----ETEYMTEYVVTRWYRAPELLLNS-SEYTSAIDVWSVGCI 233
MPK13     TNCDLKICDFGLARTSN----ETEIMTEYVVTRWYRAPELLLNS-SEYTGAIDIWSVGCI 223

MPK1      ANCDLKICDFGLARASNT---KGQFMTEYVVTRWYRAPELLLCC-DNYGTSIDVWSVGCI 223
MPK2      ANCDLKICDFGLARTSNT---KGQFMTEYVVTRWYRAPELLLCC-DNYGTSIDVWSVGCI 223
MPK7      ANCDLKICDFGLARTSQG---NEQFMTEYVVTRWYRAPELLLCC-DNYGTSIDVWSVGCI 223
MPK14     ANCDLKICDFGLART------YEQFMTEYVVTRWYRAPELLLCC-DNYGTSIDVWSVGCI 220

MPK8      ADCKLKICDFGLARVSFNDAPTAIFWTDYVATRWYRAPELCGSFFSKYTPAIDIWSVGCI 299
MPK15     ADCKLKICDFGLARVSFNDAPTAIFWTDYVATRWYRAPELCGSFFSKYTPAIDIWSVGCI 285
MPK9      SDCKLKICDFGLARVSFNDAPSAIFWTDYVATRWYRAPELCGSFFSKYTPAIDIWSIGCI 218
MPK17     ADCKIKICDLGLARVSFTDSPSAVFWTDYVATRWYRAPELCGSFYSNYTPAIDMWSVGCI 211
MPK16     ADCKLKICDFGLARVAFNDTPTAIFWTDYVATRWYRAPELCGSFFSKYTPAIDIWSIGCI 220
MPK18     ANCKLKVCDFGLARVAFNDTPTTVFWTDYVATRWYRAPELCGSFFSKYTPAIDVWSIGCI 220
MPK19     ANCKLKVCDFGLARVSFNDTPTTVFWTDYVATRWYRAPELCGSFCSKYTPAIDIWSIGCI 208
MPK20     ANCKLKICDFGLARVAFNDTPTTIFWTDYVATRWYRAPELCGSFYSKYTPAIDIWSIGCI 220
          :.  .:*: *:****             *:**.*********     ..*  :**:**:***
```

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                                   274
MAPK3      FMELMNRKPLFPGKDHVHQMRLLTELLGTPTESDLGFTHNEDAKRYIRQLPNFPRQPLAK 288
MAPK6      FMELMDRKPLFPGRDHVHQLRLLMELIGTPSEEELEFLN-ENAKRYIRQLPPYPRQSITD 312
MAPK10     FMEIMNREPLFPGKDQVNQLRLLLELIGTPSEEELG-SLSEYAKRYIRQLPTLPRQSFTE 309

MAPK4      LGETMTREPLFPGKDYVHQLRLITELIGSPDDSSLGFLRSDNARRYVRQLPQYPRQNFAA 293
MAPK11     LGEIMTREPLFPGRDYVQQLRLITEVN--------------------------------- 257
MAPK12     LGEIMTGQPLFPGKDYVHQLRLITELVGSPDNSSLGFLRSDNARRYVRQLPRYPKQQFAA 291
MAPK5      FAEIMTREPLFPGKDYVHQLKLITELIGSPDGASLEFLRSANARKYVKELPKFPRQNFSA 293
MAPK13     FMEILRRETLFPGKDYVQQLKLITEVS--------------------------------- 250

MAPK1      FAELLGRKPIFQGTECLNQLKLIVNILGSQREEDLEFIDNPKAKRYIRSLPYSPGMSLSR 283
MAPK2      FAELLGRKPVFPGTECLNQIKLIINILGSQREEDLEFIDNPKAKRYIESLPYSPGISFSR 283
MAPK7      FAEILGRKPIFPGTECLNQLKLIINVVGSQQESDIRFIDNPKARRFIKSLPYSRGTHLSN 283
MAPK14     FAEILGRKPIFPGTECLNQLKLIINVVGSQQDWDLQFIDNQKARRFIKSLPFSKGTHFSH 280


MAPK8      FAEMLLGKPLFPGKNVVHQLDLMTDFLGTPPPESISRIRNEKARRYLSSMRKKQPVPFSH 359
MAPK15     FAEMLLGKPLFPGKNVVHQLDIMTDFLGTPPPEAISKIRNDKARRYLGNMRKKQPVPFSK 345
MAPK9      FAEMLTGKPLFPGKNVVHQLDIMTDLLGTPPPEAIARIRNEKARRYLGNMRRKPPVPFTH 278
MAPK17     FAEMLTGKPLFPGKNVVHQLELVTDLLGTPSPITLSRIRNEKARKYLGNMRRKDPVPFTH 271
MAPK16     FAELLTGKPLFPGKNVVHQLDLMTDMLGTPSAEAIGRVRNEKARRYLSSMRKKKPIPFSH 280
MAPK18     FAEVLTGKPLFPGKSVVHQLELITDLLGTPKSETISGVRNDKARKYLTEMRKKNPVTFSQ 280
MAPK19     FAEVLTGKPLFPGKSVVHQLDLITDLLGTPKSETIAGVRNEKARKYLNEMRKKNLVPFSQ 268
MAPK20     FAEVLMGKPLFPGKNVVHQLDLMTDLLGTPSLDTISRVRNEKARRYLTSMRKKPPIPFAQ 280
           : * :  :.:* * . ::*: :: :.
```

**Figure III.4: Local alignments of the 20 *Arabidopsis thaliana* MAPKs:** The residue at equivalent position 144, 274, 218 and 222 in MPK6 is highlighted in yellow, green and blue respectively. MAPKs are presented according to their group, Group A, B, C and D respectively, with a line spacer in between in the alignment.

MPK6 are quite conserved (Figure III.4). Moreover they show an important catalytic activity after immuno-precipitation from yeast, we choose then to focus on Y144C, D218G/E222A mutations for further characterization. We generated by site-directed PCR the double mutant version of MPK6, $MPK6^{D218G/E222A}$ without the additional A9V mutation. The mutated gene of *MPK6*, $MPK6^{Y144C}$ and $MPK6^{D218G/E222A}$ as well as the WT gene were cloned in a protein expression vector. The single mutated genes, $MPK6^{D218G}$ and $MPK6^{E222A}$, were also created and produced in the same system in order to understand the contribution of each mutation to in the MPK6 activity. These kinases were then expressed as a hexahistidine-tagged protein in *Escherichia coli* cells, and purified using cobalt resin.

$MPK6^{WT}$ did not show a significant kinase activity on MBP (Figure III.5A). In contrast $MPK6^{Y144C}$ and $MPK6^{D218G/E222A}$ were able to phosphorylate MBP. Interestingly, the single mutants $MPK6^{D218G}$ and $MPK6^{E222A}$ did not exhibit any increased kinase activity, suggesting that the activity of $MPK6^{D218G/E222A}$ is not a result of an additive effect of each mutation, but rather of the synergic action of the two mutations together. Using *E. coli* as expression system, we demonstrated that the residues found in our screen and more specifically Y144C and D218G/E222A mutations are very important for activation of MPK6, and this activation is independent of MAP2Ks. Combining the three activating mutations (Y144C, D218G and E222A) in MPK6 kinase enhanced furthermore its activity by 2 fold compared to the activity reached by the Y144C single mutation and about 6 folds more than the double mutation D218G/E222A (Figure III.5B).

To have a precise idea on the position of the residues found to be important for MPK6 intrinsic activity, we used the secondary structure of the closest mammalian MAPK (87% protein sequence homology), ERK2 as a model for MPK6 (Figure III.6). It appears that Y144 is located in the ATP binding pocket, D218/E222 is located in the activation loop: E222 is the glutamic acid residue of the TEY phosphorylation motif, D218 is four amino acid upstream.

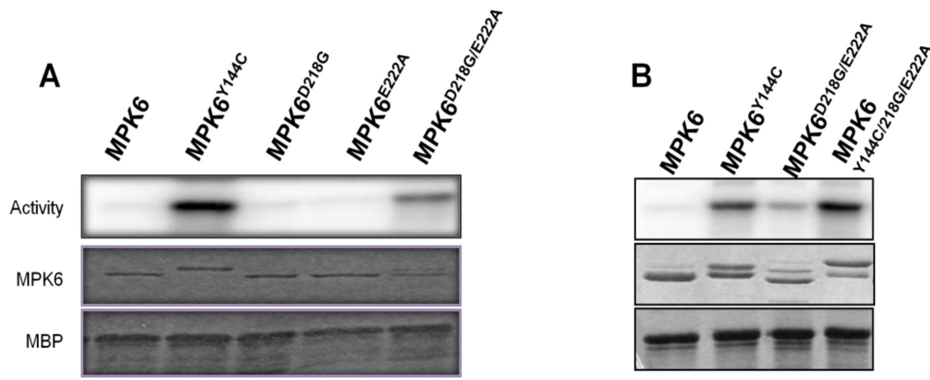



**Figure III.5: MPK6 mutants are active independently of MAP2K activation: A.** Kinase activity with purified (0.6µg) MPK6 WT and mutants identified in the yeast complementation screen. **B.** Kinase activity of MPK6 WT and combined CA mutations.

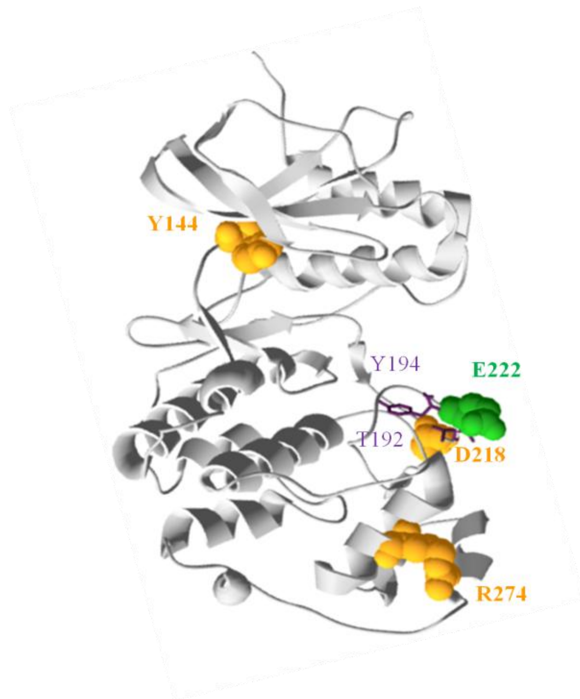



**Figure III.6: Positions of the mutations making MPK6 active based on the predicted 3D-structure of MPK6 on ERK2 model (Canagarajah *et al.*, 1997).**

## *C. **Discussion***

### *1. The rationale of choosing a yeast screen to fish up plant auto-activating mutations*

This part of my work describes a genetic screen in yeast adapted from the screen developed by Engelberg's group (Engelberg & Livnah 2006) to isolate active form of yeast MAPK. The basis of this screen is that only active forms of MAPK would induce the appropriate respective phenotype in a MAP2K null strain. The approach taken in this study is very stringent, because it screens for MAPKs which are active in the complete absence of their relevant MAP2K.

The main technical innovation in our screen compared to the one designed by Engelberg and co-workers comes from the use of a PCR technology to introduce mutations in the MAPK gene instead of using the bacterial strain of *E. coli* LE30. This strain contains the mutant allele *mutD5* triggering a proofreading defect of the DNA polymerase III and is only capable of introducing single mutation during the plasmid replication due to the low mutation frequency. The main advantage of our approach is that the level of mutations is far higher and better controlled. Frequency ranges between 1 to 10 mutations per Kb and all types of DNA mutations are supposed to be equi-probable. All positive clones are independent clones. These advantages clearly allowed us to identify double mutants. The limit of this kind of screen could be that the very active mutant might be lethal for the yeast, and subsequently lost in the screen (Yaakov *et al*., 2003).

No active plant MAPK mutants were published so far. Teige and co-workers had shown that the MAP2K MKK2 and the MAPKs MPK4 and MPK6 could replace Pbs2 and Hog1 in the double mutant *pbs2Δhog1Δ* (Teige *et al.,* 2004). A trial to complement the yeast double mutant *pbs2Δhog1Δ*, under salt stress conditions, by MPK4 with the CA mutation identified in the literature was not successful: mutant forms of MPK4 did not acquire an increased kinase activity when expressed in the yeast and do not complement the salt-dependent growth phenotype (*data not shown*). It was for that reasons that we decided to perform a de novo screen using *Arabidopsis* MPK6 because MPK6 complements more efficiently *pbs2Δhog1Δ* than MPK4.

```
MAPK6       ENIVAIRDIIPPP-LRNAFNDVYIAYELMDTDLHQIIRSNQ----ALSEEHCQYFLYQIL 174
FUS3        ENIITIFNIQRPD-SFENFNEVYIIQELMQTDLHRVISTQ-----MLSDDHIQYFIYQTL 122
Kss1p       ENIISILDKVRPV-SIDKLNAVYLVEELMETDLQKVINNQNSGFSTLSDDHVQYFTYQIL 128
ERK2        ENIIGINDIIRAP-TIEQMKDVYIVQDLMETDLYKLLKTQ-----HLSNDHICYFLYQIL 132
p38alpha    ENVIGLLDVFTPARSLEEFNDVYLVTHLMGADLNNIVKCQK-----LTDDHVQFLIYQIL 135
HOG1        ENLICLQDIFLSP-----LEDIYFVTELQGTDLHRLLQTRP-----LEKQFVQYFLYQIL 129
            **:: : :   .      :. :*:  .*  :** .::  .      * .:.  :: ** *
```

**Figure III.7: Local alignments of *Arabidopsis* MPK6 with animal (ERK2 and P38alpha) and yeasts MAPKs (FUS3, KSS1p and HOG1):** The homologous residues at position 144 in MPK6 are highlighted in pink.

### *2. We found important residues for MAP2K-independent MPK6 activity*

Using MPK6 to complement the yeast double mutant *pbs2*Δ*hog1*Δ resulted in 27 mutant candidates with around 60 mutations in total. We consider our screen as saturated for single mutations since some mutations were obtained several times, like the mutations of the Y144 residue which were identified in 21 independent clones. Moreover, this mutation alone on MPK6 was able to complement the yeast double mutant showing that this residue is very important for kinase auto-activity. Others residues that appears to be important for MPK6 activity are D218 and E222. The activity of the $MPK6^{Y144C}$ and $MPK6^{D218G/E222A}$ mutant proteins assayed *in vitro* showed enhanced MPK6 kinase activity. When expressed in a heterologous system that lacks MAPK signaling pathway component, we confirmed that the rescue of *pbs2*Δ*hog1*Δ is really due to the autophosphorylation of MPK6.

Because of the protein sequence homology between ERK2 and MPK6 (87% protein sequence homology), the secondary structure of ERK2 was used as a model to have a better view of the location of the residues found to be important for MPK6 activity. It appears then that Y144 residue is located in the ATP binding pocket, D218/E222 are located in the activation loop: E222 is the glutamic acid residue of the TEY phophorylation motif, D218 is 4 amino acid upstream.

Alignment of MPK6 with other MAPKs from yeast and animals (Figure III.7) revealed that Y144 is the homologous of ERK2 Q103 residue known as the gatekeeper residue (Emrick *et al.,* 2006). Structure-functions study of ERK2 already showed that mutations in the gatekeeper residue trigger kinase hyperactivity linked to an enhanced intramolecular autophosphorylation of the activation lip of ERK2 on the TEY motif. The gatekeeper residue is located in the nucleotide binding pocket and is involved in the binding selectivity for small-molecule inhibitors (Shah *et al.,* 1997). Evidences on the role of this particular residue on plant MAPK activity come also from the use of mutant version of *Arabidopsis* MPK4 Y124A/G to induce ATP binding pocket enlargement and to render the kinase more sensitive to specific inhibitor molecule (Brodersen *et al.,* 2006). Like in ERK2 the increased auto-activation in $MPK6^{Y144C}$ could be explained by the fact that this mutation changes a voluminous tyrosine residue to a small cysteine residue which could induce conformational changes in MPK6 protein structure making it more flexible and promoting then interactions with and auto-phosphorylation of the activation lip like in

ERK2$^{Q103A}$. We did not observe any stronger auto-phosphorylation of MPK6 CA mutants but this may be linked to technical limitations.

In my view, the most important MAPK activating mutation that came out in the screen we performed is the double mutation in the activation loop. Despite the fact that some mutations triggering hyperactivity were identified in the activation loop of human or yeast MAPK (Bell *et al.,* 2001; Diskin *et al.,* 2004), the combination of the mutations found to activate MPK6, D218G and E222A, was not shown so far. Surprisingly, these mutations convert acidic amino acid residues to neutral ones. This has an opposite effect to the modification of the activation loop which happens after the phophorylation of the kinase. A plausible scenario explaining why the MPK6 mutated in the activation loop is CA, could be that the small alanine and glycine residue that replace the D218 and E222 respectively introduces a break in the catalytic loop and enhances its flexibility, allowing autophosphorylation.

# *Chapter IV*

# *CA mutations activate other MAPKs*

# *Chapter IV - **Homologous mutations in other Arabidopsis MAPKs often render the kinase catalytically active***

In this chapter, I present the attempt to transfer the MPK6 CA mutations identified in the screen to other MAPKs. I will also try to point the particular different regulation of some MAPKs.

## *A. **Identification of residues to mutate in other MAPKs***

Our goal was to test whether mutations at the homologous residues in other MAPKs would render them hyperactive. Sequence alignment of the 20 *Arabidopsis* MAPK was performed to verify whether residues identified in our MPK6 screen were conserved (Figure III.4). At the homologous position of the MPK6 Y144, most of the MAP kinases of the groups A, B and C, have a conserved tyrosine. The notable exceptions are MPK3, MPK5 and MPK10 which have a threonine, phenylalanine and asparagine residues respectively. Interestingly, all MAPKs belonging to the atypical D group, have a phenylalanine residue at Y144 homologous position, a residue found in our screen to triggers hyperactivity in MPK6.

Although the amino acid sequence of the activation loop is not perfectly conserved, the homologous positions of the residues D218 and E222 were also identified for *Arabidopsis* MAPKs of the groups A, B and C. In many cases, the residue D218 (in MPK6) was not conserved, we rather found glutamine (Q), glutamic acid (E) or asparagine (N). The D-group MAPKs showed longer activation loop, making the identification of D218G and E222A MPK6 homologous residues uneasy.

As mention in the previous part, MPK6 R274 residue is not very well conserved therefore we did not work further on it.

## B. CA mutations often trigger MAP2K-independent kinase activity for MAPKs belonging to the groups A B and C

To test if the CA mutations were able to trigger CA activity for other MAPKs, we choose some members from each group, MPK3 and 6 from group A, MPK4 from group B and MPK7 from group C. We created the homologous mutated forms of $MPK6^{Y144C}$ and $MPK6^{D218G/E222A}$ in MPK3, MPK4 and MPK7. They correspond to $MPK3^{T119C}$, $MPK4^{Y124C}$ carrying the first mutation and to $MPK4^{D198G/E202A}$, $MPK3^{D193G/E197A}$ and $MPK7^{D188G/E192A}$ corresponding to the double mutant. The wild type and mutated forms of each kinase were expressed as a 6xHIS tagged proteins in the same expression vector used for MPK6. Since I had difficulties to purify the $MPK3^{D193G/E197A}$, we expressed it in the pDEST-periHIS-MBP vector known to help the good folding of recombinant proteins and to address them to the periplasm, which is an advantage in case of toxic protein for the bacteria (Nallamsetty *et al.,* 2005). All the MAPKs were then assayed for their kinase activity toward MBP. The mutant forms of these MAPKs acquired often increased intrinsic activity compared to their corresponding WT proteins (Figure IV.1) however they exhibit different level of phosphorylation, which is in some cases due to different protein amount: $MPK4^{Y124C}$ and $MPK4^{D198G/E202A}$ behave like MPK6 mutants (Figure IV.1A). $MPK7^{D188G/E192A}$ shows also increased kinase activity (Figure IV.1D). Surprisingly MPK3wt could remarkably phosphorylate MBP compared to the other WT MAPKs, however $MPK3^{T119C}$ did not show any increased intrinsic activity compared to its WT form (Figure IV.1A). This result might be explained by the threonine residue at the homologous position 144 instead of tyrosine in most of the other MAPKs (Figure III.4). The Peri-His-MBP-$MPK3^{D193G/E197A}$ was also tested for its activity, and was then compared to Peri-His-MBP-MPK3wt protein (Figure IV.1B). The $MPK3^{D193G/E197A}$ mutant acquired a considerable higher catalytic activity when compared to its corresponding WT protein.

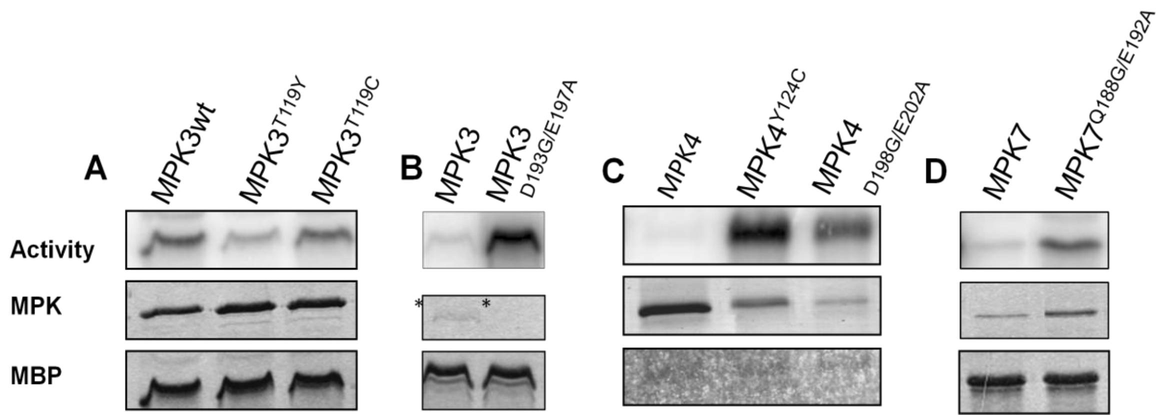



**Figure IV.1 : *In vitro* characterization of the CA mutations in other MAPKs:** MPK3 (A and B) MPK4 (C), MPK7 (D) and MPK12 (E) were produced as HIS tagged protein (B, C, E and D) or periHIS-MBP (C) and assayed as their ability to phosphorylate MBP.

## *C. Some MAPKs have residues allowing activity at homologous position Y144 of MPK6*

MPK3 but also MPK5, MPK10 and all MAPKs of the group D do not have a tyrosine residue at the homologous position Y144 of MPK6. As this residue position could be important to control the auto-activity, we wanted to test if this amino acid found in these kinases could trigger a MAP2K-independent activity. This is supported by the fact that MPK3, which as a threonine at gatekeeper postition, shows a high activity when expressed in a MAP2K free system *Echerichia coli*. To test the hypothesis that the gatekeeper T119 residue allows the MAP2K-independent activity of MPK3, the "revertent" form T119Y of MPK3, comparable to MPK6wt, was generated and tested for its phosphorylation capacity. As it's shown in Figure IV.IA, the activity of $MPK3^{T119Y}$ is decreased by about 50% compared to the activity of the WT or the $MPK3^{T119C}$. This suggests that the MPK3 T119 explains partially the activity of the WT protein.

Sequence alignment showed that other MAPK members, including MPK5 and all the kinases of the group D, have a phenylalanine residue at the gatekeeper position (Figure III.4), phenylalanine being a residues identified in our screen as allowing hyperactivity of MPK6. As for MPK3, this "natural mutation" could increase their catalytic activity. Two MAPKs of the group D, MPK16 and MPK19, were chosen for *in vitro* activity studies. They were cloned in pDEST17 as hexahistidine-tagged recombinant protein for purification. Unfortunately all my attempts to purify the full length proteins failed, although it was possible to induce their expression in Rosetta cells. In a pull down assay using cobalt resin (Figure IV.2A), the MPK19 protein was degraded when ran on SDS-gel, it was then difficult to conclude whether the elevated activity observed after kinase assay with this sample is due to activity of the full length protein or to the degraded one. I decided then to express only their kinase domain and check their activity. MPK16 truncated kinase, referred as MPK16Δ, was expressed in *E. coli* cells and easily purified. At the same time the "revertant version" $MPK16\Delta^{F106Y}$ was created and purified in the same way. In an *in vitro* kinase assay, MPK16Δ showed an important kinase activity on MBP. Interestingly, the mutant $MPK16\Delta^{F106Y}$ form lost completely its activity compared to MPK16Δ (Figure IV.2B). This result is similar to what I observed with $MPK3^{T119C}$, and could indicate that like for MPK6 the equivalent position 144 is important for regulating auto-activity in other MAPKs. However the regulation of the kinase activity through the gatekeeper residue seems to

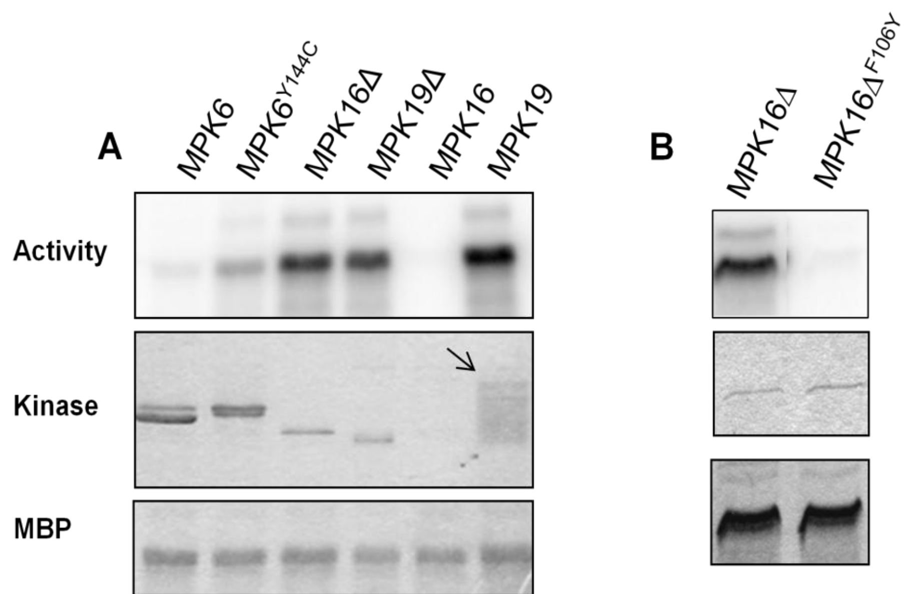



**Figure IV.2 : *In vitro* characterization of MAPK of the group D: A.** Proteins pull down using anti-His antibody followed by a kinase assay on MBP. **B.** Activity assay using purified proteins.

not to be the same for all MAPKs, as some residues are naturally mutated into activating residues.

## *D. Double mutation in the activation loop renders the mammalian MAPK ERK2 constitutively active*

Given the successful utilization of MPK6 mutations to produce CA mutants of *Arabidopsis* MAPKs, we assumed that it could be possible to use the mutations we found to activate MAPKs from different kingdoms.

The mutation in the ATP binding pocket, Y144C/F, that we identified in our screen have been already described in the mammalian MAPK ERK2 as a gatekeeper residue (Emrick *et al*., 2006). And when mutated to Alanine or Glycine residue, ERK2 displays enhanced autoactivity due to intramolecular auto-phosphorylation (Emrick *et al.,* 2006). Because the mutation in the ATP binding pocket can activate MAPKs from different kingdom, we wanted to know if the double mutations of the activation loop (D218A/E222G) found to activate *Arabidopsis* MAPKs in our screen could render ERK2 active. For this purpose, we created in *ERK2* ORF the mutations G180A and E184G, identified as homologous to MPK6 D218A and E222G (Figure IV.3). The gatekeeper mutant $ERK2^{Q103A}$ was used as a control. The wild type proteins as well as the mutant forms were produced in *E. coli* cells (BL21 strain) as His tagged recombinant protein. ERK2 WT protein and the mutant versions were subjected to *in vitro* kinase activity assay (Figure IV.3). Both $ERK2^{Q103A}$ and $ERK2^{G180A/E184G}$ acquired increased catalytic activity toward MBP when compared to WT protein. This result suggests that the residues we identified in the activation loop can be used to create CA MAPKs from other kingdoms.

**AtMAPK6**

```
gggctagctcgaGTC   ACTTCTGAGAGTGATTTCATG         ACTGAATATGTTGTCacgagatggtaccgtgc
 G  L  A  R  V     T  S  E  S  D  F  M           T  E  Y  V  V  T  R  W  Y  R  A
 G  L  A  R  V  A  D  P  D  H  D  H  T  G  F  L  T  E  Y  V  A  T  R  W  Y  R  A
ggccttgcccgtGTTGCAGATCCAGACCATGATCATACAGGGTTCTTGACAGAGTATGTAGCCacgcgttggtacagagc
```

**RnERK2**

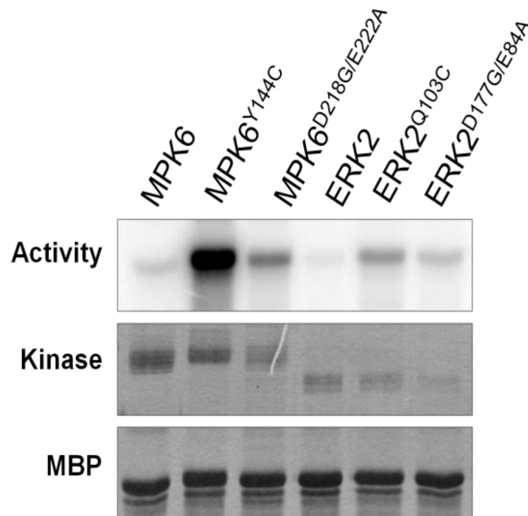



**Figure IV.3: CA mutations are able to activate ERK2:** (A) Creation of D177G/E184 in ERK2, homologous to MPK6 D218G/E222A mutations, (B) MBP kinase assay with ERK2 mutant forms and MPK6 wt and MPK6$^{Y144C}$ and MPK6$^{D218G/E222A}$ as control.

## E. Discussion

I identified the homologous residues of the mutated residue identified to activate MPK6 among the 19 other *Arabidopsis* MAPKs. I then showed that creation of these mutations into some MAPKs from different groups often resulted in increasing their catalytic activity. This was true for MPK4 that belongs to the group B, MPK7 from group C and partially for MPK3 from group A suggesting that the mutations we identified define a useful tool to biochemically study the MAPKs.

### *1. Some MAPKs have residues at the gatekeeper position allowing MAPK-independent activity*

We confirmed previous work showing that the nature of the residue at the gatekeeper position control MAP2K-independent activity (Emrick *et al.,* 2006): tyrosine triggers a weak basal activity (MPK4, MPK6 and MPK7) whereas we showed that threonine (for MPK3) and Phenylalanine (for MPK16) allow a higher basal activity. Since these proteins were produced as recombinant protein in *E. coli*, we may question if the residue-based activity has a function *in planta*. At our knowledge, no one reported any MPK3 activity without PAMP activation and MAPK from the group D are poorly known proteins.

MPK3, MPK5 and MPK10 do not have a tyrosine at the homologous position Y144. We did not go for the biochemical characterization of MPK5 and 10 although they were both cloned with the two different mutations. For MPK3, we first realized that the wild type protein is active when produced in *E. coli* while we know that it's not *in vivo* without stress (*data not shown*). We then suspected that this observation could be linked to the threonine residue at the position 119. From *in vitro* kinase assay experiment realized on the MPK3$^{T119Y}$, called MPK3 revertant form, the kinase activity was decreased compared to both MPK3wt and MPK3$^{T119C}$ forms. This observation leads us to conclude that threonine residue like cysteine or phenylalanine at the equivalent position 144 in MPK6 could control MAPK activity. However we also observed that Peri-His-MBP-MPK3wt protein have no detectable activity compared to His-MBP MPK3$^{D193G/E197A}$ when tested in an *in vitro* kinase assay. This could be linked either to the important activity of MPK3$^{D193G/E197A}$ which in the experiment conditions leads to the non

detection of the wt protein activity or to the phosphorylation of the 6His-MPK3wt in the bacteria, which a very improbable hypothesis. Protein expressed using the Peri-HIS-MBP are addressed to the bacterial periplasm (Nallamsetty *et al.,* 2005) and could then escape to the phosphorylation by intracellular kinase. Moreover MPK3wt activity might not be detectable *in vivo* because of the presence of phosphatases in the cell that could permanently regulate its elevated basal activity. A simple experiment that could help understanding MPK3 behavior is to perform a phosphatase (CIP for example) treatment on MPK3wt expressed with the two different tags and see whether it looses its activity.

### *2. How does Group D MAPK function?*

The exception for Y144 residue conservation concerns also all the MAPKs from the group D, which have a phenylalanine residue instead. Phenylalanine at the gatekeeper position is one of the mutations that activate MPK6 in a MAP2K-independent way. By mutating this residue to tyrosine, I showed that it was responsible for the high catalytic activity of the WT protein. This finding enhanced our interest to understand the regulation of MAPKs from this group as not much data is available on their role in the plant cell and the way they are regulated. Implication of MAPK from the group D in signaling was reported only recently. MPK9 was proposed to have a role in the ROS mediated ABA signaling (Jammes *et al.,* 2009), and MPK18 could have a role in cortical microtubule related functions (Walia *et al.,* 2009). Evidence for activation of MAPK from this group came last year with a study in which the authors reported that MPK8 could be activated by different stresses like wounding, $H_2O_2$ and JA (Takahashi *et al.,* 2010).

Interestingly MAPKs from D group are longer kinases because of a long carboxyl terminal domain which is not really conserved between the different members of the group (Figure III.4). We compared the secondary structure of MPK9, a MAPK from group D with MPK4. Both MAPKs have a classic globular kinase structure with similar concatenation of β sheets and α helixes, MPK9 however shows a longer less structured C-terminal tail (Figure IV.4). This configuration is reminiscent of the plant MAP3K proteins which present a long amino-terminal unstructured domain, thought to be an auto-inhibitory domain (Suarez-Rodriguez *et al.,* 2010). Unfortunately, I was not able to answer the question of whether the phenylalanine residue at the gatekeeper residue could really activate MAPK from this group, since it was not possible to test

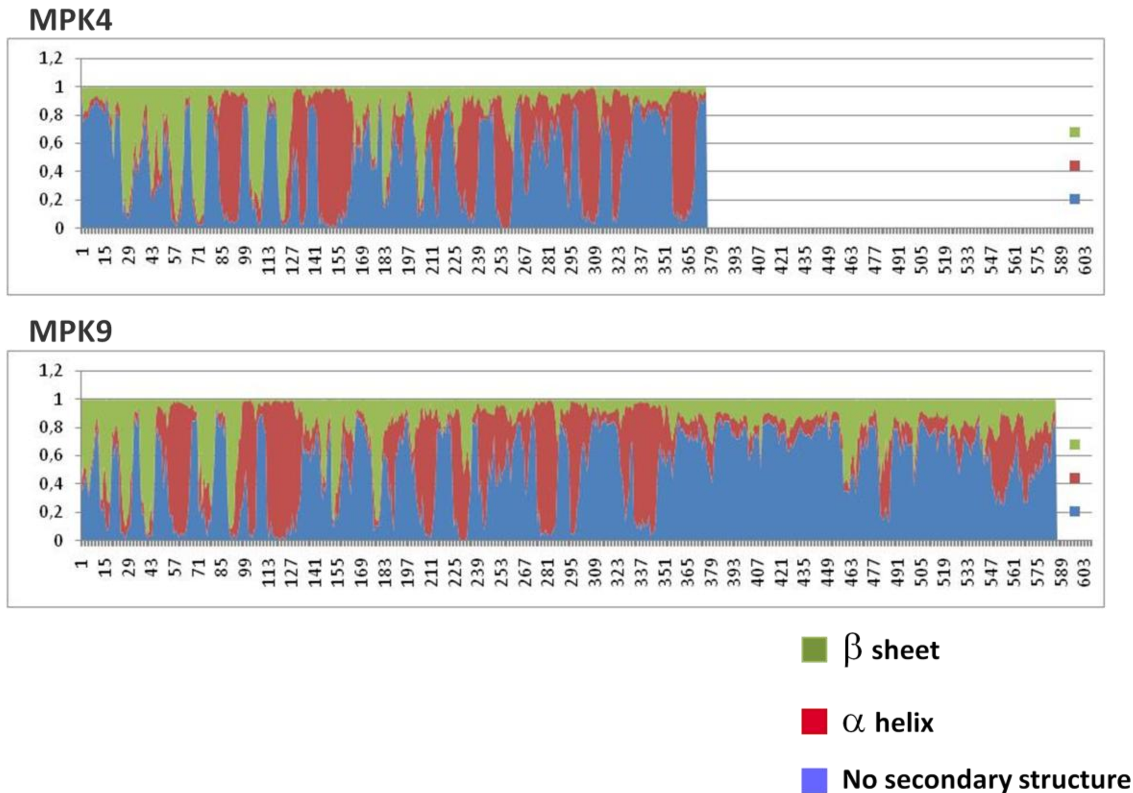



**Figure IV.4: Secondary structure prediction of MPK9 from group D compared to a canonical MPK4 (group B) of *Arabidopsis*.** The software (Expasy, Jufo) we used calculates the probability of each residue of the protein sequence to belong either to a α helix or to a β sheet structure. We could observe using this program that MPK4 as well as MPK9 have a classic MAPK structure with two lobes, an N terminal lobe constituted of β sheet with some α helix, and a C terminal lobe essentially constituted of α helix. MPK9 have an addition a C terminal unstructured domain.

the activity of the full length protein. We can build a working model though: the kinase domain is active independently of MAP2K phosphorylation, activity allowed by phenylalanine residue at the gatekeeper position. Negative kinase regulation could occur through its C-terminal inhibitory domain. We need to bring more evidence to support this model. It could be very informative to express and obtain the full length protein to test its kinase activity. Expressing it in a plant transient system might be a solution to test this promising working model.Double CA mutations in the activation loop could activate MAPKs from different kingdoms

The combination of the double mutations in the activation loop triggers hyperactivity in the human MAPK ERK2. This finding suggests that the mechanism by which they activate MAPKs is conserved. This lead us to the conclusion that depending on the region where the mutation occurs, once they could emulate the same conformational changes as the MAPK phosphorylation their transfer to, other MAPK even from different *Philae* could be successful in increasing intrinsic kinase activity. The lack of plant MAPK structure is also limiting for as to predict the conformational changes induced by the amino acid substitutions. Regarding the importance of MAPK signaling in plant (Suarez-Rodriguez *et al.,* 2010) the scientific community should put more interest and invest in this aspect.

# *Chapter V*

# *CA MAPK mutants retain WT specificity*

# *Chapter V - CA-MAPK mutants retain WT kinase specificity*

In this chapter I describe the characterization of the CA mutants with respect to phosphorylation motif preferences, specificity of docking interaction with substrates and upstream activators and protein subcellular localization.

In the case of animal MAPKs, it has been demonstrated that the phosphorylation of a target is controlled by both the residues surrounding the phosphorylation site and to specific protein-protein interactions occurring through other domains in the kinase and in the interactor (Ubersax & Ferrell 2007). For plant MAPKs, it's still unclear whether targeting of distinct protein substrates by different MAPKs is due to specific characteristics in their respective phosphorylation site motif or of other interacting domains on the kinase surface. Animal MAPKs usually phosphorylate their targets on serine (S) or threonine (T) residue followed by proline (P), the so called SP/TP sites. They could also have a preference for Pro at position -2 (Clark-Lewis *et al.,* 1991; Songyang *et al.,* 1996). In addition of the residues surrounding the phosphorylation site, MAPK specificity depends on direct physical encounter between the enzyme and its target protein occurring through distinct kinase domains. Many studies on animal and yeast MAPK signaling pathways showed that the modular protein-protein interactions are mediated by regions designed "docking sites" (Figure I.2). These sites enhance specificity by recruiting a low-affinity catalytic domain to their proper substrates and by promoting the formation of pathway-dedicated signaling complexes (Bardwell 2006). Direct protein-protein interactions have been reported between a MAPK and specific activators MAP2Ks or substrates, and this in various signal transduction pathways in animals and in yeast.

## *A. WT and CA MAPKs phosphorylation preferences are not affected*

### *1. WT and CA forms of MPK3, MPK4 and MPK6 phosphorylate peptides on a canonical SP/TP site*

To investigate whether the kinase preference toward the sequence surrounding the phosphorylation site is not affected by the CA mutations, particularly the mutations in residues of the activation loop, we used a peptide array technique. The peptide array consists of 198 peptide mixtures. Each peptide has as a phosphorylation site an equimolar mixture of serine and threonine at its central position. It also contains a fixed residue corresponding to one of the 20 amino acids at one of the nine positions (between -5 to +4) around the phosphorylation site (Hutti *et al.,* 2004; Vlad *et al.,* 2008). The phosphorylation of this peptide array gives a picture of the preferred amino acid between position -5 and +4 around the phosphorylation site. In Figure V.1 the spot intensities indicate the impact of the corresponding fixed amino acid on the phosphorylation of the peptide by the kinase.

The active versions of MPK6, MPK6$^{Y144C}$ and MPK6$^{D218G/E222A}$ were tested on the peptide array (Figure V.1A and B). This experiment was preceded by a kinase assay using a mixture of peptides with a serine or threonine at their central position as substrate. This allowed to know whether the CA MPK6 are able to phosphorylate this size of peptide and recognize the potential phosphorylation site and whether it has a preference for a serine or threonine. It also allows identifying the most relevant time point for the peptide phosphorylation, since this preliminary assay is performed over a time point scale. MPK6$^{Y144C}$ and MPK6$^{D218G/E222A}$ phosphorylation pattern show a high phosphorylation signal on peptides with a proline at +1 and in a less extend on peptide with proline at -2 and charged amino acids at position +2 (Arginine or Lysine). This is a classical MAPK preference pattern and very close of the one already published for MPK6 (Stulemeijer *et al.,* 2007).

We also compared using this technique the phosphorylation profile of MPK3wt to its corresponding mutated version MPK3$^{T119C}$ (Figure V.1C and D). This comparison was possible because the wild type 6His-MPK3 produced in bacteria could phosphorlate MBP. As it's shown in the Figure, the phosphorylation profile of MPK3wt is very similar to the mutated version

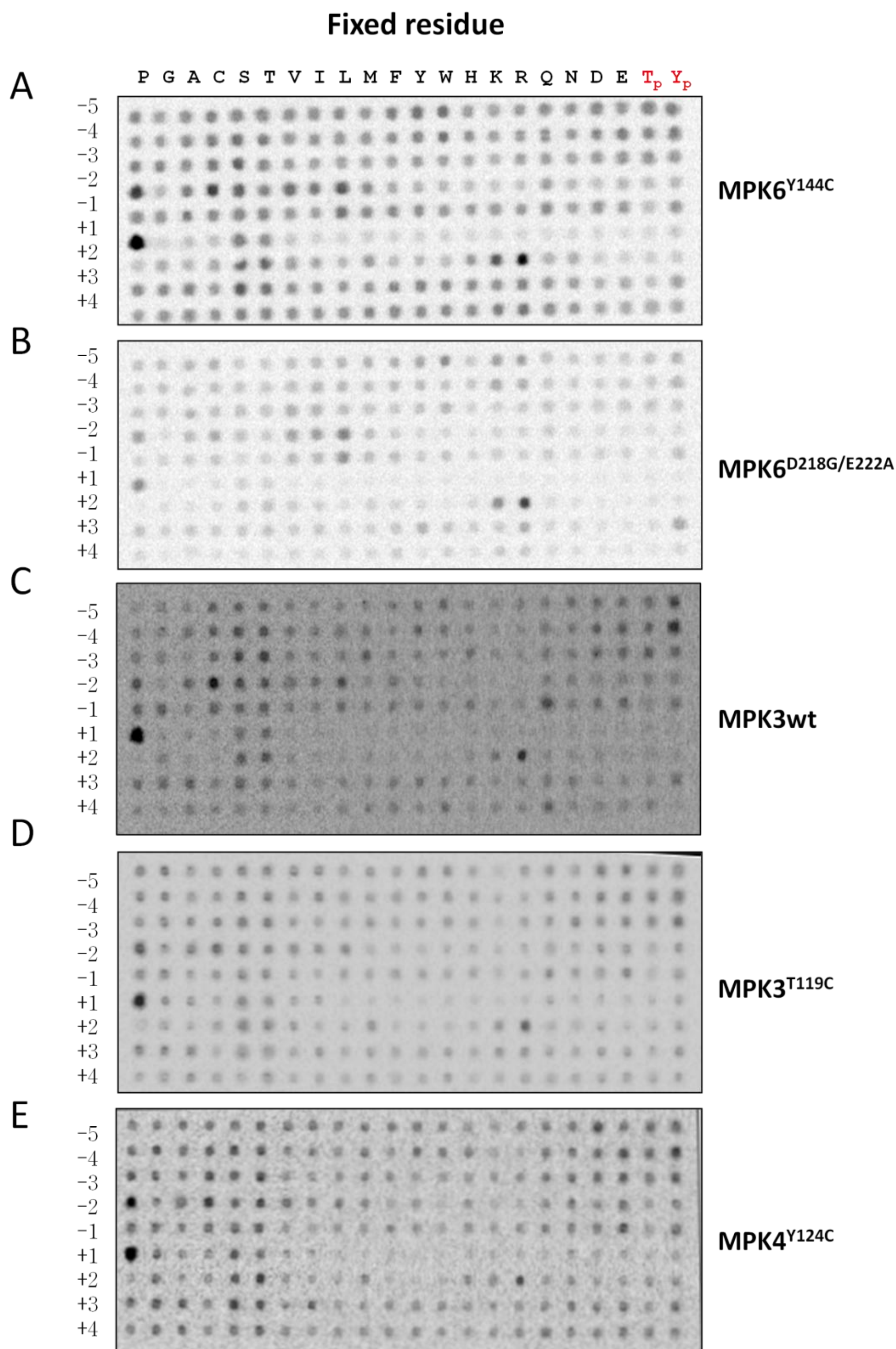



**Figure V.1: Phosphorylation of the semi-degenerate peptide arrays by MAPKs active forms.** (A and B) MPK6$^{Y144C}$ and MPK6$^{D218G/E222A}$ 1 µg/µl of reaction, (C) 0.7µg/µl of MPK3 WT, (D) 0.86 µg/µl of MPK3$^{T119Y}$ and (D) 1.5µg/µl of reaction of MPK4$^{Y124C}$. Phosphorylated peptides were spotted onto SAM2 streptavidin-coated membranes and radioactivity detected after washing of unincorporated radiolabeled ATP using phosphor imaging.

**Table V.1**: Quantification of phosphorylation of the semi-degenerate peptide array by the MPK3

**MPK3 WT**

| | P | G | A | C | S | T | V | I | L | M | F | Y | W | H | K | R | Q | N | D | E | pST | pY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| -5 | 0,50 | 0,84 | 0,60 | 1,44 | 1,45 | 1,39 | 0,79 | 0,62 | 0,65 | 0,72 | 0,96 | 1,11 | 1,44 | 0,70 | 0,72 | 0,94 | 0,96 | 1,31 | 1,48 | 1,38 | 1,65 | 3,04 |
| -4 | 0,67 | 0,81 | 1,09 | 1,35 | 1,94 | 1,76 | 0,94 | 0,82 | 1,16 | 0,93 | 0,44 | 1,19 | 0,86 | 0,53 | 0,31 | 0,47 | 0,92 | 0,79 | 1,33 | 1,70 | 1,34 | 3,64 |
| -3 | 0,88 | 0,86 | 0,44 | 1,02 | 2,23 | 2,48 | 0,79 | 0,73 | 0,77 | 1,45 | 0,64 | 0,61 | 0,54 | 1,03 | 0,43 | 0,44 | 0,97 | 0,84 | 1,44 | 1,41 | 1,67 | 1,49 |
| -2 | 1,74 | 0,33 | 1,23 | 3,52 | 1,86 | 1,52 | 1,29 | 0,94 | 1,70 | 0,46 | 1,01 | 0,51 | 0,27 | 0,39 | 0,11 | 0,18 | 0,72 | 0,70 | 0,72 | 0,80 | 0,86 | 0,83 |
| -1 | 1,35 | 1,46 | 0,57 | 1,48 | 1,30 | 1,52 | 0,74 | 0,65 | 0,99 | 0,60 | 0,74 | 0,74 | 0,58 | 0,87 | 0,44 | 0,54 | 2,37 | 0,75 | 1,05 | 1,26 | 0,72 | 1,16 |
| 0 | 0,00 | 0,00 | 0,00 | 0,00 | 20 | 20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 1 | 13.36 | 0,29 | 0,33 | 0,34 | 1,28 | 1,24 | 0,24 | 0,18 | 0,02 | 0,13 | 0,17 | 0,30 | 0,06 | 0,29 | 0,28 | 0,18 | 0,28 | 0,34 | 0,35 | 0,35 | 0,45 | 0,39 |
| 2 | 0,11 | 1,25 | 0,44 | 0,27 | 2,81 | 2,90 | 0,64 | 0,43 | 0,35 | 0,65 | 0,29 | 0,40 | 0,32 | 0,69 | 1,29 | 4,09 | 0,82 | 0,78 | 0,91 | 0,56 | 0,93 | 0,66 |
| 3 | 1,22 | 1,62 | 1,82 | 0,87 | 2,03 | 1,35 | 0,85 | 0,59 | 0,72 | 0,68 | 1,01 | 1,04 | 0,70 | 0,70 | 0,47 | 0,85 | 1,12 | 1,10 | 0,61 | 0,65 | 0,70 | 1,55 |
| 4 | 0,63 | 0,64 | 1,29 | 0,86 | 1,23 | 1,09 | 0,69 | 0,47 | 0,99 | 0,86 | 1,33 | 0,66 | 1,57 | 0,50 | 0,78 | 0,82 | 2,40 | 0,85 | 1,24 | 1,11 | 1,29 | 0,44 |

**$MPK3^{T119C}$**

| | P | G | A | C | S | T | V | I | L | M | F | Y | W | H | K | R | Q | N | D | E | pST | pY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| -5 | 1,52 | 1,78 | 0,71 | 0,94 | 1,39 | 1,39 | 1,05 | 1,04 | 0,56 | 0,80 | 0,62 | 1,14 | 1,00 | 0,48 | 0,12 | 0,68 | 0,86 | 0,55 | 1,73 | 1,64 | 1,13 | 1,27 |
| -4 | 1,40 | 1,44 | 0,74 | 0,86 | 1,87 | 1,70 | 0,71 | 1,31 | 0,83 | 0,67 | 0,74 | 0,76 | 0,83 | 0,60 | 0,32 | 0,32 | 0,91 | 0,79 | 1,57 | 1,63 | 1,70 | 2,17 |
| -3 | 1,10 | 1,36 | 1,19 | 1,45 | 2,11 | 1,93 | 0,72 | 0,58 | 0,64 | 0,88 | 0,54 | 0,68 | 0,79 | 0,51 | 0,16 | 0,60 | 0,76 | 1,55 | 1,00 | 1,44 | 1,42 | 2,49 |
| -2 | 4,04 | 0,89 | 1,68 | 2,75 | 1,75 | 1,37 | 1,22 | 1,04 | 1,29 | 0,27 | 0,29 | 0,20 | 0,27 | 0,30 | 0,12 | 0,25 | 0,46 | 0,35 | 0,74 | 0,73 | 0,37 | 0,79 |
| -1 | 1,71 | 1,79 | 0,71 | 1,21 | 1,73 | 1,60 | 0,70 | 0,41 | 0,81 | 0,57 | 0,93 | 0,72 | 0,63 | 0,71 | 0,28 | 0,58 | 1,37 | 0,86 | 0,91 | 1,76 | 0,26 | 0,99 |
| 0 | 0,00 | 0,00 | 0,00 | 0,00 | 20 | 20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 1 | 12,43 | 1,07 | 0,79 | 0,44 | 1,64 | 1,07 | 0,47 | 0,50 | 0,08 | 0,08 | 0,08 | 0,10 | 0,10 | 0,08 | 0,04 | 0,11 | 0,36 | 0,20 | 0,22 | 0,15 | 0,17 | 0,50 |
| 2 | 0,29 | 0,85 | 0,54 | 0,91 | 2,77 | 2,19 | 1,05 | 0,36 | 0,51 | 1,58 | 0,20 | 0,30 | 0,29 | 0,41 | 1,32 | 4,61 | 0,47 | 0,42 | 0,63 | 0,28 | 0,43 | 0,92 |
| 3 | 2,13 | 1,85 | 1,86 | 0,41 | 1,60 | 1,27 | 0,61 | 1,13 | 0,52 | 0,83 | 0,64 | 0,70 | 0,79 | 0,49 | 1,15 | 1,20 | 0,69 | 0,54 | 0,84 | 0,75 | 0,42 | 1,18 |
| 4 | 0,93 | 1,52 | 1,94 | 1,12 | 0,77 | 1,82 | 0,64 | 0,86 | 0,68 | 1,28 | 0,71 | 0,75 | 1,11 | 0,44 | 0,65 | 1,07 | 0,88 | 0,83 | 0,88 | 1,10 | 0,95 | 1,15 |

MPK3$^{T119C}$, quantification of the spot intensities gives a correlated data between the two version of MPK3 (see Table V.1).

These features seems to be common for MAPKs (Figure V.1) because we did not found any significant differences between the profile of CA MPK4 and the mutant versions of MPK3 or MPK6, we rather observe the same MAPK peptide phosphorylation signature. These results lead to the conclusion that the CA mutations do not change the kinase preferences around the phosphorylation site. It also suggests that plant MAPKs specificity is mainly under the control of target interaction with surface of the kinase, distinct from the active site, as they are in animal and yeast.

### *2. MAPK from group D have a different phosphorylation motif*

All the CA MAPK that we tested appears to have a similar phosphorylation motif. We wanted then to investigate whether this motif is conserved in the atypical MAPKs from group D. We examine in a preliminary assay whether the wt kinase domain of MPK16 and the "revertant" one MPK16Δ$^{F106Y}$ could phosphorylate peptide mix. Like in MBP phosphorylation assay, the truncated MPK16 could phosphorylate degenerate peptide mix but not the MPK16Δ$^{F106Y}$ form (Figure V.2A). MPK16Δ was then assayed on peptide chips and revealed a particularly unexpected pattern (Figure V.2B): the active protein phosphorylates all the peptide with a cysteine residue at any 9 position in the peptides.

## B. <u>CA MAPK mutants interact with their natural substrates and upstream regulators in yeast two-hybrid assay</u>

To examine whether the known MAPK interactions are still conserved between the CA MAPKs versions and some of their known upstream activators and substrates, we used the yeast two hybrid (Y2H) system. We know from the literature that MKK4 interacts in Y2H with MPK3 and MPK6 and that MKK2 interacts specifically with MPK4 and MPK6 (Ichimura *et al.,* 1998; Teige *et al.,* 2004; Lee *et al.,* 2008). Based on *in planta* assays or Y2H data, other studies

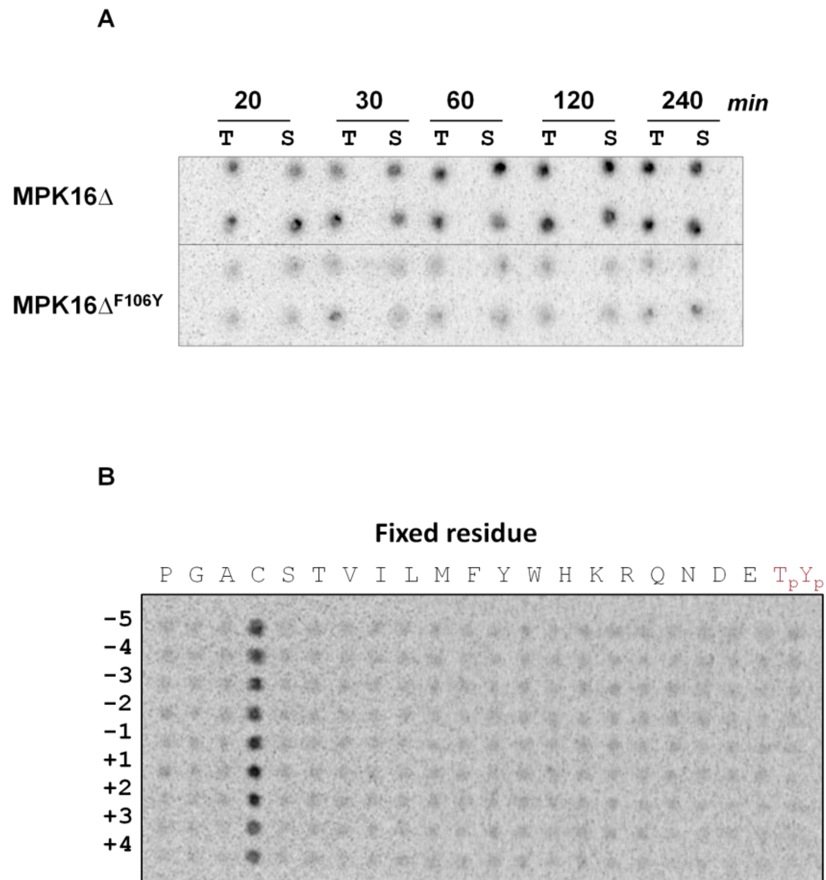



**Figure V.2: Phosphorylation of the semi-degenerate peptide array by MPK16Δ. A.** MPK16Δ and MPK16Δ$^{Y144C}$ phosphorylation of peptide mix (mixed peptides with serine (S) or threonine (T) as phosphoacceptor). Peptide chips profile after phosphorylation with 1.2 µg/µl MPK16Δ.

demonstrated the interaction between MAPKs and their specific substrates: MPK3 with VIP1 (Djamei *et al.,* 2007), MPK4 with MKS1 (Andreasson *et al.,* 2005) and MPK6 with ERF104 (Bethke, Scheel *et al.,* 2009).

I first tested the interactions between MAPKs and some of their known upstream regulators, the MAP2Ks. For that purpose, the reporter yeast strain (MaV203 strain) was co-transformed with MAP2K expression vectors -encoding the *MKK2* and *MKK4* ORFs fused to the GAL4 activation domain- (AD) and MAPK expression vector -encoding MAPK WT and CA forms of *MPK3*, *MPK4* and *MPK6* ORF fused to the GAL4 binding domain- (BD). Interactions between MKK2 and MPK4 WT and both mutants forms (MPK4$^{Y124C}$ and MPK4 $^{D198G/E202A}$) were detected based on growth on histidine medium supplemented with increased concentration of 3-AT (3-Amino-1,2,4-triazole). I noticed that this interaction was slightly weaker in the case of the double mutant MPK4$^{D198G/E202A}$ when tested on high stringent media (starting at 65mM of 3-AT). MKK2 interacts also with MPK6 WT and CA, the interaction being stronger in case of MPK6$^{Y144C}$ form (Figure V.3A). We confirmed that MKK4 can interact specifically with MPK3 and MPK6 WT and CA. As published, we did not detect any interactions between MKK2 and MPK3 WT and CA, and none between MKK4 and MPK4 WT and CA. Proteins did not interact with AD and BD alone which were used as controls. These results suggest that the mutations rendering the kinases active do not change the specificity toward their activating kinases.

In a second experiment, I tested the interaction of our CA mutants of MAPKs against their known substrates (Figure V.3B). In this case, yeast cells were transformed with WT and CA forms of MPK3, MPK4 and MPK6 fused to GAL4 binding domain, and the corresponding substrates, VIP1, MKS1 and ERF104, respectively, fused to GAL4 activation domain. VIP1 was specifically interacting with the three MPK3 forms (WT, MPK3$^{T119Y}$ and MPK3$^{D193G/E197A}$). A specific interaction between the three MPK4 forms and MKS1 was also observed. MPK6 WT protein and MPK6$^{D218G/E222A}$ interact specifically with ERF104, this interaction being relatively weak (30 mM 3AT histidine supplemented medium). Interaction between MPK6$^{Y144C}$ and ERF104 was barely detectable. Technical problem left aside (protein expression level, protein folding…), this observation could be explained by the fact that MPK6 doesn’t need a very important physical interaction with its substrate, as the interaction between MPK6 wt protein and ERF104 remains slight compared to the other MPK-Substrate interactions.

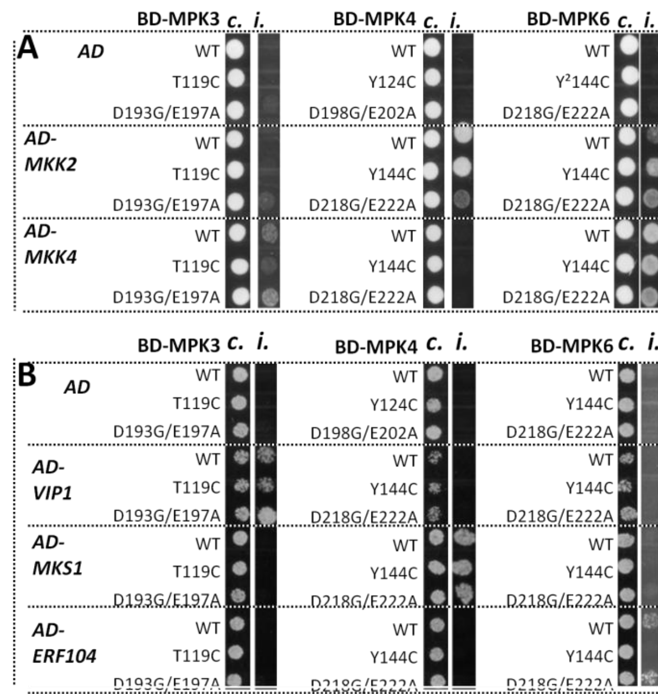



**Figure V.3: Y2H interaction between wild type and active forms of MPK3/4/6 with (A) activator MKK2, MKK4 and (B) substrate, VIP1, MKS1 and ERF104:** Combinatory interaction in yeast 2 hybrid of MPK3, 4 and 6 WT and CA forms with MKK2 and MKK4 (**A**) and with VIP1, MKS1 and ERF104 **(C**). Co-transformed single yeast colonies were spotted on control medium (c.) and selective medium supplemented with 65mM for (A) or 30mM for (B) of 3AT (i.).

All these data together indicate that the CA mutations do not change the specificity of the kinase toward its cognate MKK and to its specific substrate.

## C. Transient expression of CA MAPKs in Nicotiana benthamiana

MAPK localization is important for their proper functioning. In order to investigate a possible change in the sub-cellular localization of the CA MAPKs, we cloned MPK3, MPK4, MPK6 and MPK7 fused in frame with the DsRED fluorescent protein in C-term in a binary vector. We realized transient expression of the chimerical proteins in *Nicotiana benthamiana* leaves by agro-infiltration and monitor their sub-cellular localizations using confocal microscopy (data not shown). MPK7 was barely detectable and therefore it is impossible to conclude yet. MPK4 WT and CA showed a similar localization mainly in the nucleus but also weakly in the cytoplasm. MPK3wt and MPK3$^{T129C}$ were largely localized in the nucleus but no signal was observed for MPK3$^{D193G/E197A}$, which could be linked to the apparent death of the *Nicotiana* cells. For these preliminary results, we did not succeed to clone WT MPK6, but the two CA mutant forms, MPK6$^{Y144C}$ and MPK6 $^{D218G/E222A}$ also triggered cell death. Repetitions are necessary to validate this data.

## D. Discussion

By acquiring intrinsic activity, the kinase mutants may have lost their specificity toward downstream substrate and recognition by upstream elements. In this part, the results based on two complementary strategies were exposed to test this. I first used the degenerate peptide array to identify the preference of the MAPKs toward amino acid residues around the phosphorylation site. And second, by using yeast two hybrid system, I ensured that docking interactions are conserved between the kinase and substrate or activating kinase. Additionally, preliminary experiments to test whether the sub-cellular localization of the MAPKs was affected by the CA mutations, were not very conclusive, but gave interesting functional information as some CA forms triggered plant cell death.

### 1. Plant MAPK of the groups A, B and C recognize a classical MAPK sites

The phosphorylation of a protein by a kinase depends in part on the presence of a motif efficiently recognized and phosphorylated by this kinase (Vlad *et al*., 2008). Semi degenerated peptide array allowed us to measure activate kinase MPK3, MPK4 and MPK6 preferences for substrate. Measuring the phosphorylation rate of a peptide mixture with one fixed amino acid at one position, enable determining the contribution of a particular residue at a particular position in the phosphorylation of serine or threonine situated in the middle of the peptide.

It was advantageous to have a wild type active form of MPK3 that made the comparison with the mutant version $MPK3^{T119C}$ possible, even if this T119C mutation does not further increase MPK3 activity. Comparing the phosphorylation profile showed no significant differences, as all the phosphorylation score are correlated (Table V.1). This means that mutation at the equivalent residue 144 in MPK6 does not change the phosphorylation preference of the kinases and therefore could similarly recognize their substrates. The kinase preferences were the same for the other MAPKs we tested and bearing the same mutation ($MPK4^{Y124C}$ and $MPK6^{Y144C}$) or the double mutations in the activation loop ($MPK6^{D218A/E222G}$). The peptide array profile matches the reported MAPK substrate phosphorylation sites with an absolute requirement for proline residue in position +1 of the serine or threonine phospho-acceptor. This result is in agreement with the studies on MAPK substrates (Liu & Zhang 2004; Caspersen *et al.,* 2007;

Djamei *et al.,* 2007). A weaker preference for proline or cysteine residues at position -2 was also observed and reported for other animal MAPKs (Sheridan *et al.,* 2008).

In conclusion, our results confirm what was determined for animal MAPKs (ERK2, p38α, p38δ, JNK2) using the same approach. Active site specificity appears to be general conserved feature for all MAPK and is then insufficient to direct a particular kinase to a specific protein substrate. Protein substrate affinity and specificity is controlled by interactions that occur outside of the active site (Biondi & Nebreda 2003; Goldsmith *et al.,* 2007; Raman *et al.,* 2007). Apart from studying the conservation of the MAPK phosphorylation preferences and whether they change with the CA mutations, we wished also to use this technique to bio-informaticaly identify new targets, querying plant protein databases with the calculated matrix of phosphorylation motif preferences (Vlad *et al.,* 2008). However, with our results and the published data on animal MAPKs showing that all MAPKs share the same preferences for the phosphorylation sites, this technique is not appropriate to identify new MAPK substrates.

### *2. The unexpected result of MAPKs from group D…*

A result difficult to explain is the phosphorylation motif of the MPK16 kinase domain. This protein appears to exclusively show a stronger phosphorylation signal on peptides containing a cysteine residue. Astonishingly peptides with proline residue at position +1 which is a minimum requirement for a MAPK to phosphorylate its target were not more phosphorylated in the matrix. This may be a technical problem: what is measured is not the kinase preference but rather the fact that the activity is modified depending on the redox environment affected by cystein residue. A report on the effect of various sulfur amino acid on tyrosyl and serine/threonine phosphorylation was published by Kodama group (2009), in which they claim that depending on the cystein compound the phosphorylation could be enhanced. This could alternatively indicate that MAPK from group D have a completely different phosphorylation motif than the canonical other MAPKs.

### *3. Docking interaction is conserved between CA MAPK and their substrate and MAPKK*

We investigated also whether the CA mutations could change protein-protein interaction specificities using the Y2H assay. We globally found that interaction between the CA MAPK and their cognate MAP2K and substrate where conserved and that the structural changes induced by CA mutation does not interfere with docking regions. Although in some cases, the strength of the interactions could be affected by the CA mutations. A quantification of this data might be useful to understand whether the modifications of the interactions are really due to the mutations. Alternatively, they could reflect a mechanistic effect: we may expect that the affinity of a given interactor for its cognate kinase is different depending on the activation state of the kinase.

This result suggests also that CA MAPK could be used *in planta* to study MAPK function. Even though using these forms to complement a mutant for instance could be the best proof that the CA mutation doesn’t alter the signaling pathways. This is what I will describe in the following chapter.

# Chapter VII
# MPK4 function in plant pathogen responses

# *Chapter VI - Generation and activity of Arabidopsis lines expressing constitutively active forms of MPK4*

Whereas *mpk3/mpk3* and *mpk6/mpk6* do not have an obvious morphological alteration, *mpk4/mpk4* plants are dwarfed and sterile, likely because they overproduce salicylic acid and reactive oxygen species (Petersen *et al.,* 2000). We took advantage of this morphological phenotype to test whether the CA MPK4 isoforms, mutated in the residues identified in the yeast complementation screen, were able to complement *mpk4-2* knock-out plants. This would bring a clear proof that CA MPK4 retains the specificity features of the WT protein and at the same time, create a new tool to better understand the ambiguous function of MPK4 in plant stress response and development.

## *A. Expressing CA-MPK4 under the control of MPK4 endogenous promoter in mpk4 transgenic lines leads to morphological complementation*

To avoid any silencing phenomenon linked to the use of a strong expression promoter but also to evaluate the effect of the CA mutation in a background as WT as possible, we choose to work with the native *MPK4* locus, including promoter, introns, exons, UTRs and terminator. We cloned the locus fused to a PC2 tag in pGREEN0229 to generate pGREEN0229-MPK4-PC2 (Figure IV.1) (cloning details in Materiel and Methods). The PC2 tag is constituted of three different epitope-tags: 9xMyc, 8xHIS and 1xStrepII allowing biochemical studies. pGREEN0229-MPK4-PC2 was mutated to create pGREEN0229-MPK4$^{Y124C}$-PC2 and pGREEN0229-MPK4$^{D198G/E220A}$-PC2. Vectors were transformed by floral dipping in *mpk4-2* background. We transformed the progeny of a *mpk4-2/MPK4* plants because the homozygous *mpk4-2/mpk4-2* plants are sterile. Transgenic homozygous *mpk4-2* plants with a segregating transgene were isolated expressing:

| | |
|---|---|
| promMPK4:MPK4wt-PC2- | 6 independent lines |
| promMPK4:MPK4$^{Y124C}$-PC2- | 10 independent lines |
| promMPK4:MPK4$^{D198G/E220A}$-PC2- | 7 independent lines |

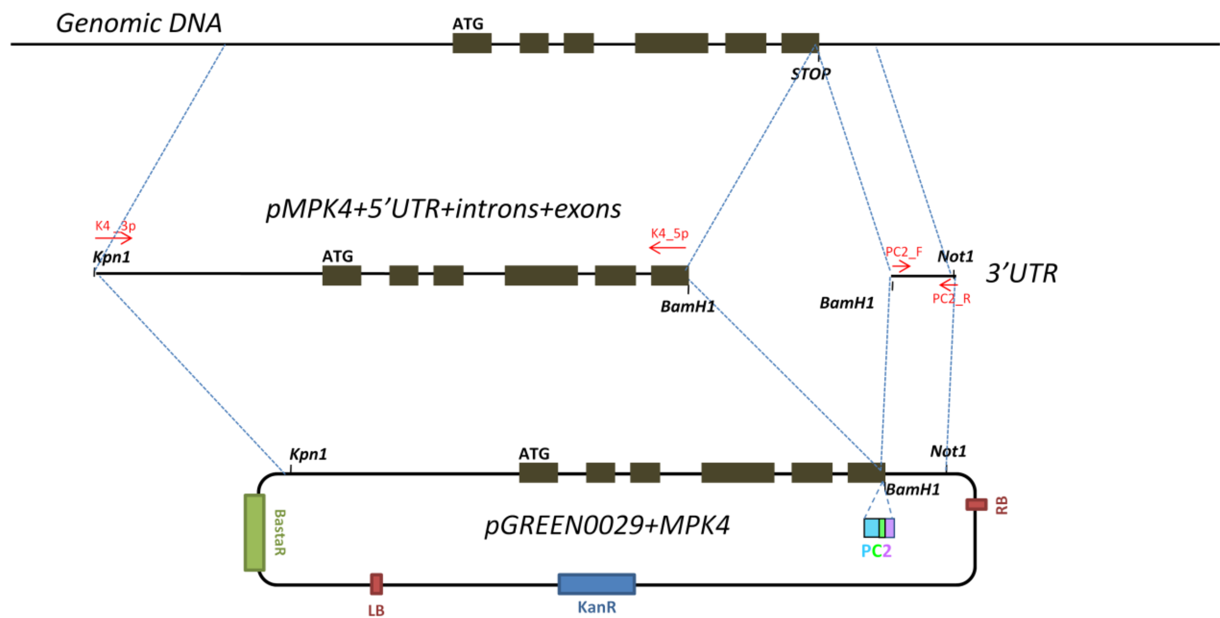



**Figure VI.1**: **Cloning strategy of *MPK4* locus in pGREEN0229**. The MPK4 -open reading frame upstream (2693 bp) and downstream (258 bp) of the termination codon was amplified from Col-0 genomic DNA with specific primers and cloned in pGEMTeasy. We reassembled the *MPK4* locus in pGREEN0229 and introduced the PC2 tag to generate pGREEN0229-MPK4-PC2.

MPK4$^{Y124C}$ and MPK4$^{D198G/E202A}$ loci were both able to complement the developmental defects of *mpk4-2* mutant as did MPK4wt locus (Figure VI.2A). All the lines recovered a normal growth suggesting the functional complementation. This indicates that CA MPK4 are functioning in the *mpk4* background. Two lines expressing MPK4$^{D198G/E202A}$ isoform produced less seeds. Given the fact that we have only few lines, it was difficult to conclude between a true CA double mutation effect, or a normal morphological variability that we can observe in a transformation process.

In further analysis, the progeny of T1 plants were used: they are homozygous for *mpk4-2* and hemizygous for the *MPK4* transgene. A western blot analysis was performed, and showed that all the transgenic lines have a detectable protein amount which means that the transgene is expressed and the protein is stable (Figure VI.2B). All the lines selected for further characterization have similar expression level. We realized that the anti-MPK4 antibody available in the lab raised against the last 16 Carboxyl terminal amino acids of MPK4 protein was not able to recognize MPK4-PC2 proteins in our complemented lines (not shown). I rather used anti-c-myc antibody that recognize the c-myc tag in the PC2 cassette at the C-terminal part of the protein.

## B. ***Not all the CA mutations allow enhanced MPK4 activity in planta***

Since the CA-MPK4 lines (mpk4/pMPK4::MPK4$^{Y124C}$ and mpk4/pMPK4::MPK4$^{D198G/E202A}$) did not showing any obvious morphological phenotype compared to WT-complemented lines, we were wondering whether the mutated kinase were indeed CA *in planta*. To test if the kinase activity of CA-MPK4 fusion proteins in the mpk4/pMPK4::CA-MPK4 lines is enhanced compared to transgenic plants expressing the WT MPK4, I performed MBP kinase assay on c-myc immuno-precipitated proteins and compared the activity of CA MPK4 tagged proteins to that of WT MPK4 tagged protein (Figure VI.2). We surprisingly notice that only the lines expressing MPK4 form with mutations in the activation loop D198G/E202A exhibit a higher MPK4 activity. MPK4 activity in the MPK4$^{Y144C}$ lines was barely detectable, which is comparable to the basal activity of WT MPK4 transformed plants.

A

B

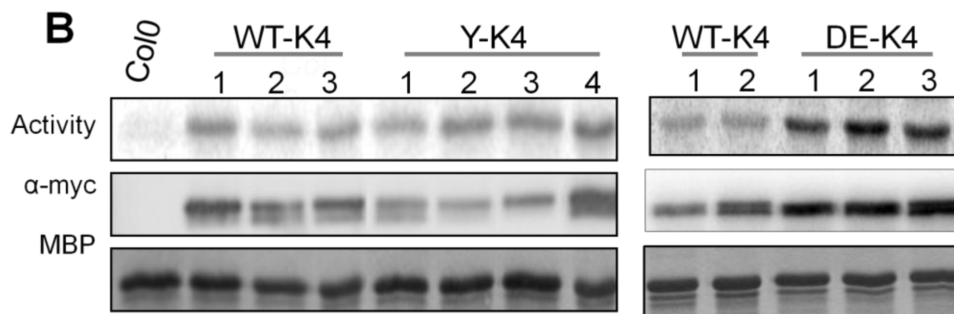



**Figure VI.2 : MPK4 expression and activity in MPK4$^{Y124C}$ and MPK4$^{D198G/E202A}$ lines.** MPK4 was immunoprecipitated with an anti c-myc antibody from independent lines expressing WT-MPK4 (WT-K4 1, 2 and 3), MPK4$^{Y124C}$ (Y-K4 1, 2, 3 and 4) and MPK4$^{D198G/E202A}$ (DE-K4 1,2 and 3).

This suggests that only double mutation in the activation loop can activate MPK4 *in vivo*. For the next part of this work we concentrated our efforts on studying mpk4/pMPK4::MPK4$^{D198G/E202A}$ lines because of their enhanced kinase activity.

## *C. MPK4 activation in CA-MPK4 expressing lines*

PAMPs can activate MPK4 through the two MAP2K MKK2 and MKK1, and the MAP3K MEKK1 (Ichimura *et al.,* 2006; Nakagami *et al.,* 2006; Suarez-Rodriguez *et al.,* 2007; Gao *et al.,* 2008; Qiu, Zhou *et al.,* 2008). Flg22 activation of MPK4 is well characterized in *Arabidopsis* (Suarez-Rodriguez *et al.,* 2010). To evaluate the level of activation of MPK4 in the MPK4$^{D198G/E202A}$ lines and whether MPK4 is still activable after stress perception, we compared the activity of 10-12 days old plantlets of the MPK4$^{D198G/E202A}$ and WT-MPK4 lines after 15 and 30min flg22 stress (1µM) (Figure VI.3). MPK4 protein was immuno-precipitated with anti-c-Myc antibody and assayed against MBP. MPK4$^{D198G/E202A}$ activity in resting conditions was about 10% (Figure VI.3B) of the flg22 induced MPK4 activity in a MPK4 WT line. Additionally, MPK4$^{D198G/E202A}$ activity was increased by flg22 treatment, and its activation was twice more important than WT-MPK4. This result shows that the CA mutations do not hinder the phosphorylation by the upstream MAP2K(s) in flg22 response. After 30 min of flg22 stress, MPK4 de-activation was the same in the MPK4$^{D198G/E202A}$ and the WT-MPK4 lines, meaning that both forms of the kinase have the same sensitivity to phosphatases.

## *D. MPK4$^{D198G/E202A}$ transgene complements the mutation in MEKK1*

MEKK1 is proposed to be the main MAP3K acting upstream of MPK4 in the stress cascade (Ichimura *et al.,* 2006; Nakagami *et al.,* 2006; Suarez-Rodriguez *et al.,* 2007; Gao *et al.,* 2008; Qiu, Zhou *et al.,* 2008). In fact *mpk4*, *mkk1mkk2* and *mekk1* plants have very similar phenotypes, including a very strong dwarfism. Additionally in *mekk1* and *mkk1mkk2* background, MPK4 is not activated anymore by PAMP treatment. If this model is valid and

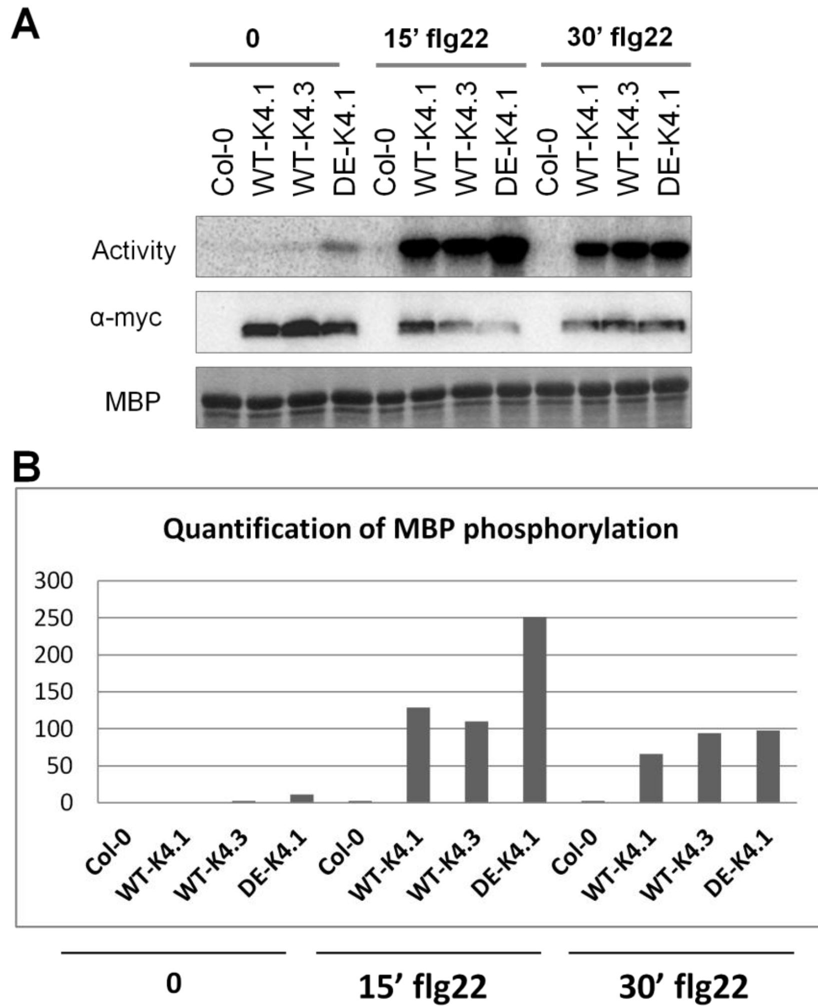



**Figure VI.3 : MPK4 kinase activities immuno-precipitated from MPK4$^{D198G/E202A}$ lines after 15 and 30min of 1µM flg22 treatment.** **A.** MPK4 was immuno-precipitated with an anti c-myc antibody from independent lines expressing MPK4wt (WT-K4 line 1 and 3) and MPK4$^{D198G/E202A}$ (DE-K4 line1) as control together with Col-0. No MPK4 is immunoprecipitated with anti c-myc antibody from Col0 plants. **B.** Quantification of the MBP phosphorylation by MPK4 with the same line.

MPK4$^{D198G/E202A}$ is functionally over active in the CA-MPK4 lines, *MPK4$^{D198G/E202A}$* transgene should complement the morphological growth defect of mutants of the upstream kinases. *mekk1-1* heterozygous plants were crossed with the *MPK4$^{D198G/E202A}$* plants. The F2 plants were genotyped to select for *mekk1-1* homozygous plants containing *MPK4$^{D198G/E202A}$* transgene. The *mekk1* morphological phenotype appears to be largely reverted in the *mekk1-1/mekk1-1 MPK4/MPK4 MPK4$^{D198G/E202A}$* and *mekk1-1/mekk1-1 mpk4-2/mpk4-2 MPK4$^{D198G/E202A}$* plants. Plants of both genotypes had curly leaves and *mekk1-1/mekk1-1 mpk4-2/mpk4-2* MPK4$^{D198G/E202A}$ appears to be less reverted (Figure VI.4). Both lines were able to produce seeds. These plants showed an almost normal growth phenotype when compared to *mekk1-1* plants which are few millimeter tall at maximum and dying after 2-3 weeks in our growth conditions. This confirms that MPK4$^{D198G/E202A}$ is (1) functionally replacing MPK4wt and (2) constitutively active *in planta*. This result brings also more genetic evidence that MEKK1 is an important upstream step in the pathogen related MPK4 cascade and provide a very promising tool to better understand MEKK1-MKK1/MKK2-MPK4 pathway functions.

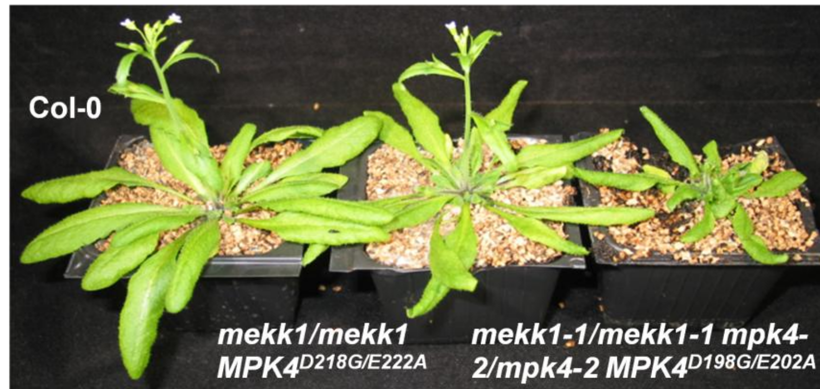



**Figure VI.4 : Morphological phenotype of *mekk1-1/mekk1-1 MPK4$^{D198G/E202A}$* (middle) and *mekk1-1/mekk1-1 mpk4-2/mpk4-2 MPK4$^{D198G/E202A}$* (right) lines compared to wild type Col-0 plants (left).** Plants are 5 weeks old. In our conditions, mekk1-1 homozygous plants do not succeed to develop further 1 cm tall and dies after 3 weeks.

# E. Discussion

### 1. Not all CA MPK4 could trigger hyperactivity in planta

Both forms of CA MPK4 locus (Y119C and D198G/E202A) complemented *mpk4-2* dwarfed phenotype but only MPK4$^{D198G/E202A}$ plants showed an increased MPK4 activity. This morphological phenotype rescue was not necessary the consequence of an increased MPK4 activity, since the MPK4$^{Y119C}$ form which did not increase the level of MPK4 further than the WT kinase complemented the morphological alterations of *mpk4* mutant plants. It was previously shown that *mpk4* mutant phenotype is not only due to the loss of MPK4 protein but also to the lack of basal level of the MPK4 activity in the cell. *mpk4* mutant cannot be complemented by the catalytically inactive and non phosphorylable mutant MPK4$^{T201A/Y203F}$, neither by the catalytically inactive and phosphorylable mutant MPK4$^{K72R}$ (Petersen *et al.,* 2000; Brodersen *et al.,* 2006). And this non complementation concerns not only the dwarfism, but also the SA accumulation and the PR gene expression which remains high. All this data suggest that MPK4 basal activity is important for non stress dependant phenotype.

The fact that MPK4$^{Y124C}$ does not have a higher activity than MPK4wt, whereas MPK4$^{D198G/E202A}$ does *in vivo*, could be linked to the mutation location. Phosphatase activities are thought to be involved in negative feedback mechanisms down regulating MAPK activity in plants. Expressing a CA MAPK in plants could turn on this negative regulatory process which may explain that some CA MAPK mutants do not show apparently an increase activity level. The fact that MPK4$^{D198G/E202A}$ is not affected by this negative regulation could come from the position of the D198G/E202A mutations in the vicinity of the TEY motif, direct target of the phosphatases.

Interestingly, the MPK4 Y124 residue was found in a chemical-genetic screen to induce ATP binding pocket enlargement when mutated to glycine residue and enhance their affinity to specific inhibitors. MPK4$^{Y124G}$ mutant form was used to conditionally complement *mpk4* mutant (Brodersen *et al.,* 2006)MPK4$^{D198G/E202A}$ activity increase in response to flg22

D198G/E202A mutations resulted in only 10% of the flg22-elicitated MPK4wt activity. It was actually expected to not obtain mutations that fully activate the MAPK in our screen since very active mutant of MAPK are known to kill the yeast (Yaakov *et al*., 2003).

In the CA-MPK4 lines, $MPK4^{D198G/E202A}$ activity is increased by flg22 stress. This suggests that D198G and E202A mutations, that we suspected to modify phosphatase ability to dephosphorylate MPK4, do not hinder the phosphorylation by the MAP2Ks. We already showed that the physical interaction between $MPK4^{D198G/E202A}$ and MKK2, the upstream activator of MPK4 is maintained. The second scenario could be that D198G/E202A mutations might induce an intermolecular phosphorylation in stress conditions, increasing further its activity. It was shown in animal system that active MAP2K could further increase the activity of CA-MAPKs (Diskin *et al*., 2004). Interestingly, preliminary results suggest that $MPK4^{D198G/E202A}$ had also a stronger activity in response to 15 min flg22 than WT-MPK4 (Figure VI.3).

The increase of $MPK4^{D198G/E202A}$ activity with flg22 treatment is an important difference with CA MAP2K lines published so far in which phospho-mimicking mutations were used to render the MAP2Ks CA (Brader *et al*., 2007). Since the MAP2K are not phosphorylable anymore, they cannot be further activated when the cascade is switched on. In our case, MPK4 is active in resting conditions but can be further activated by flg22. The MEKK1-MKK1/MKK2-MPK4 cascade is primed but not constitutively activated. The interpretation of phenotypic results should consider this particular point. Alternatively, in *mekk1-1/mekk1-1* $MPK4^{D198G/E202A}$ plants, the output activity of MEKK1-MKK1/MKK2-MPK4 module is only dependant on $MPK4^{D198G/E202A}$ activity but should not be increased anymore by flg22.

### *2. CA MPK4 lines have a WT phenotype*

As far as we know, $MPK4^{D198G/E202A}$ plants are morphologically similar to WT Col-0 plants. This was not really expected if we consider all the data published on MPK4 function (Brodersen *et al*., 2006; Gao *et al*., 2008; Petersen *et al*., 2000; Su *et al*., 2007). *mpk4* mutant plants are constitutively stressed and therefore resistant plants (at least to biotrophic pathogen) and MPK4 basal activity appears to be important to restore this phenotype (Brodersen *et al*., 2006; Petersen *et al*., 2000). MPK4 is considered as a negative regulator of basal immunity. Complementing *mpk4* with a CA-MPK4 would result in down-regulation of the defense responses controlled by MPK4 and constitutively sick plants. This phenotype would have been obtained if MPK4 was fully constitutively activated in our plants. However because we choose to mimic a natural MPK4 expression we opt for a native promoter expression. In this case

expressing CA-MPK4 under the 35S strong promoter might be useful to try to rich a maximum MPK4 activation. It could be also interesting to generate lines that strongly express CA-MPK4 but under inducible promoter, to be able to directly induce the expression of an active MPK4 without being in particular stress conditions.

### 3. *MPK4$^{D198G/E202A}$ rescues the phenotype of its cognate MAP3K mutant*

Complementing *mpk4* mutant with MPK4$^{D198G/E202A}$ proved that we can use the CA mutations identified in the screen we performed to activate MAPKs *in vivo* and to restore alterations due the loss of a kinase activity. Using the CA-MPK4 we were able to phenotypically restore a close to normal growth the *mekk1* dwarfed phenotype (Suarez-Rodriguez *et al*., 2007). This gives more genetic evidence that MEKK1 and MPK4 are in the same signaling cascade, since the MPK4 activity is the responsible of *mekk1* complementation. This strategy was previously used to decipher the MAPK cascade downstrem of YODA in stomatal patterning (Wang 2007). Though this is only a preliminary data, we need to verify that CA-MPK4 is indeed active in the *mekk1-1/mekk1-1* CA-MPK4 even if it is strongly expected. As it is a very interesting tool, I will give details on what we are planning to investigate with these lines in a specific section of my perspectives.

# Chapter VII
# MPK4 function in plant pathogen responses

# *Chapter VII - Utilization of MPK4$^{D198G/E202A}$ lines to address MPK4 function in pathogen responses*

The lines we created, are interesting tool to investigate MPK4 function *in planta*. As mentioned in the introduction, MPK4 has a negative role in regulating Systemic Acquired Resistance (SAR). *mpk4* mutant plants over-accumulate ROS and Salicylic Acid (SA), constitutively express SA responsive marker genes like PR1 and PR2 and therefore exhibit higher resistance to biotrophic pathogens (Petersen *et al*., 2000). In *mpk4* mutants, the Methyl Jasmonate induced expression of PDF1.2 is abolished (Petersen *et al*., 2000). This finding was supported by the enhanced sensitivity of *mpk4* towards infection with *Alternaria brassicicola* showing that MPK4 is also required for ET/JA mediated defense pathways. Regarding all the data published on *mpk4*, we hypothesized that MPK4$^{D198G/E202A}$ lines would behave in an opposite way to *mpk4* mutant plants when challenged by pathogen.

## *A. PAMP inhibition assay in MPK4$^{D198G/E202A}$ lines*

MPK4 have been extensively shown to be an important player in basal immunity (Peterson *et al*., 2000). MPK4 activity is important for these responses since the complementation of *mpk4* mutant plants with a dead version of MPK4 (MPK4$^{AEF}$) was not successful to restore wild type phenotype (Brodersen *et al*., 2006). Growth inhibition was measured in response to flg22 in order to investigate long term PAMP responses. 7 days old seedlings grown in Agar MS media were transferred into liquid MS medium supplied with increasing flg22 concentrations (0, 10, 100 and 1000 nM). Plant weight was measured 7 days after. We observed that flg22 in the media reduced the growth of roots, leaves and cotyledons (Figure VII.1A). This inhibition was dose dependant, about 50% growth decrease with 100nM flg22 for Col-0 plants and lines expressing either MPK4$^{D198G/E202A}$ or WT-MPK4 (Figure VII.1A). This observation was confirmed by fresh weight measurements (Figure VII.1B). Prolonged incubation of the seedlings in the presence of flg22 resulted in dwarfed plants at higher concentrations. Similar weight values were established for Col-0, WT MPK4 and MPK4$^{D198G/E202A}$ plants. However, whereas the seedlings remained

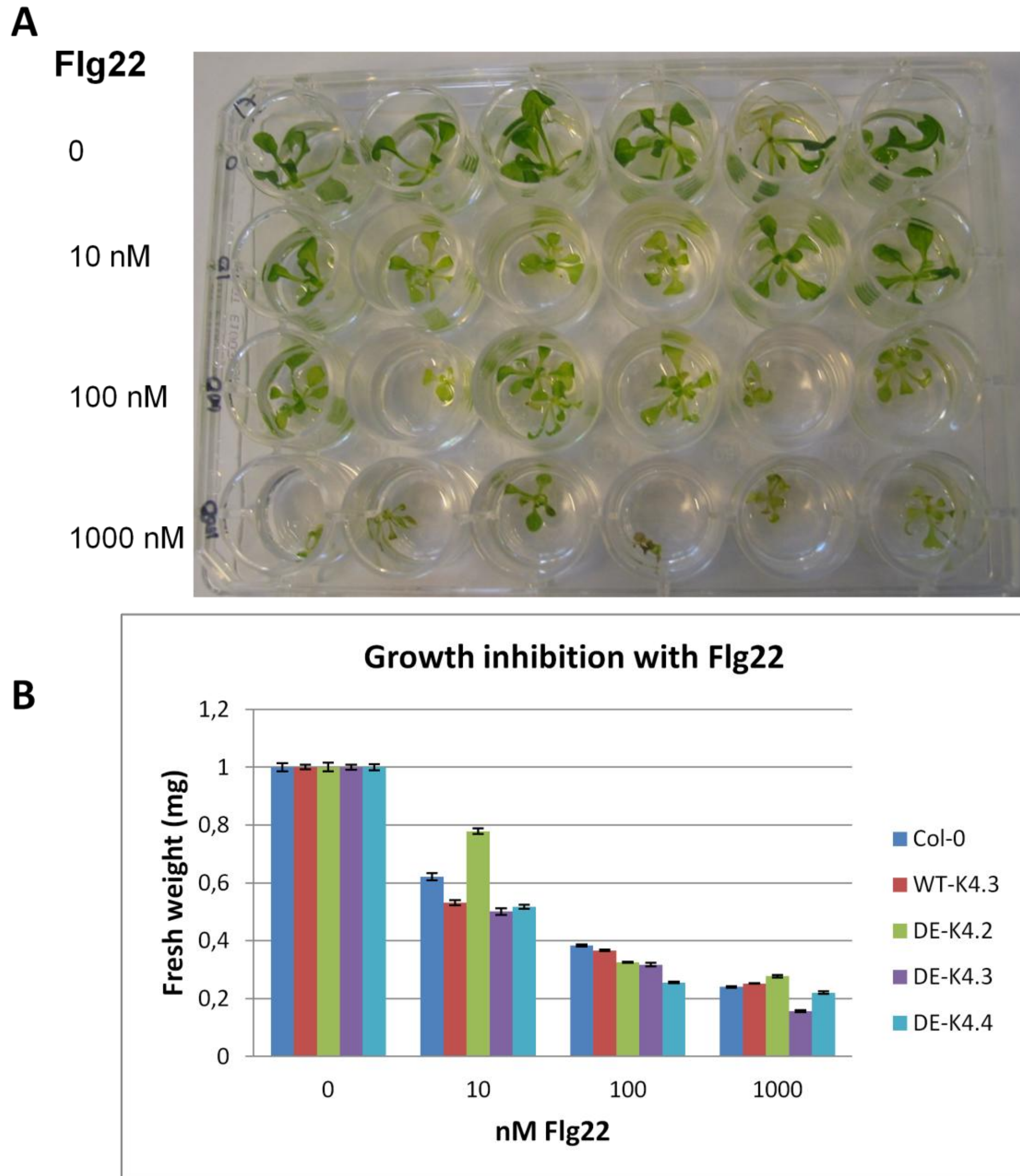
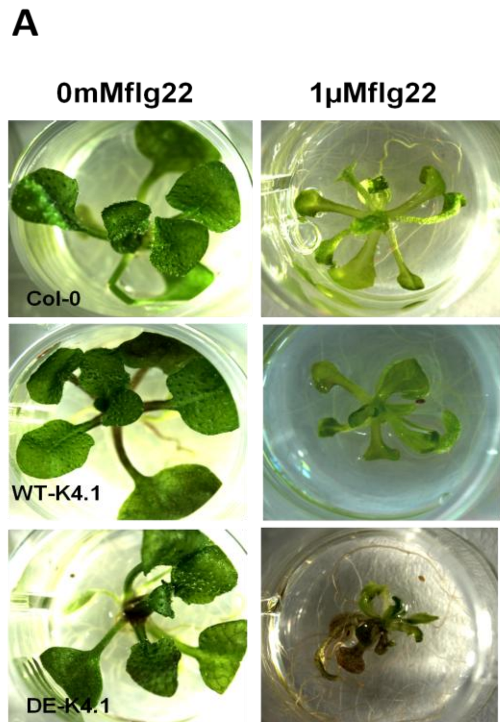



**Figure VII.1: Effect of flg22 on CA-MPK4 seedlings growth. A.** 6 days old seedlings of CA-MPK4 ($MPK4^{D198G/E202A}$ independent lines referred as DE-K4.2, 3 and 4), WT-MPK4 and Col-0 were incubated for 7 days in liquid MS medium supplemented with increased flg22 concentration (0, 10, 100, 1000nM). The plate picture represents the growth inhibition observed with line DE-K4.3. **B.** Dose dependant of growth inhibition caused by flg22, after 7 days of treatment the fresh weight of individual seedlings was measured. Bars show average and standard deviation of n=6 seedlings/ treatment. The same results were obtained for at least 3 independent replicates.

green for Col-0 and WT-MPK4 lines, the plants complemented with *MPK4*$^{D198G/E202A}$ forms, were dwarfed as well, but displayed brown areas on the cotyledons and on the leaves (Figure VII.2A). Staining of these plantlets with lactophenol trypan blue showed that these areas were dead tissues. The coloration was more pronounced in the MPK4$^{D198G/E202A}$ lines compared to WT-MPK4 (Figure VII.2B) suggesting that cell death induced by flg22 is increased in the MPK4$^{D198G/E202A}$ lines.

## B. Plants expressing MPK4$^{D198G/E202A}$ have an increased susceptibility to the biotrophic pathogen Pseudomonas syringae

To investigate further MPK4 function in plant pathogen responses, homozygous *mpk4* plants expressing *MPK4*$^{D198G/E202A}$ were used to assess their resistance to the hemi-biotrophic bacterial pathogen *Pseudomonas syringae* pv *tomato (Pst)* DC3000 which causes leaf speck disease both on tomato and *Arabidopsis* (Hoon Sohn *et al*., 2009). 4 week old plants grown under short day conditions were spray-inoculated with virulent *Pst* DC3000 ($1\times10^7$ cfu/ml in 10 mM $MgCl_2$). Bacterial titers were measured at 0 and 3 days post inoculation (dpi) in the MPK4$^{D198G/E202A}$ and WT-MPK4 lines(Figure VII.3). As positive control, we used the *enhanced disease susceptibility 1* (*eds1*) mutant which is impaired in pathogen-induced SA accumulation and therefore in the SA-dependant immune responses (Falk *et al*., 1999; Clarke *et al*., 2001). After infection, no striking differences were observed in the development of disease symptoms between the different transgenic lines and Col-0, whereas *eds1* mutant plants showed enhanced disease symptoms as expected (Figure VII.3). However, bacterial quantification at day 3 post inoculation showed that the two WT-MPK4 lines behaved like Col-0 plants whereas MPK4$^{D198G/E202A}$ lines showed significantly higher growth of *Pst* DC3000 ($P \leq 0.05$, Student's *t* test). The observed susceptibility of CA lines is not as pronounced as *eds1* plants though. These data indicate that MPK4 is involved in defense response to the biotrophic pathogen *Pseudomonas syringae,* which could be related to the deregulation of SA pathway present in the *mpk4* mutant (Brodersen *et al.,* 2006).



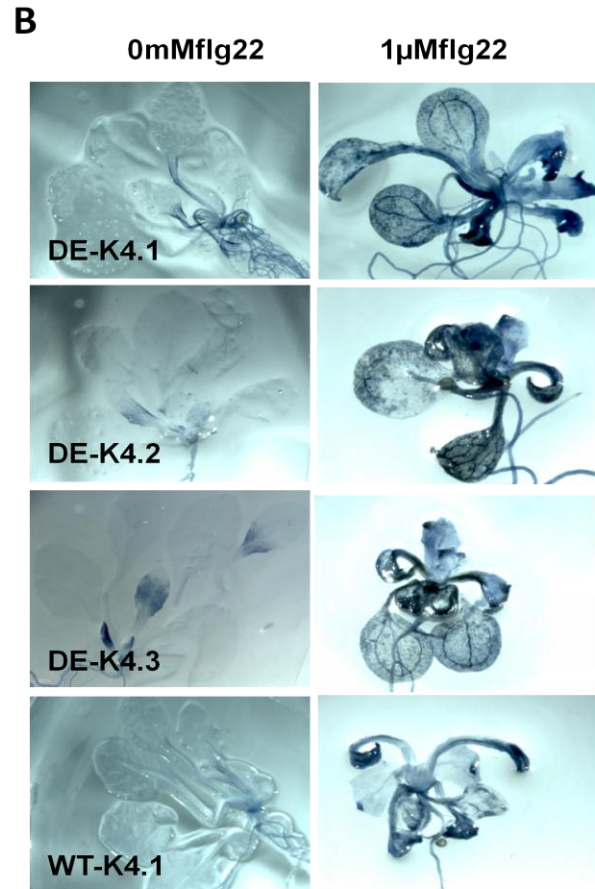




**Figure VII.2: Growth defects occasioned by flg22 observed on CA-MPK4 seedlings. A.** Six days old seedlings of lines expressing $MPK4^{D198G/E202A}$ (DE-K4.1, 2 and 3) or WT MPK4 (WT-K4.1) and Col-0 incubated for one week in MS medium supplemented or not with 1µM of flg22. **B.** Visualization of seedlings damage occasioned by flg22 with lactophenol trypan blue staining.

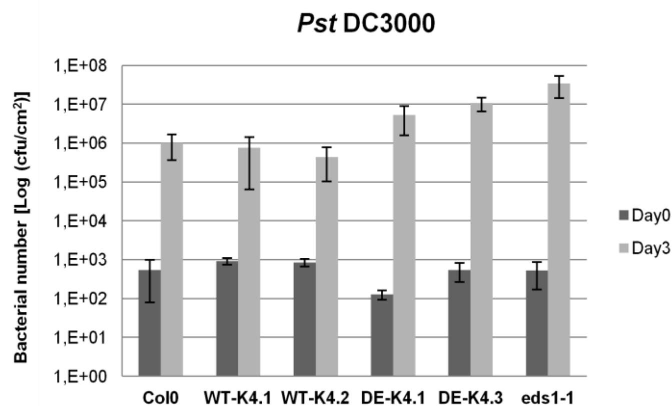



**Figure VII.3: CA-MPK4 response to infection with *Pst* DC3000.** Four week-old plants expressing WT (WT-K4.1 and 2) or $MPK4^{D198G/E202A}$ (DE-K4.1 and 3) together with Col-0 and *eds1-2* mutant plants were spray inoculated with *Pst* DC3000 and bacterial titers from 4 technical replicates were determined 2h (dark grey bars) or 3 days (light grey bars) post infection (at least 2 biological replicates were performed for each line).

## C. MPK4$^{D198G/E202A}$ lines have an increased susceptibility to both pathogen mutated in the T3SS and avirulent pathogens

Plants defend themselves against microbial invasion by two layers of defense responses. The first line of active defense is induced after PAMPs perception (PTI). The second is induced after effector recognition by the plant through resistance proteins (ETI) (Hein *et al.,* 2009). Different pathogen strains impaired in the first or the second kind of pathogen elicitor are available to specially trigger one particular type of resistance response in plants. In order to dissect further MPK4 function in plant defense responses, I used *Pst* DC3000 *hrcC* mutant defective in the T3SS, and is then impaired in effector delivery. Pst DC3000 *hrcC* induces mainly PTI. I used also an avirulent strain of *Pst* DC3000 expressing the effector AvrRps4. AvrRps4 is a bacterial effector known to suppress PTI and recognized in *Arabidopsis* by the TIR-NB-LRR RPS4 receptor to mainly trigger ETI (Wirthmueller *et al.,* 2007). Using these two strains, we aimed to investigate further in which layer(s) of the plant defense response MPK4 is involved.

Similarly than with virulent *Pst* DC3000, 4 week old plants were spray-inoculated with a bacterial suspension of *Pst* DC3000 *HrcC* ($1\times10^8$ cfu/ml in 10 mM $MgCl_2$) and bacterial titers quantified at 0 and 3 days post inoculation. MPK4$^{D198G/E202A}$ plants were significantly more susceptible ($P \leq 0.05$, Student's *t* test) than WT plants (Figure VII.4). In a second set of infection assay, I used the *Pst* DC3000 AvrRps4 ($1\times10^8$ cfu/ml in 10 mM $MgCl_2$) bacterial strain. At 3 days post inoculation, the MPK4$^{D198G/E202A}$ lines showed more disease symptoms such as leaf yellowing and necrosis than WT-MPK4 lines or Col-0 (Figure VII.5A). This phenotype was more pronounced in *eds1* mutant plants. Bacterial quantification confirmed that MPK4$^{D198G/E202A}$ lines are, like *eds1,* significantly more susceptible to *Pst* DC3000 AvrRps4 ($P \leq 0.01$, Student's *t* test) which allowed between 1.5 and 2 log unit increase of bacterial growth compared with Col-0 and the WT-MPK4 complemented lines (Figure VII.5B). The recognition of the AvrRps4 protein in MPK4$^{D198G/E202A}$ lines does not result in a resistance reaction like in WT plants. The development of cell death was further examined in the different lines using lactophenol trypan blue staining of leaves at 3 days post inoculation (Figure VII.5C). MPK4$^{D198G/E202A}$ plants displayed enhanced staining usually corresponding to cell death compared to Col-0 plants and WT-MPK4 lines. The necrosis observed might be due to the resistance triggered by RPS4-

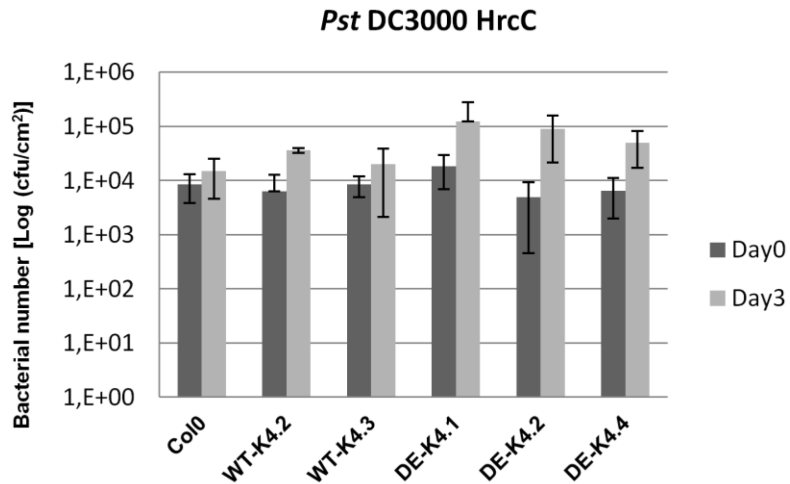



**Figure VII.4: PTI response in CA-MPK4:** Four week-old plants expressing WT (WT-K4.2 and 3) or MPK4$^{D198G/E202A}$ (DE-K4.1, 2 and 4) were spray inoculated with *Pst*DC3000 *HrcC* and bacterial titers from 4 technical replicates were determined 2h (dark grey bars) or 3 days (light grey bars) post infection (at least 2 biological replicates were performed for each line).

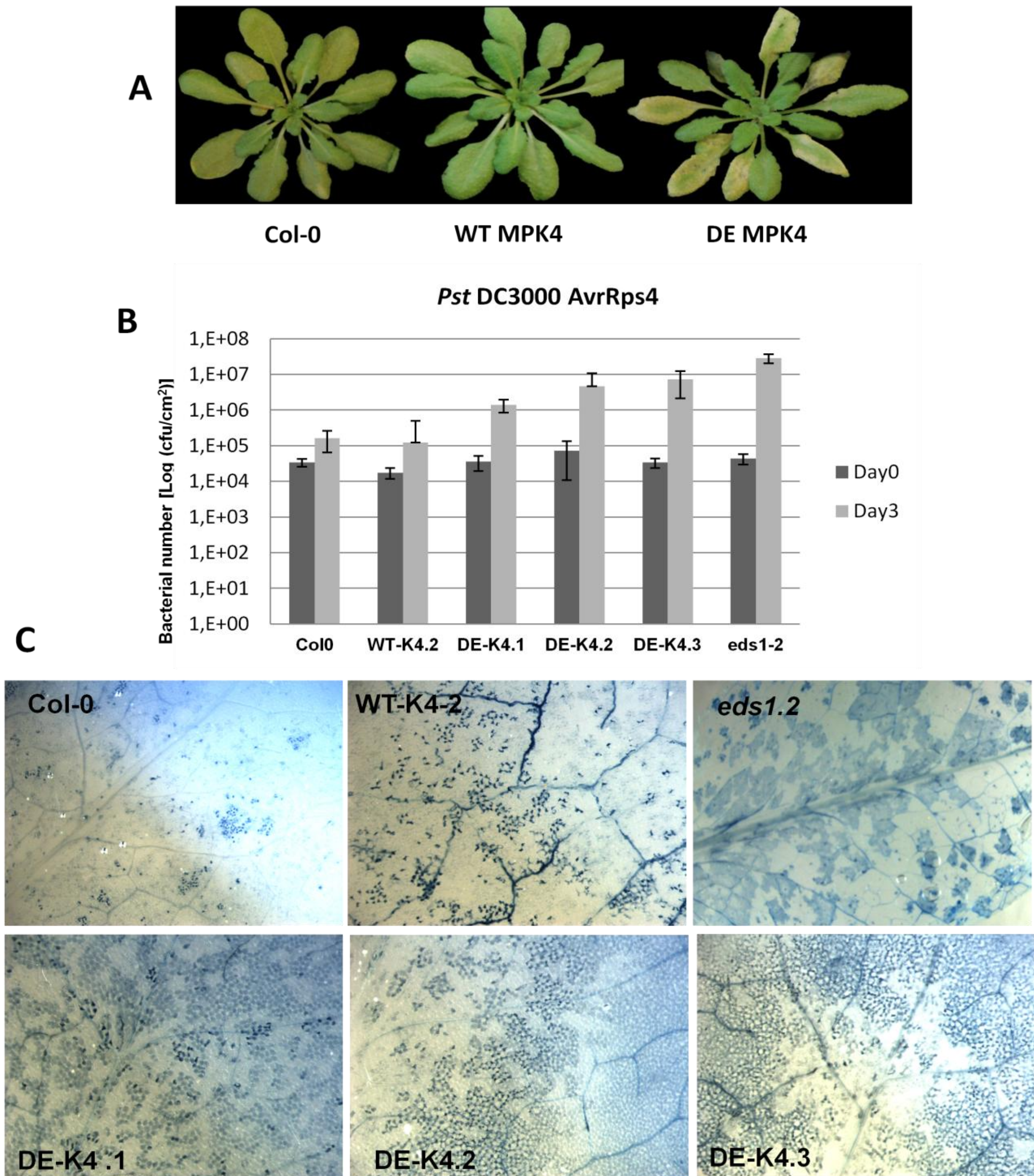



**Figure VII.5: ETI response in CA-MPK4: A.** Disease symptoms observed on 4 week-old plants at days 3 post infection with *Pst*DC3000 AvrRps4. **B.** Bacterial titers determined 2h (dark grey bars) and 3 days (light grey bars) on WT (WT-K4.2) or MPK4$^{D198G/E202A}$ (DE-K4.1, 2 and 3) together with Col-0 and *eds1-2* mutant after spray inoculation with *Pst*DC3000 AvrRps4. **C.** Visualization of dead cells with trypan blue staining in leaves of 4 week old plants after infection with *Pst*DC3000 AvrRps4.

mediated recognition, and therefore reflect an enhanced HR, it could alternatively reflect the enhanced disease symptoms due to higher bacterial colonization.

To answer this question, I examined the reaction of the different MPK4$^{D198G/E202A}$ lines to a TIR-NB-LRR mediated resistance of a distinct biotrophic pathogen, the Oomycete *Hyaloperonospora arabidopsidis (Hpa)*. MPK4$^{D198G/E202A}$ lines were tested for RPP4-mediated resistance by inoculating with Emwa1 isolate of *Hpa*. The *RPP4* gene is present in the *Arabidopsis* Col-0 ecotype and absent in Ws-0, and therefore *Hpa* Emwa1 is avirulent in Col-0 and virulent in Ws-0 (Slusarenko & Schlaich 2003). 2 weeks-old seedlings were spray inoculated with an *Hpa* conidiospore suspension and 6 days after inoculation leaves were stained with lactophenol trypan blue to visualize pathogen growth and plant cell death. As shown in Figure VII.6, WT-MPK4 lines produced discrete HR lesions as well as some trailing necrosis at pathogen infection sites, similar to those observed in the Col-0 leaves. MPK4$^{D198G/E202A}$ lines showed reduced resistance to *Hpa,* manifested by increased trailing necrosis surrounding pathogen hyphae. Cell death lesions in *mpk4* mutant were very important, demonstrating, as previously reported (Petersen *et al.,* 2000), the deregulation of cell death programs. As expected, *eds1* mutant and Ws-0 plants showed enhanced susceptibility manifested by the free hyphae growth and spore production (Holub *et al.*, 1994; Coates and Beynon, 2010). These results lead us to conclude that the enhanced cell death observed after *Pst* DC3000 AvrRps4 infection on MPK4$^{D198G/E202A}$ lines is not due to a deregulation of the hypersensitive response but rather reflects the hyper susceptibility of the plant resulting in a cell necrosis.

Taken together, these results indicate that MPK4 is not only involved in PTI like it was described before (Petersen *et al.,* 2000), but could also play a role in ETI.

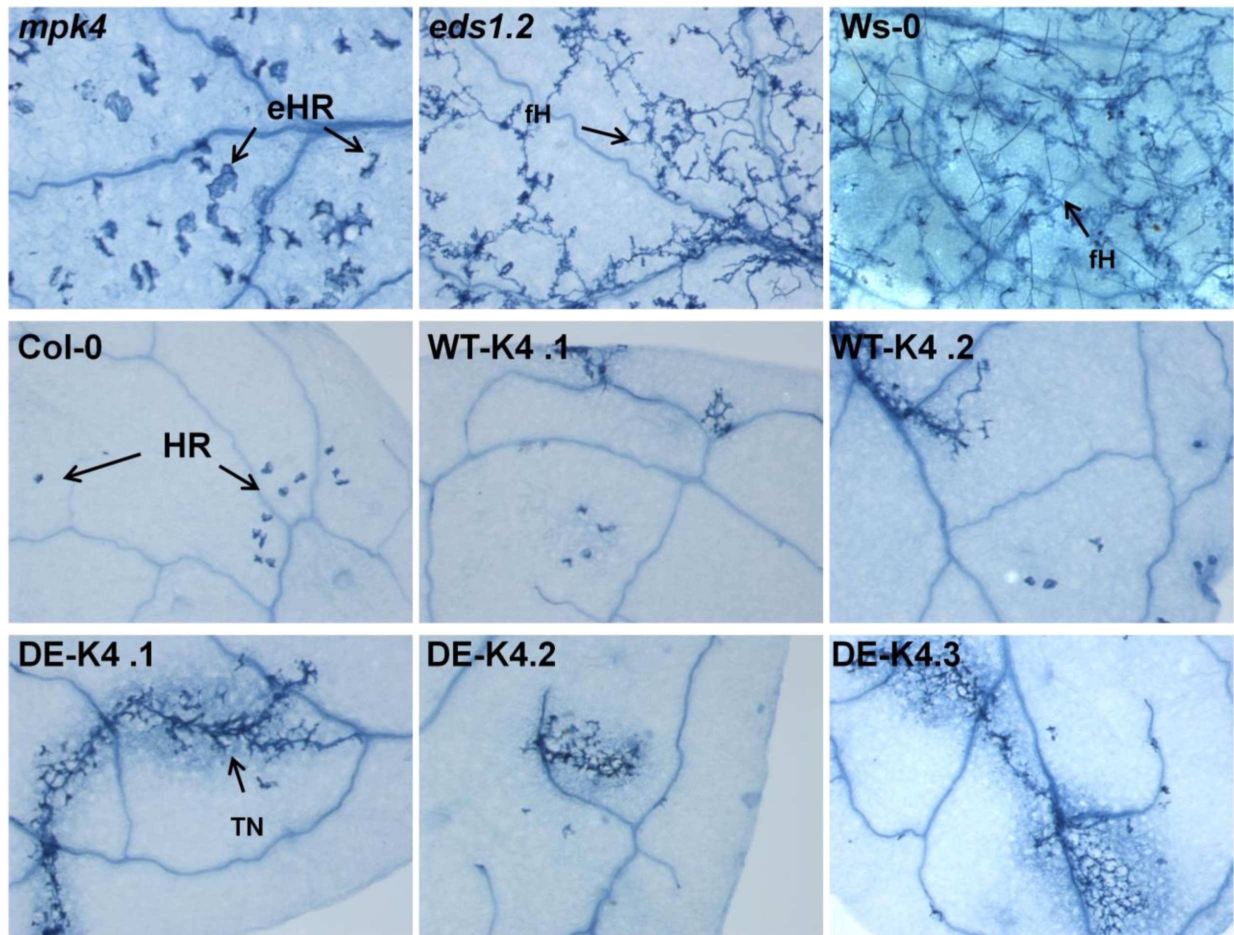


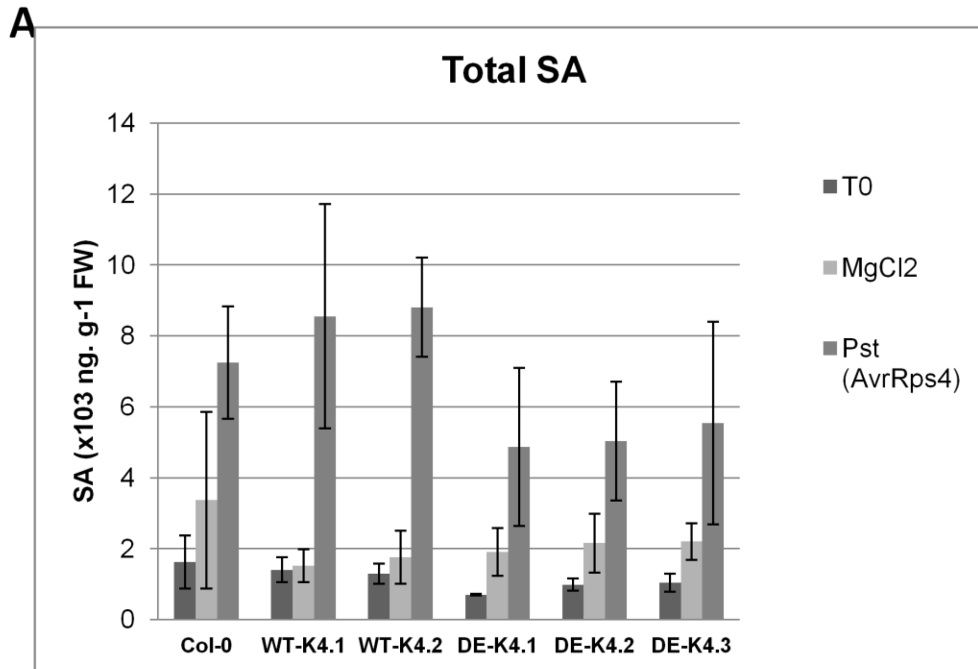

**Figure VII.6: Resistance phenotypes of MPK4$^{D198G/E202A}$ lines and the corresponding controls (*mpk4* Col-0, Ws-0, *eds1.2* and WT MPK4: lines WT-K4.1 and 2) to *H. parasitica* Emwa1 (RPP4):** Visualization of pathogen growth and dead cells with lactophenol trypan blue staining in leaves of 2 week-old plants, 6 dpi with avirulant *H. parasitica* Emwa1. HR: hypersensitive response associated cell death; her: enhanced HA; TN: trailing necrosis; fH: free pathogen hyphae.

## D. *CA-MPK4 transgenic lines under-accumulate SA in response to pathogen challenging*

*mpk4* plants are severely dwarfed and exhibit constitutive systemic acquired resistance (SAR) linked to an elevated amount of salicylic acid (SA). The fact that reducing the endogenous SA levels via the expression of a bacterial salicylate hydroxylase gene (nahG) rescues the majority of the *mpk4* mutant phenotypes, indicates that the elevated SA levels largely account for these phenotypes (Petersen *et al.,* 2000).

We then tried to understand if the impaired defense responses in the MPK4$^{D198G/E202A}$ expressing lines were linked to SA hormone miss-accumulation. SA amount was monitored in 4 week old plants without any treatments, 24 hours after *Pst* DC3000 AvrRps4 inoculation or after buffer spraying (mock $MgCl_2$) (Figure VII.7A). In untreated plants, the SA levels were half less in MPK4$^{D198G/E202A}$ lines compared to Col-0 and WT-MPK4 lines. This indicates that constitutive activation of MPK4 decreased furthermore the residual SA level below the normal average. 24 hours after *Pst DC3000 AvrRps4* spray inoculation, we observed a 30% decrease of SA accumulation in MPK4$^{D198G/E202A}$ lines compared to control plants. This result points to the negative effect of MPK4 over-activation on SA accumulation and could therefore explain the enhanced disease response to the biotrophic pathogen we observed.

SA accumulation in *mpk4* is known to induce PR1 gene expression (Petersen *et al*., 2000). The expression of some PR gene was then investigated using semi-quantitative PCR technology, before and after *Pst* DC3000 infection in MPK4$^{D198G/E202A}$ and WT-MPK4 plants. PR1 transcript appears to accumulate half less in the MPK4$^{D198G/E202A}$ lines compared to Col0 and to WT-MPK4 plants (Figure VII.7B). More genes were tested in a quantitative RT-PCR like PDF1.2, SID2, PDF1.2 and EDS1, however the results were not really convincing and not reproducible. This might be due to high expression variability between the different lines hiding the effect triggered by CA mutations. Nevertheless a tendency to a reduced expression of PDF1.2, PAD4 and EDS1 was noticed (data not shown).



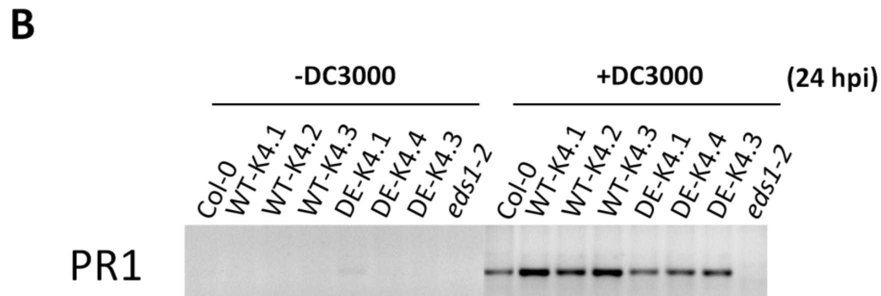




**Figure VII.7: Quantification of salicylic acid (SA) content and PR1 gene expression in CA-MPK4 lines: A.** SA (ng/g fresh weight+/-SE) was measured in leaves of 4 week-old plant expressing WT MPK4 (WT-K4.1 and 2) or MPK4$^{D198G/E202A}$ (DE-K4.1, 2 and 3) with Col-0 as control before (T0) and after spray inoculation with *Pst* DC3000 AvrRps4 or the mock treatment ($MgCl_2$). **B.** Accumulation of mRNA in Col-0 and lines expressing WT MPK4 and MPK4$^{D198G/E202A}$ 24h after *Pst*DC3000 infection.

## ***E. CA-MPK4 lines have an increased susceptibility to the nectrotrophic pathogen Altenaria***

In contrast to SA pathway, the induction of jasmonate responsive genes is blocked in *mpk4* plants (Petersen *et al*., 2000), suggesting that while MPK4 negatively regulates SA-mediated defense, the SA/JA balance being affected in favor of SA in the mutant, results in negative regulation of JA-mediated defense responses. To answer the question whether the decreased SA hormone level in $MPK4^{D198G/E202A}$ lines, due to the increased activity MPK4 could affect the JA mediated resistance pathway, I investigated the response of $MPK4^{D198G/E202A}$ lines to the necrotrophic fungus *Altenaria brassicicola* (Figure VII.8).

Infection was performed on 6 week old plants to have large rosette leaves by dropping spore suspension (ca. $5x10^5$ spores/ml) on the leaf blades. Leaves were then cut at the base of the petiole and kept in agar media to ensure high humidity. Col-0 plants, which are resistant to *A. brassicicola*, developed small necrotic spots at the infection sites. To quantify the level of damage, the size of the necrotic area around every infection site was estimated 7 days post infection (dpi). Four different categories of damage were distinguished based on a modified evaluation system published (Brader *et al.,* 2007):

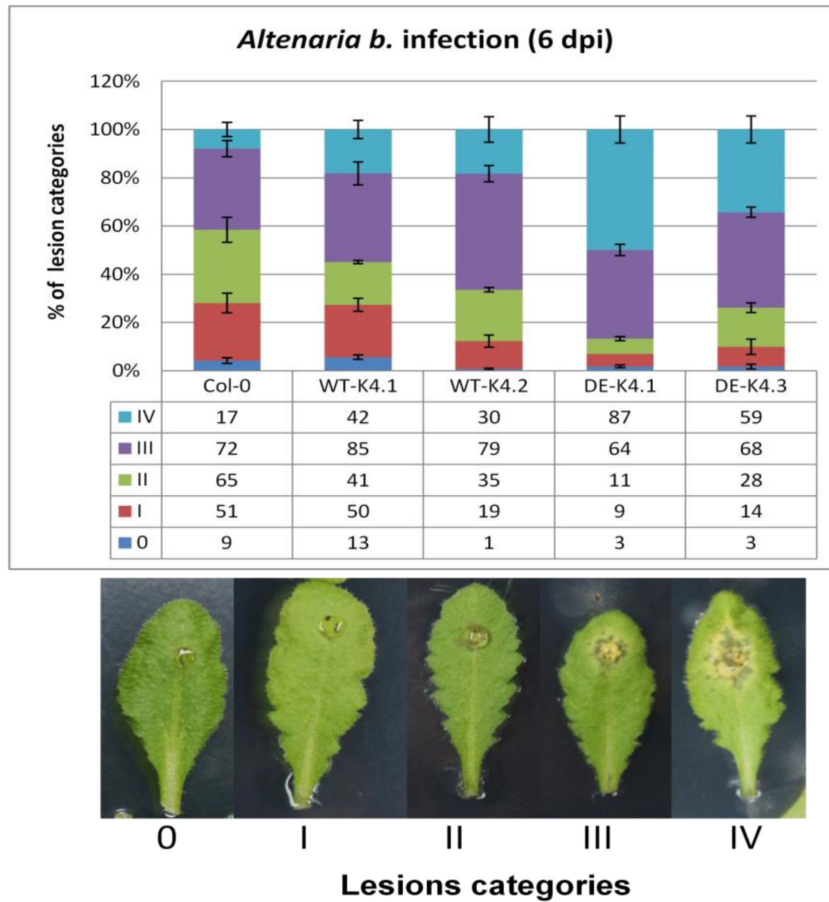
Necrosis type I: the necrotic area is smaller than the droplet size

Necrosis type II: the necrotic area covers the infection site

Necrosis type III: the necrotic area expand the infection site

Necrosis type IV: about 25% of the leaf area start show necrosis

We observed that both $MPK4^{D198G/E202A}$ lines tested tend to display an increased sensitivity towards *A. brassicola* compared to Col-0 and WT-MPK4 plants. They both have developed more necrosis of type III and IV compared to the WT plants indicating that the pathogen can easily grow on the leaves.




| | Col-0 | WT-K4.1 | WT-K4.2 | DE-K4.1 | DE-K4.3 |
|---|---|---|---|---|---|
| IV | 17 | 42 | 30 | 87 | 59 |
| III | 72 | 85 | 79 | 64 | 68 |
| II | 65 | 41 | 35 | 11 | 28 |
| I | 51 | 50 | 19 | 9 | 14 |
| 0 | 9 | 13 | 1 | 3 | 3 |

**Figure VII.8: CA MPK4 lines to *Altenaria brassicola* infection:** quantification of damage by evaluation of the necrotic area size (see lesions categorie). Shown is a summary of the results of 3 independent experiments.

## *F. Discussion*

In this last chapter I describe my experiments using the MPK4$^{D198G/E202A}$ lines to understand MPK4 function in defense responses using mainly some well characterized strains of biotrophic and necrotrophic pathogens. My results concerning the negative role of MPK4 in response to PAMP confirm the previously published data on MPK4 function. These studies took mainly advantage of *mpk4* and related mutants but as their phenotypes are very severe, suggesting a long cascade of events triggered by the KO mutations, the results are still matter of debate in the scientific community. My study correlates with these data, but is based on WT-looking complemented lines in which the mutation-induced perturbation is less deleterious. This is a proof of concept that CA mutations in MPK4 are very useful tool to address their function *in planta*.

### *1. CA-MPK4 lines respond to PAMP*

Growth inhibition by flg22 was not statistically affected in these lines, if we consider the weight of the plantlets after 7 days. Given the response variability between plants of the same genotype, a mild difference between lines might be difficult to observe. However, MPK4$^{D198G/E202A}$ lines exhibited reproducibly brown tissue compared to the WT lines when grown with 1µM flg22. Using a cell death specific dye, the brownish tissues were all stained but we don't know whether this represent really a disease necrosis or plant programmed cell death in response to the PAMP. Characterization of molecular marker of specific cell death or the use of specific mutants could help to clarify this feature.

It is not clear in the literature how is growth inhibition related to the induction of defense response. The stress inhibition of growth could be related a redirection of the available cellular resources to defense processes or to a programmed repression of processes important for growth (Gomez-Gomez *et al*., 1999). It was already proposed that some elicitors could increase the secondary metabolites (Chaudhuri *et al.,* 2009), the brownish tissues suggest an increased accumulation of flavonoid and phenolic compounds. Flavonoids and polyphenols are important antioxidants compounds acting as signal molecules and have defensive or stimulant roles in the plant. MYC2 transcription factor, which is an important modulator of JA-mediated responses,

was shown to be a positive regulator of important components in JA-mediated flavonoid biosynthesis (Dombrecht *et al.,* 2007).

It could be interesting, to test, after having directly confirmed the metabolite over-accumulation in $MPK4^{D198G/E202A}$ lines, if this response is dependent on JA and related to a balance change in the SA/JA content. These questions could be answered by measuring the JA content in the $MPK4^{D198G/E202A}$ plantlets after flg22 inhibition.

## *2. MPK4 is a negative regulator of PTI*

Although MPK4 is activated by PAMPs, the phenotype of *mpk4* suggests that MPK4 is a negative regulator of defense responses. *mpk4* mutant has an elevated level of SA, accumulates PR transcripts and has increased resistance toward biotrophic pathogens like *Pseudomonas syringe* and *Hyloperonospora parasitica*, but increased susceptibility to the necrotrophic pathogen *Altenaria brassicola* (Petersen *et al.,* 2000; Brodersen *et al.,* 2006). Biochemical studies (Qiu, Fiil *et al.,* 2008) also suggest that MPK4 in resting conditions sequesters its substrates MKS1 and the transcription factor WRKY33. After elicitation, MKS1 is phosphorylated and WRKY33 released. WRKY33 can then activate camalexin biosynthesis pathway via the regulation of *PAD3* gene expression. However, this module is also functional as in the absence of MPK4, WRKY33 is permanently released from the complex.

Using the hemibiotrophic pathogen *Pst* DC3000 WT strain and the T3SS mutant strain *hrcC,* we saw an opposite phenotype of *mpk4* in the $MPK4^{D198G/E202A}$ lines. This finding brings more evidence that MPK4 is a negative regulator of basal defense because in CA lines MPK4 is already active. This result does not really fit Mundy's model, because, in our transgenic lines, we would expect MPK4 to constitutively phosphorylate its substrate MKS1 and WRKY33 to be permanently released from this complex to activate PAD3 expression. In this situation $MPK4^{D198G/E202A}$ lines would have been more resistant to *Pseudomonas* like the mutant plants, because of the importance of camalexin secondary metabolites in the resistance to biotrophs (Glazebrook *et al.,* 1996; Glazebrook *et al.,* 1997).

### *3. Is MPK4 targeted by effectors to shut down defense responses?*

An avirulent strain of *Pst* DC3000 (AvrRpm1) activates MPK4 in the same way a virulent strain does (Qiu, Fiil *et al.,* 2008). This result was never interpreted by the possible implication of MPK4 in ETI response.

MPK4$^{D198G/E202A}$ transgenic lines were more susceptible toward all strains of *Pst* DC3000 including the avirulent strain *Pst* DC3000 AvrRps4. Because MPK4 is constitutively active in these lines, we concluded that MPK4 could also function in RPS4-AvrRps4 mediated resistance. Moreover MPK4$^{D198G/E202A}$ lines sustain the development of the avirulent *Hpa* Emwa1 hyphea and exhibited trailing necrosis. These results suggest that CA-MPK4 lines are impaired in ETI response mediated by RPP4 receptor too.

To circumvent PTI successful pathogens have evolved T3SS system to inject effector proteins. However virulent pathogens face the parallel evolution of plant receptors and continuously have to develop new mechanisms to shut down basal defense (Jones & Dangl 2006). Recently several studies pointed to a suppression of PTI by many effectors and this very often via targeting component leading to the suppression of MAPKs activation (MAP3K or plasma membrane receptor kinase associated protein) in response to PAMPs (He *et al.,* 2006; Crabill *et al.,* 2010; Wu *et al.,* 2011). Some effector proteins can even straightforwardly target MAPKs. MPK4 was recently shown to be directly targeted by the effector protein AvrB from *P. syringae* (Cui *et al.,* 2010). AvrB interacts with and phosphorylates MPK4 leading to hormone signaling disturbance in favor of JA signaling.

Furthermore RPS4 is the only *Arabidopsis* TIR-NB-LRR protein which has been shown to act in the nucleus and signal entirely through EDS1 for activation of downstream events (Wirthmueller *et al.,* 2007). In our case MPK4 is active and EDS1 gene is likely down regulated due to the lack of SA. The MPK4$^{D198G/E202A}$ sensitivity in response to *Pst* DC3000 AvrRps4 could be then explained by the under-accumulation of SA. Whether AvrRps4 plays with hormone balance like in the case of AvrB is not known, because gene expression data are not really reliable to draw conclusion regarding this aspect. It could be more informative to monitor JA hormone content in our lines in order to understand whether MPK4 has a role in hormone balance making it a target of pathogen effectors. *mpk4* phenotype could also be a pleiotropic consequence of the constitutive SA accumulation like in many mutants (Kirik *et al.,* 2001;

Yoshida *et al.,* 2002; Meng *et al.,* 2009). Unfortunately gene expression data are not really conclusive so far, even if we saw a tendency of down regulation of PDF1.2, PAD4 and EDS1 upon challenging with *Pst*DC3000 AvrRps4. A full genome transcriptome analysis of MPK4$^{D198G/E202A}$ plants in resting conditions has also been performed in order to have a clue about downstream events controlled by MPK4 activation. In the MPK4$^{D198G/E202A}$ line, only 9 genes were miss-regulated compared to MPK4wt lines (*data not shown*). This result was surprising considering that *mpk4* mutant showed about 900 genes miss-regulated (*unpublished data*). A plausible explanation for this result could be that the level of activation of MPK4 in MPK4$^{D198G/E202A}$ lines did not reach the threshold necessary to induce gene expression changes. We were although able to see that these lines behave in an opposite way compared to the mutant regarding resistance to biotrophic pathogens.

***4. Since SA is lower in CA-MPK4 why these lines are more susceptible to necrotrophs?***

MPK4$^{D198G/E202A}$ lines appear to be more sensitive to *Altenaria* infection than WT-MPK4 plants. This result is not in accordance with previous data published by Mundy's group, showing that *mpk4* mutant is more susceptible to *Altenaria* due the decreased JA level (Brodersen *et al.,* 2006). One obvious explanation is that Mundy and co-worker used *mpk4-1* mutant in the Landsberg ecotype whereas our line are built in Col-0 ecotype, both ecotypes behaving differently. Another explanation is that *mpk4* mutants being very sick, the development of necrotrophic pathogen, despite constitutively risen defenses, is favored by the spontaneous cell death phenotype.

MPK4 is proposed to be involved in the regulation of ET/JA dependant defenses. And this based on the reduced PDF1.2 mRNA induction in response to JA and that MPK4 activity is required for this induction (Brodersen *et al.,* 2006). Moreover it was shown that reducing the SA content in *mpk4* mutant plants with NahG expression does not recover the sensitivity of *mpk4* to *Altenaria* infection. Additional support indicating that MPK4 could be somehow a positive regulator of the JA pathway comes from investigation of AP2C1, an *Arabidopsis* Ser/Thr phosphatase of type 2C. This phosphatase is considered as novel stress signal regulator that inactivates MPK4 and MPK6. AP2C expression is pathogen and wound induced. *Arabidopsis*

*ap2c1* mutant plants produce significantly higher amounts of JA upon wounding and are more resistant to phytophagous mites (Schweighofer *et al.,* 2007). Resistance to *A. brassicicola* was shown to be JA dependent, as *coi1* mutants defective in JA signaling (Feys *et al.,* 1994) shows an increased sensitivity against the fungal pathogen (Thomma *et al.,* 1998; Vijayan *et al.,* 1998). MPK4$^{D198G/E202A}$ plants appear to be slightly more susceptible to *Altenaria* infection. This result is unexpected, since the reduced SA levels in MPK4$^{D198G/E202A}$ lines, inducing a normal or even elevated JA hormone levels, should triggers resistance to necrotroph pathogens. This result is in agreement with the sensitive phenotype of MKK2$^{EE}$ in response to *Altenaria* (Brader *et al.,* 2007), MKK2 being one of the activators of MPK4 in the MEKK1-MKK1/2-MPK4 cascade. The authors of this study showed that this phenotype is linked to compromised SA and JA production even if the genes involved in these hormones were found to be up-regulated in the MKK2$^{EE}$ plants (Brader *et al.,* 2007). The picture of necrotrofic pathogen interaction with plant is complex. The fact that some pathogens can manipulate hormonal balance for their own purposes, by producing coronatine for instance, which is a JA mimicking molecule (Loake & Grant 2007) or through effector proteins (Cui *et al.,* 2010) has to be taken into account.

Several studies pointed to the importance of camalexin, an indolic secondary metabolite whose amount is controlled by MPK4 activity, in the resistance to biotrophic pathogen (Ren *et al.,* 2008) and more precisely to *Altenaria* (Thomma *et al*., 1999; vanWees *et al*., 2003). As attenuation of resistance of *A. thaliana* against *Alternaria* was shown to be linked to a deficiency of the phytoalexin, camalexin, or insensitivity to jasmonate (Thomma *et al.,* 1998; Thomma *et al.,* 1999). Additionally comparative analysis using 24 *Arabidopsis* ecotypes indicated that there was a close relationship between the resistance against *Alternaria* and camalexin production (Kagan & Hammerschmidt 2002). All this raise the question to whether the presumed model of the constitutive regulation of PAD3 by MPK4 could explain the MPK4$^{D198G/E202A}$ susceptibility phenotype toward necrotrophic pathogens.

This leads us to conclude like it was recently proposed by Robert-Seilaniantz and co-workers that hormonal balance and cross talk are not a simple story of SA inhibiting JA production and *vise versa* (Robert-Seilaniantz *et al*., 2011). So far, studying SA and JA/ET-mediated defense responses was often based on the translation of expression levels of marker genes to predict the trade-offs between necrotroph and biotroph resistances. This expression is not always correlated with increase in hormone level (Brader *et al.,* 2007).

# *Chapter VIII*

# *Conclusions and Perspectives*

# Chapter VIII - General conclusions and perspectives

## A. Main results of my work

In this work, I developed a strategy to identify constitutively active variant of plant MAPKs with MAP2K-independent increased activity. In a genetic screen in yeast, we used *Arabidopsis* MPK6 as a model to identify mutations which trigger constitutive MAPK activity. We identified three sets of mutations. Among these interesting mutations, the mutations in MPK6 residue Y144 was known. The residue is located in the ATP-binding pocket and is homologous to the well described Gatekeeper residue of the mammalian MAPK ERK2 (Emrick *et al.,* 2006). We found also a new combination of two mutations D218G and E222A which activates intrinsically the kinase and does not depend on upstream activation. Introduction of the corresponding mutations in MAPKs belonging to different other subgroups often resulted in constitutive activation. This suggests that the mutations that we identified provide a general strategy to render MAPK constitutively active.

To show that this strategy is useful to understand MAPK function, I choose to work on MPK4 and generated lines in which the endogenous MPK4 gene is replaced by gene carrying CA mutations. Inexplicably, only the $MPK4^{D198G/E202A}$ lines showed a systematic increase of MPK4 activity *in planta*. This result could come from the cellular phosphatase activities able to dephosphorylate the activation loop of $MPK4^{Y124C}$ but not the diverging one of $MPK4^{D198G/E202A}$. This suggests that all CA mutations in a *MAPK* gene will not always activate the kinase *in planta* and will need to be carefully characterized before doing any further functional investigation. The final proof that the MPK4 mutants are functionally constitutively active in plant cells, come from the fact that $MPK4^{D198G/E202A}$ construct is also able to complement the *mekk1* phenotype, which is defective in the upstream activation of MPK4.

This work on model plant MAPKs is now presented in a manuscript (Chapter IX). It also led me test the CA mutations in a MAPK belonging to other kingdoms: I showed that mutations of the activation loop of the mammalian ERK2 also confer CA. Collaborations were established

with Robert Hipskind (Institut de Génétique Moléculaire de Montpellier) to test whether this mutant is useful to understand downstream events controlled by ERK2.

## ***B. Using CA MPK4 plants to better understand MPK4 function in stress and development…***

My proof-of-concept work on CA MPK4 lines provides also information on MPK4 function in stress responses. MPK4 is a well studied kinase but its exact function remains unclear: on one hand, together with MPK3 and MPK6, MPK4 is activated by pathogens and abiotic stresses (Suarez-Rodriguez *et al.,* 2010). On the other hand, genetic evidence suggests that MPK4 negatively regulates pathogen defenses (Petersen *et al.,* 2000; Brodersen *et al.,* 2006). The fact that *mpk4* mutants but also *mekk1* and *mkk1mkk2*, are dwarfed without being challenged by any stress suggests that the activity of MEKK1-MKK1/MKK2-MPK4 cascade is required for a stress-independent processes. The picture is even more complex since MPK4 belongs also to a second independent cascade involved in cytokinesis its cognate MAP3K and MAP2K being ANP1/ANP2/ANP3 and MKK6 (Kosetsu *et al.,* 2010; Takahashi *et al.,* 2011; Zeng *et al.,* 2011). New reports points also to its physical association with microtubule to regulate their bundling (Beck *et al.,* 2010). CA-MPK4 lines provide interesting data to better decipher the kinase function. We are actually starting collaboration with Jozef Samaj (Palacky´ University, Czech Republic) to examine the microtubule organization in the $MPK4^{D198G/E202A}$ lines.

The WT aspect of CA-MPK4 plants confirms the new role of MPK4 in developmental processes, this new function might not require the same level of activity as for stress response. We showed that $MPK4^{D198G/E202A}$ lines show an opposite phenotype to some of the *mpk4* traits published previously: plants expressing $MPK4^{D198G/E202A}$ instead of the WT form are more susceptible to *Pst* DC3000 and to two strains used to evaluate the relative contribution of PTI (*Pst* DC3000 HrcC) and ETI (*Pst* DC3000 AvrRPS4) in the plant responses to pathogens. This suggests that MPK4 has a role in both types of defense responses or/and that PTI and ETI are not independent as they are usually presented. Other hormone measurements will help to clarify MPK4 function in hormonal balance (Brodersen *et al.,* 2006). Similar results were indirectly obtained with the constitutive expression of the *P.syringae* effector avrB in *Arabidopsis*, which

appears to activate MPK4 and therefore increase susceptibility to *Pst* DC3000 (Cui *et al.,* 2010). With the necrotrophic pathogen *Altenaria*, both CA-MPK4 and *mpk4* knock out behave in the same way. Suggesting that even though the SA level in CA-MPK4 decreased, it did not affect JA accumulation.

The *mekk1 MPK4*$^{D198G/E202A}$ lines generated at the end of my PhD constitute one the most promising tools developed during my work. It allows the genetic dissection of MPK4 pathways. The fact that these plants have a WT phenotype suggests that MPK4$^{D198G/E202A}$ activity, despite reaching only 10% of the stress activated level, is enough to complement all the MPK4-dependent developmental functions. Interestingly, in the *mekk1 MPK4*$^{D198G/E202A}$ background, the output activity of MEKK1-MKK1/MKK2-MPK4 cascade should not be increased anymore by stresses. Thereby, these plants constitute the first true mutant of this PAMP-activated MEKK1-MKK1/MKK2-MPK4 cascade in a background for which all the other MPK4 pathways are functional: these lines are a useful tool to characterize further the MEKK1-MKK1/2-MPK4 cascade in PAMP response. We will investigate whether the PAMP response, in terms of MPK4 further activation (observed in the CA-MPK4 lines), maintain or not the regulation of the genes previously proposed to be downstream of MPK4, basal immunity responses (ROS production, callose deposition…) in the absence of MEKK1.

One step further, the strategy to complement a mutant by a downstream CA MPK4 is a very promising strategy to provide genetic argument that two elements, one being MPK4, are working in the same signaling pathway. In the laboratory, this strategy will be used in two scientific projects:

- We are working on BAKl and BKK1, two receptor-like-kinases of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE family. The *bakl/bkk1* double mutant shows a phenotype of spontaneous cell death and seedling lethality (He *et al.,* 2008) similar to the mutants in MEKK1-MKK1/2-MPK4 cascade (Bueso and Hirt, unpublished result). This suggests that these receptors could be involved in the same linear pathways, as BAK1-BKK1 receptors were indeed demonstrated to be part of the PAMP receptors activating MAPK pathways (Ranf *et al.,* 2011; Roux *et al.,* 2011). But given the multiple functions of BAK1-BKK1 (including hormonal perception (He *et al*., 2008) and the fact that once again the double mutant is very sick, the demonstration is not totally done yet. *bak1 bkk1 MPK4*$^{D198G/E202A}$ plant phenotype would provide very interesting information regarding their function in defense response.

- Another similar promising experiment would allow clarifying the relationships between MAPKs and AGC kinases. Our laboratory works on lines over-expressing OXI1 (Fozani *et al*., 2011). These plants show a very sick *mpk4*-like phenotype and some contradictory experiments suggested that OXI1 and MAPK could regulate each other. The cross of OXI1 over-expressing lines and CA-MPK4 lines could also help to better understand the relationship between these signaling modules.

## C. Will the CA MAPK strategy be helpful for the two other stress activated MPK3 and MPK6?

In response to PAMPs it was extensively shown that together with MPK4 pathway a second MAPK cascade involving MAP2K4/5- MPK3/6 is activated. This cascade is often presented as independent but is likely not. CA-MPK4 lines may be also used to answer the question of how is this cascade working in absence or presence of PAMP. It was recently shown that WRKY33 transcription factor which was proposed to be an effector of MPK4, is directly phosphorylated and modulated by MPK3 and 6 to control PAD3 expression in response to biotrophic infection (Mao *et al*., 2011).

Moreover, we are actually generating and characterizing CA-MPK3 and CA-MPK6 lines under native and inducible promoters in order to investigate the part of defense response controlled by each of these MAPKs in more details. We expect these plants to be more resistant to stresses including biotic ones. They will bring new information about the way plants deals with biotrophic versus necrotrophic pathogen challenging at the PTI level. These plants, if they are significantly more tolerant to stresses, are also promising material for application in agriculture. During my PhD, we tried to patent the CA mutations but the proof of concept on a MAPK which regulates positively defence responses remains to be done.

## ***D. 17 other MAPKs without clear functions…***

One of the main goals of my PhD work was to find a strategy to activate all *Arabidopsis* MAPKs. CA mutations are a very nice tool that we have now in hands and that we will use to study the function of the 17 remaining MAPKs of *Arabidopsis*. One of the major advantage of studying MPK3, MPK4 and MPK6 was the possibility to activate them using PAMPs. The function of the remaining MAPKs is not really characterized, mainly because of the difficulty to monitor their activity *in planta* and because the signal activating their pathways is not identified yet. Studying mutants gives obviously important information but the results are based on the absence of events, consequence of the loss-of-function allele. As shown for MPK4, using the mutant it is still not easy to understand its real function in defense response, for instance. By using the CA mutations, it will be possible to directly distinguish what are the downstream events controlled by a single MAPK. I initiated at the end of my PhD the cloning of all the remaining MAPKs and created for each of them the corresponding CA mutants. Vectors for protoplast expression are ready and the strategy will consist to identify the downstream events controlled by their activity using genome wide transcriptome analysis.

# *Supplemental material*

# *Chapter IX - Supplemental Material – Manuscript of the article "Constitutively active kinases as a new tool for analyzing MAPK pathways: proof-of-concept that Arabidopsis MPK4 is a negative regulator of defense"*

Souha Berriri, Ana Garcia, Wilfried Rozhon, Stephanie Pateyron, Jeffrey Leung, Heribert Hirt, Jean Colcombet

**ABSTRACT**

Plant Mitogen Activated Protein Kinases (MAPKs) are known to be involved in various important processes including stress signaling and development. However, for the majority of the 20 MAPK genes found in *Arabidopsis* genome no function has been assigned yet. In the past, mutations triggering constitutively active protein kinase were a very successful approach to decipher their role. This approach failed for MAPKs since the necessary residues remained unclear. In order to identify mutations triggering constitutive MAPK kinase-independent activity in MAPKs, we developed a functional genetic yeast screen. We identified several mutations that render MAPKs constitutively active (CA). Importantly, CA-MAPKs maintain their specificity towards both upstream activators and downstream substrates. As a proof-of-concept, MPK4 was investigated. In contrast to *mpk4* knock out plants, which show increased levels of salicylic acid (SA) and pathogen resistance against Pseudomonas syringae, CA-MPK4 plants show decreased SA and resistance than wild type plants. These results prove that MPK4 is a negative regulator of plant defense and that the use of constitutively active MAPKs adds an important tool for signal transduction research.

**INTRODUCTION**

Plants are subjected to a large number of environmental challenges and have to adapt their metabolism, growth and development accordingly. For this purpose, plants contain many genes encoding for proteins involved in signal perception and transduction (1). Among which, kinases and phosphatases are particularly abundant: 4% of Arabidopsis thaliana genes code for kinases (2) compared with 1,7% in human and 2% in yeast. It is estimated that at least 30% of proteins are phosphorylated, but this value is probably underestimated by far. MAPKs (Mitogen Activated Protein Kinase) pathways define key functional signaling modules conserved between kingdoms, and are usually composed of 3 kinases able to sequentially activate each other by phosphorylation: a MAP3K (MAP2K Kinase) activates a MAP2K (MAPK Kinase) which in turn activates a MAPK. In plants, MAPK signaling cascades are involved in various processes like development, hormone signaling and stress responses (3, 4). Among the 20 putative MAPKs found in Arabidopsis, extensive studies showed that MPK3, MPK4 and MPK6 are major regulators of innate immune response. Several studies revealed that MAPK cascades are branched pathways (3-5) and that a given MAPK can be involved in multiple processes. MPK4 for instance, originally described as a stress activated kinase (6-9), was recently shown to be involved in cytokinesis and cytoskeleton organization (10-12).

Pathogen elicitors are able to activate MPK3, 4 and 6. Flg22, a 22 amino acid peptide derived from Pseudomonas flagellin, provided a very powerful model to decipher these signaling

pathways and exemplify the complexity of MAPKs cascades. Flg22 is able to initiate plant defense responses through its binding to the receptor FLS2-BAK1 and activate two MAPK pathways. One of these MAPK cascades is defined by the MAP2Ks MKK4 and MKK5, which act redundantly to activate the MAPKs MPK3 and MPK6 (13). Through phosphorylation of several identified targets (14-17), this pathway leads to the activation of plant defense such as Pathogen-Related (PR) gene expression, ethylene synthesis, callose deposition, ROS production, etc.

Flg22 activates also a parallel cascade defined by MEKK1, which activates MKK1 and MKK2, that act redundantly on MPK4. These kinases were demonstrated to interact together both in yeast (9, 18) and *in planta* (19). Additionally, in *mekk1* or *mkk1mkk2* backgrounds, the flg22-induced activation of MPK4 is abolished (19-21). The double-mutants *mkk1mkk2* and the two single mutants *mekk1* and *mpk4* exhibit similar phenotypes: they are dwarf plants over-accumulating Salicylic Acid (SA) and Reactive Oxygen Species (ROS) and show spontaneous cell death on leaves and constitutive expression of *PR* genes (19). The dwarf phenotype is partially reversed by expression of the bacterial SA hydrolase nahG or by mutations in genes of SA biosynthesis (8, 21-23). Transcriptomic analysis confirmed the similarities of the mutants and their functions in SA and ROS signaling (24). This molecular phenotype largely accounts for the mutant phenotypes to pathogen infection. For example, *mpk4* is resistant to the biotrophe *Pseudomonas syringae* but sensitive to the necrotrophe *Altenaria brassicola* (6, 8).

Recently, the number of publications revealing a function of MAPK cascades in pathogen signaling increased greatly. A set of three MAPKs -MPK3, MPK4 and MPK6- was found to be activated upon pathogen interaction, and converge to the same defense mechanisms relaying on common regulating components (6, 17, 23). The functions of the 17 others MAPKs are often still unclear. A way to sort out the role of a particular kinase in physiological processes is the use of constitutively active (CA) kinase versions. So far the extensively used stress-independent way of activating MAPKs is *hitherto* the utilization of CA MAP2Ks. CA-MAP2Ks are obtained when the two phospho-sites in the activation loop are mutated into acidic amino acids (25). Unfortunately, this strategy is not successful for MAPKs. In this work, we adapted a genetic screen in yeast to isolate constitutively active MAPKS. We identified two amino acid residues which render MAPKs constitutively active in bacteria, yeast and *in planta*. As a proof of concept, this strategy was used to investigate the physiological function of MPK4.

## RESULTS

### Functional complementation of the yeast *pbs2Δhog1Δ* allows the identification of CA-MPK6

To identify mutations triggering constitutive kinase activation, we adapted a yeast functional expression screen developed by Engelberg and co-workers (26). Our laboratory has previously shown that AtMPK6, together with the MAP2K AtMKK2, rescues the salt sensitivity of a yeast strain mutated in the MAP2K PBS2 and the MAPK HOG1 (9). In the absence of MKK2, MPK6 does not restore the *pbs2Δhog1Δ* growth on salt, indicating that MPK6, without its upstream MAP2K, is not functional. In these conditions, mutations triggering MPK6 CA should restore the *pbs2Δhog1Δ* salt growth. We performed a screen on *pbs2Δhog1Δ* yeast expressing a randomly mutated *MPK6* ORF population. When replicated on 400-600 mM NaCl, only very few colonies were able to grow. To exclude MPK6-independent recovery of salt tolerance, their plasmids were extracted, re-transformed in *pbs2Δhog1Δ* and the growth on salt confirmed. Finally, 27

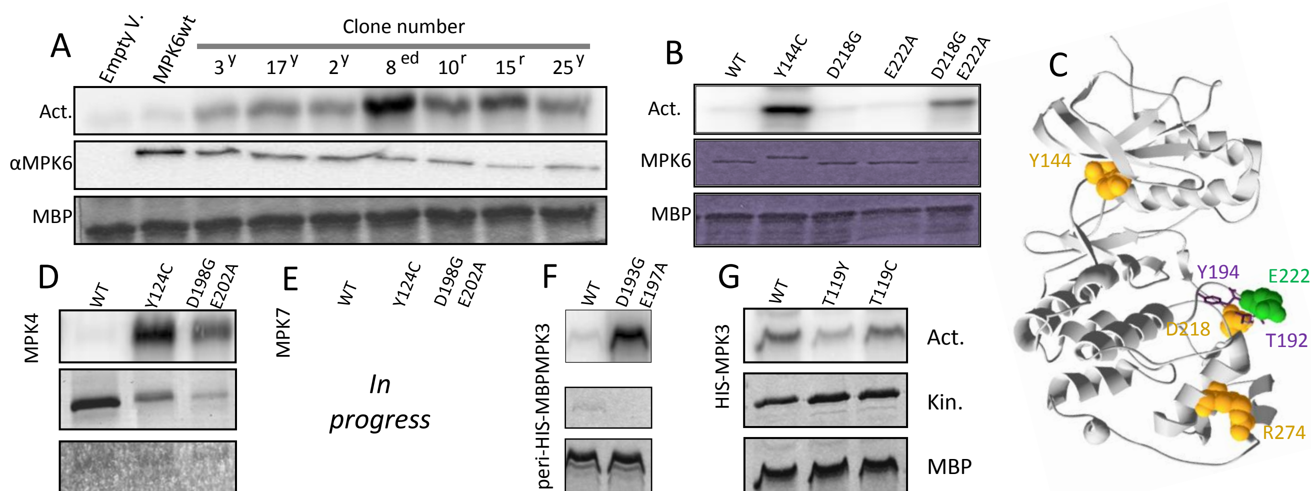



**Figure 1**

Characterization of CA MAPK activity

A. Kinase activity toward MBP of MPK6 WT and some candidate mutants (containing Y144 (**y**), R274 (**r**) or D218 E222 (**ed**) mutations) after immuno-precipitation from *pbs2Δhog1Δ* yeast cells using MPK6 antibody. Clone numbers refer to supplemental text.

B. Kinase activity toward MBP of recombinant MPK6 WT and CA mutants.

C. Ribbon diagram of ERK2-based MPK6 structure with space field residues identified as CA mutations in the yeast screen. Sticks represent T192 and Y194 of the TEY motif.

D. Kinase activity toward MBP of recombinant MPK4 (C), MPK7 (D), and MPK3 (E and F) with CA mutations produced as HIS- (A,B and C) or PERI-HIS-MBP- (D) tagged proteins.

MPK6 candidate clones were identified and sequenced, revealing between 1 to 6 residue mutations in *MPK6* (Supplemental Table 1). Y144C, Y144F and R274H mutations were found in 12, 9 and 4 clones respectively, indicating that they are important for *pbs2Δhog1Δ* complementation.

To confirm that *pbs2Δhog1Δ* complementation is linked to an increased MPK6 activity, some MPK6 mutants were immuno-precipitated from yeast using specific anti-MPK6 antibody. The MPK6 activity was subsequently assayed for the ability to phosphorylate Myelin Basic Protein (MBP), a common heterologous substrate for MAPKs. Under these conditions, the activity of wild type (WT) MPK6 was barely detectable whereas all MPK6 mutants phosphorylated efficiently MBP (Fig. 1A). This shows that the MPK6 mutants that are able to complement *pbs2Δhog1Δ* have a higher catalytic activity.

**CA-MPK6 candidates have a MAP2K-independent activity**

Sequence analysis of the candidate clones pointed to Y144 and R274 as important residues for kinase auto-activity because their mutations were identified in 25 out of 27 clones (Fig. 1B). One of the two remaining clones exhibited three mutations including D218G and E222A in the kinase activation loop (Fig. 1B). Since the double mutant in theses residues showed high intrinsic activity after immuno-precipitation from yeast, we chose to work on Y144C and D218G/E222A mutations for further characterization (Fig. 1C).

To confirm the importance of these residues, we generated the three MPK6 single mutants $MPK6^{Y144C}$, $MPK6^{D218G}$ and $MPK6^{E222A}$ and the double mutant $MPK6^{D218G/E222A}$ without the additional mutations obtained in the yeast screen. The respective MPK6 kinases were purified as recombinant proteins from *Escherichia coli* cells, an expression system lacking MAPK activators. Whereas MPK6wt did not show significant kinase activity, $MPK6^{Y144C}$ and $MPK6^{D218G/E222A}$ strongly phosphorylated MBP (Fig. 1B). In contrast, the single mutations D218G and E222A did not increase MPK6 activity suggesting that $MPK6^{D218G/E222A}$ activity is not a result of an additive effect of each mutation, but rather of the synergistic combination of the two mutations.

**Corresponding CA mutations in other Arabidopsis MAPKs trigger hyperactivity**

We performed sequence alignment of the 20 *Arabidopsis* MAPKs (Supplemental fig. 1). At the equivalent position of MPK6 Y144, most MAPKs of the groups A, B and C have a tyrosine residue. Despite the fact that the activation loop sequences are not very conserved among MAPKs, we also identified the equivalent position of the MPK6 D218G and E222A for the groups A, B and C. In order to test if the corresponding mutations trigger autoactivity in the other MAPKs, we mutated the corresponding residues of MPK3, MPK4 and MPK7 which are MAPKs belonging to sub-groups A, B and C respectively (see Supplemental fig. 1 for positions). Hexa-histidine tagged MPK4 and MPK7 WT and mutants were purified from *E. coli*. We failed to purify the $6xHIS\text{-}MPK3^{D193G/E197A}$, and we expressed it as PERI-His-Maltose Binding Protein (Peri-HIS-MBP-) tagged protein. MPK4 and MPK7 CA forms acquired higher intrinsic activity than the WT (Fig. 1D and E). The $Peri\text{-}His\text{-}MBP\text{-}MPK3^{D193G/E197A}$ acquired a high catalytic activity compared to WT protein (Fig. 1F). But MPK3 activity was not increased by the T119C mutation (Fig. 1G). Since MPK3 does not have a tyrosine residue in the MPK6 Y144 homologous position but a threonine residue instead, we hypothesized that this threonine, like the cysteine and phenylalanine substitutions of Y144 in MPK6, allows MAP2K-independent MPK3 activity. To test this hypothesis, we created the $MPK3^{T119Y}$ mutant, mimicking the MPK6

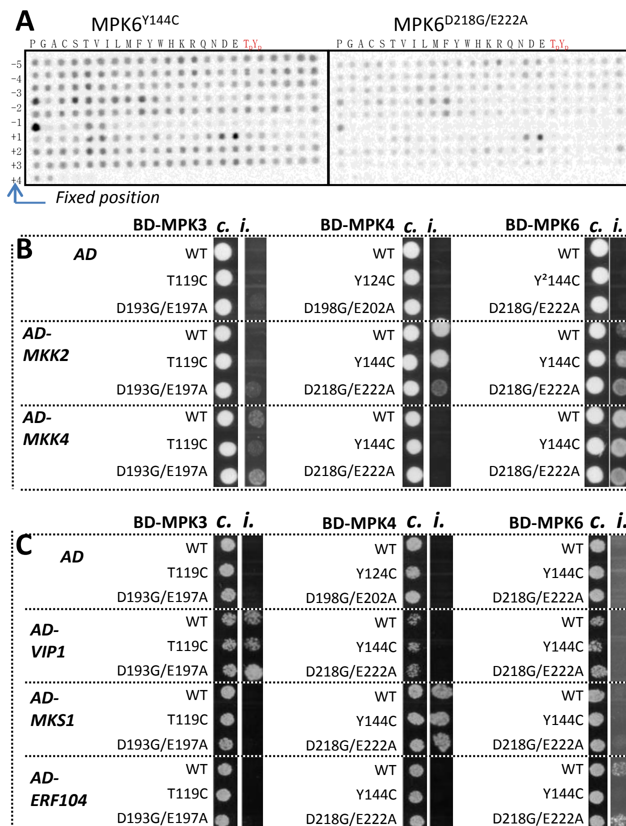



**Figure 2**

Substrate preferences and interaction specificities of WT and CA MAPKs

A. Phosphorylation of the semi-degenerate peptide array by MPK6$^{Y144C}$ and MPK6$^{D218G/E222A}$ (1µg.uL of reaction).

B and C. Combinatory interaction in yeast 2 hybrid of MPK3, 4 and 6 WT and CA forms with MKK2 and MKK4 (B) and with VIP1, MKS1 and ERF104 (C). Co-transformed single yeast colonies were spotted on control medium (c.) and selective medium supplemented with 65mM (B) or 36 mM (C) 3AT (i.).

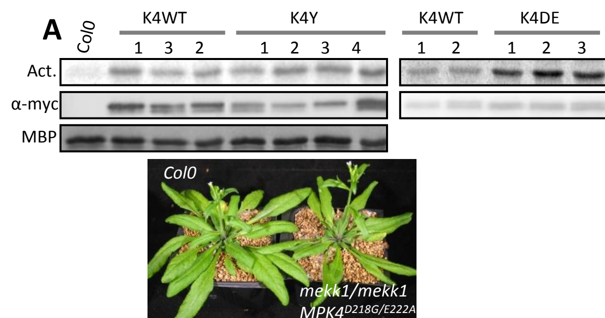



**Figure 3**

Characterization of *mpk4-2* plants complemented with *CA MPK4* locus

A. and B. Kinase activity toward MBP of MPK4-Myc immuno-precipitated from *mpk4-2/mpk4-2* lines complemented with *MPK4*$^{WT}$ (A and B), *MPK4*$^{D198G/E202A}$ (A) and *MPK4*$^{Y124C}$ (B).

C. Morphological phenotype of 5 weeks old *mekk1-1/mekk1-1 MPK4*$^{D198G/E202A}$ plants grown in long days. Arrows point curly leaves. In the growth conditions, *mekk1-1* mutants barely survive two weeks.

WT form, which was expected to have decreased kinase activity. Kinase assays showed a decrease in MPK3$^{T119Y}$ intrinsic activity compared to MPK3wt and MPK3$^{T119C}$ (Fig. 1G).

Overall these results indicate that the residues identified in the yeast screen to activate MPK6 provide a general strategy to render MAPKs constitutively active.

**CA-MAPKs phosphorylate conventional MAPK sites**

To test whether the kinase preference toward the residues surrounding the phosphorylation site was not affected by the CA mutations, we took advantage of a semi-degenerate peptide array tool (27). This array consists of 198 peptide pools and each pool has as a putative phosphorylation site (Ser/Thr) at its central position and is degenerated for all other positions except a fixed residue at one of the nine positions surrounding the phospho-site (between -5 to +4). The level of phosphorylation of each peptide by the kinase indicates the preferred residues for each position surrounding the phosphorylation site. The active MPK6$^{Y144C}$ and MPK6$^{D218G/E222A}$ were first tested (Fig. 3A). They both showed an increased phophorylation on peptides with proline at position +1 and to a lesser extent to peptides with proline at the -2 position. Moreover, we saw a preference for charged amino acids at position +2. This result is in agreement with MPK6 WT preference determined previously (28). Assays for MPK3, MPK3$^{T119C}$, MPK3$^{D193G/E197A}$ and MPK4$^{Y124C}$ gave similar results indicating that the CA mutations do not significantly change the preferences of MAPKs around the phosphorylation site (Supplemental fig. 2).

**CA-MAPK mutants retain binding specificity toward substrates and activators**

We next used the yeast two-hybrid technique to examine whether the specific interactions between MAPKs and known interactors are affected by CA mutations of the MAPKs. WT and CA forms of MPK3, 4 and 6 were fused to the GAL4 binding domain and co-expressed in the reporter strain together with putative interactors fused to the GAL4 activation domain. In accordance to previous reports (14, 15, 29), we observed that CA-MPK3 interacts with VIP1, CA-MPK4 with MKS1 and CA-MPK6 with ERF104. In some cases, CA mutations appeared to affect the strength of the interactions but not the specificity (Fig. 3B). Similar results were obtained with their corresponding activating MAP2Ks (Fig. 3C): CA-MPK3 interacted with MKK4, CA-MPK4 with MKK2, and CA-MPK6 with both MKK2 and MKK4. Taken together these data indicate that the CA mutations do not change the specificity of the kinases toward their cognate MAP2Ks nor to their specific substrates.

**MPK4$^{D198G/E202A}$ complements *mpk4* and is hyperactive in planta**

Whereas *mpk3* and *mpk6* plants do not have obvious morphological phenotype, *mpk4* homozygous plants are dwarfed (8). We took advantage of this phenotype to test whether CA-MPK4 mutants were able to functionally replace endogenous MPK4. We generated *mpk4-2* lines transformed with myc-tagged full genomic loci of *MPK4wt*, *MPK4$^{Y124C}$* and *MPK4$^{D198G/E202A}$*, (further referred to as K4WT, K4Y and K4DE lines, respectively). WT and CA mutant *MPK4* loci restored a WT morphological phenotype (n=6, 10 and 6 respectively, results not shown), suggesting that the cascade specificity is conserved in CA-MPK4 plants. To test if the CA-MPK4 kinase is constitutively active in these lines, we immuno-precipitated MPK4-myc from the corresponding lines using anti-myc antibody. All K4DE lines showed increased MPK4 activity when compared to K4WT lines (Fig. 3A) but surprisingly not the K4Y ones (Fig. 3B). We calculated that increased activity of MPK4 in K4DE lines was about 10% of the total flg22-

activated MPK4wt activity, indicating that the activity of CA-MPK4 in untreated lines lies well below the full activation levels upon pathway stimulation.

As MEKK1 is thought to be the main MAP3K acting upstream of MPK4 (21, 22, 30), we hypothesized that the *MPK4$^{D198G/E202A}$* transgene might complement the *mekk1* dwarf phenotype by reactivating downstream events controlled by the MEKK1-MKK1/2-MPK4 cascade. By crossing heterozygous *mekk1-1* plants with MPK4$^{D198G/E202A}$ lines, we generated *mekk1-1/mekk1-1 MPK4$^{D198G/E202A}$* lines. These plants showed an almost normal growth phenotype when compared to *mekk1* or WT plants (Fig. 3D), confirming that CA-MPK4$^{D198G/E202A}$ is functional *in planta*.

### MPK4$^{D198G/E202A}$ lines show increased susceptibility to *Pseudomonas syringae*

For further investigation, we focused our work on *mpk4-2* lines complemented with *MPK4$^{D198G/E202A}$* (*K4DE*) because of their higher MPK4 autoactivity. *mpk4* mutants are resistant to the hemi-biotrophic pathogen *Pseudomonas syringae*, suggesting that MPK4 is a negative regulator of the plant defense (8). According to this model, K4DE lines would be expected to be hypersensitive to *Pseudomonas*. Col-0, K4WT and K4DE plants were spray-inoculated with the virulent *Pseudomonas syringae* pv. tomato strain *DC3000* (*Pst DC3000*). 3 days post-inoculation (Fig. 4A), similar *Pst DC3000* levels were found in Col-0 or K4WT leaves but increased levels were observed in K4DE leaves, suggesting that the higher MPK4 activity in K4DE lines triggered an increased susceptibility to Pseudomonas. To distinguish between PAMP-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI) phenotypes, we used the type III secretion system-defective *Pst DC3000* mutant strain *HrcC* which is impaired in effector delivery and induces mainly PTI. After spray-inoculation, bacterial quantification showed that K4DE lines are significantly more susceptible to *Pst DC3000 HrcC*. (Fig. 4B). Similar results were obtained with the avirulent Pst DC3000 expressing the *AvrRps4* effector and triggering ETI through RPS4-mediated recognition (Fig. 4C). Disease symptoms were also more pronounced on leaves of the K4DE lines 3 days post inoculation (Fig. 4D).

As the *mpk4* resistant phenotype is linked to constitutive accumulation of SA, we hypothesised that *mpk4* lines complemented with *MPK4$^{D198G/E202A}$* would behave in an opposite way and not accumulate SA as efficiently as WT lines in response to pathogen infection. 24 hours after spray-inoculation, Col-0 and the K4WT lines accumulated similar amounts of SA, whereby the K4DE lines showed lower accumulation of SA (Fig. 4E). This result also shows that the CA-MPK4 mutation reduces the level of SA and could be responsible for the increased susceptibility of K4DE lines to *Pseudomonas*. Taken together, these data confirm that MPK4 controls SA amounts in response to biotrophic pathogen interaction and plays a role in both PTI and ETI.

## DISCUSSION

### CA mutations are a useful tool to activate MAPKs

In this article, we identified mutations which increase MAPK activity independently of MAP2K activation. Using a genetic screen in yeast and *Arabidopsis* MPK6 as a model, we identified 3 sets of mutations that trigger constitutive MAPK activity. Introduction of the corresponding mutations in MPK3, 4 and 7, belonging to different MAPK subgroups similarly resulted in constitutive activation. These results suggest that these mutations provide a general strategy to render MAPKs constitutively active.

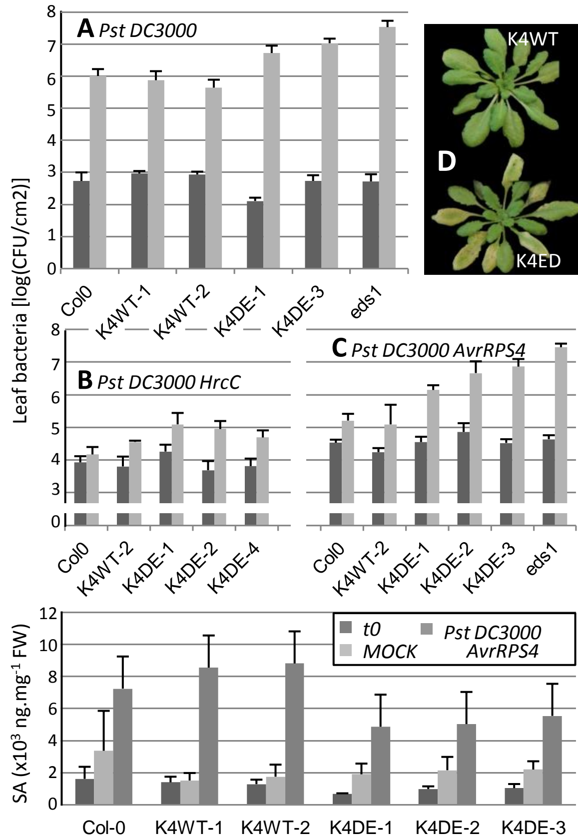



**Figure 4**

Characterisation of pathogen responses of *mpk4-2* plants complemented with *MPK4*$^{D198G/E202A}$ locus.

A to C. Example of patho-assay after 3 days of spray-inoculation with *Pst DC3000* (A), *Pst DC3000 HrcC* (B) and *Pst DC3000 AvrRPS4* (C).

D. Disease symptoms observed on K4ED line 3 day post spray-inoculation with *Pst DC3000 AvrRPS4*.

E. SA content after 3 days of spray-inoculation with *Pst DC3000 AvrRPS4*.

The MPK6 residue Y144 is located in the ATP-binding pocket and is homologous to the well described Gatekeeper residue of the mammalian MAPK ERK2 (31). Mutation of this residue is known to allow ERK2 intra-molecular auto-phosphorylation and therefore its activation in a MAP2K independent manner. MPK6 Y144C or Y144F mutations might have a similar effect. Despite the fact that some activating mutations were identified in the activation loop of human or yeast MAPKs (26, 32), the combination of the MPK6 mutation D218G and E222A is novel. Interestingly, these mutations change two acidic amino acid residues for neutral ones, i.e. they have an opposite effect to the effect of the phosphorylation of the TEY motif. Although one would expect that these changes in charge should decrease the residual activity of MAPKs, an alternative explanation could be that these mutations might change the activation loop flexibility, resulting in higher auto-phosphorylation and kinase activation.

Because we have no clear evidence on the way these mutations could enhance MAPK catalytic activity, we were concerned to bring evidence on their proper functioning: in this work, we showed that CA mutations do not change neither the MAPK binding specificities for known interactors nor their preferences toward the amino acids surrounding the phosphosites. Additionally, the use of a semi degenerate peptide array allowed to confirm that MPK3, 4 and 6 share common substrate preferences with animal MAPKs (33). The final proof that the CA mutants fully retain the WT kinase specificity comes from the fact that both MPK4$^{Y124C}$ and MPK4$^{D198G/E202A}$ were able to complement a *mpk4* knock out mutant line. Oddly, only the MPK4$^{D198G/E202A}$ lines showed a systematic increase of MPK4 activity *in planta*. This result could come from the cellular phosphatase activities able to de-phosphorylate the activation loop of MPK4$^{Y124C}$ but not the diverging one of MPK4$^{D198G/E202A}$ and suggests that all CA mutations will not always work *in planta* and need to be carefully characterized first. Strikingly, *MPK4$^{D198G/E202A}$* construct was also able to complement the phenotype of *mekk1* plants, which are defective in the upstream activation of MPK4. This result provides additional evidences that MPK4$^{D198G/E202A}$ retains activation-independent activity *in planta*.

**MPK4$^{D198G/E202A}$ *in planta* triggers pathogen susceptibility**

We choose to work on MPK4 to provide a proof-of-concept that the CA strategy can be used to understand the physiological role of MAPKs. MPK4 is a well studied kinase but its exact function remains unclear: on one hand, together with MPK3 and MPK6, MPK4 is activated by pathogens and abiotic stresses. On the other hand, genetic evidence suggests that MPK4 negatively regulates pathogen defenses (8). The fact that *mpk4* plants are dwarfed without being stressed suggests that MPK4 activity is required for a stress-independent process. CA-MPK4 lines could provide interesting data to better decipher the kinase function. Interestingly, MPK4$^{D198G/E202A}$ lines show an opposite phenotype to some of the *mpk4* traits published previously: plants expressing MPK4$^{D198G/E202A}$ instead of the WT form are more susceptible to *Pst DC3000* and the two strains used to evaluate the relative contribution of PTI (*Pst DC3000 HrcC*) and ETI (*Pst DC3000 AvrRPS4*) in the plant responses to pathogens. This suggests that MPK4 has a role in both types of defense responses. Consequently, and opposite to *mpk4* phenotype, SA accumulation is reduced in response to pathogen in the CA-MPK4 lines. Despite our efforts, we did not succeed to measure consistent gene expression modulations, likely because the inter-line variability is higher than the effect of CA mutations. Other hormone measurements will help to clarify MPK4 function in hormonal balance (6). Similar results were indirectly obtained with the constitutive expression of the *P.syringae* effector avrB in

Arabidopsis, which appears to activate MPK4 and therefore increase susceptibility to *Pst DC3000* (34).

In conclusion, we succeed to generate CA MAPKs and showed that these mutations can help to decipher MAPK function in planta. This strategy will be helpful to address the question of the relative contribution of each MAPK in complex stress signaling processes and to tackle the function of the poorly known other members of the family.

**MATERIEL AND METHODS**

**Complements to the material and methods are presented in supplemental text file.**

*MPK6* ORFs were LR-recombined in *pDR195gtw* to generate *pDR195gtw-MPK6*. The PCR fragment of randomly mutated *MPK6* ORF was produced using mutazyme II kit (Stratagene), *pDR195gtw-MPK6* as a matrix and the following primers (TTT CTC TTT CTT TCC TAT AAC ACC AAT AG TG / GTG TCA ACA ACG TAT CTA CCA ACG ATT TGA CC). pDR195 was linearized using BamH1 and co-transformed with MPK6 randomly mutated PCR fragments into the yeast *hog1Δpbs2Δ* using a classical Lithium/PEG/Heat chock method. The yeast cells having reconstructed the *pDR195gtw-MPK6* plasmids were selected on Synthetic Complete (SC: 0.17% YNB w/o carbohydrate and amino acids, 0.5% NH4SO4, 2% D-glucose, 2% BactoAgar) medium lacking uracil (SC + 0.2% Drop out minus Uracil (US Biological)), pH 5.6 adjusted with NaOH,). Colonies were replicated on YPD supplemented with 400-600 mM NaCl.

**ANKNOWLEDGMENTS**

The authors thank Markus Teige, Hakim Mireau and Sébastien Thomine who kindly provided the *pbs2Δhog1Δ* yeast strain, *pPC2* and the *pDR195gtw* vectors respectively. They are thankful to Sylvain Merlot and Florina Vlad for the peptide array technology, Elodie Hudik for establishing screening protocols and the URGV laboratory for daily help on experiments.

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