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DISSERTATION

Titel der Dissertation

The Role of the MAPKKK Raf36 in MKK2 mediated stress signalling in *Arabidopsis thaliana*

Angestrebter akademischer Grad

Doktor/in der Naturwissenschaften (Dr. rer.nat.)

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Zusammenfassung

Ziel des Projekts war die Aufklärung der Funktion der MAPK (mitogen activated protein kinase) Kinase Kinase Raf36 in MKK2-vermitteltem MAPK signalling in *Arabidopsis thaliana*. Raf36 gehört zu den 60 RAf-ähnlichen Kinasen in *A.thaliana* und wurde in einem *Yeast-two-Hybrid* (YTH) *Screen* als MKK2 Interaktor isoliert. MKK2, eine der 10 *A.thaliana* MAPKK's, hat Funktionen in Stress Signalwegen aktiviert durch Salz und Kälte Stress (Teige, 2004) sowie durch die Pathogene *Pseudomonas syringae* und *Alternaria brassicicola* (Brader, 2007).

Die Interaktion zwischen Raf36 und MKK2 konnte im Hefesystem sowie durch *in vitro* pulldown verifiziert werden. In *in vitro* Kinase-Assays konnte die Kinase Aktivität von Raf36 nachgewiesen werden. Das rekombinante Protein zeigte schwache Autophosphorylierung. Diese war in einer mutierten, Kinase-inaktiven Form des Proteins nicht mehr detektierbar. Da MKK2 von Raf36 *in vitro* nicht phosphoryliert wurde, lag die Existenz einer autoinhibitorische Domäne am Amino-Terminus des Proteins nahe. In diesem Fall wäre ein spezifischer Stimulus erforderlich um Raf36 zu aktivieren. Um die Autoinhibierung zu umgehen wurden mehrere Raf36-Versionen mit verkürztem Amino-Terminus generiert. Diese verkürzten Proteine waren in der Lage, rekombinantes MKK2 *in vitro* zu phosphorylieren.

Zwei *knockout*-Linien, unabhängige *raf36*-Allele aus der SALK T-DNA Insertions Kollektion, wurden charakterisiert zur Untersuchung der Raf36 Funktionen *in planta* verwendet. Transgene Raf36-überexprimierende Linien konnten nicht generiert werden, deshalb beschränkten sich die *in planta* Versuche auf *knockout* Pflanzen.

Keimungsversuche zeigten, dass die *raf36*-1 Linie, wie *mkk2* Pflanzen auch, in der Keimung auf Salz-haltigem Medium inhibiert sind, genauso auf Sorbitol und Methylviologen, einem oxidativen Stress Agens. Diese Ergebnisse wiesen auf eine Rolle von Raf36 zusammen mit MKK2 in osmotischem /oxidativem Stress Signalling hin. Da die zweite *raf36* Linie keine signifikante Keimungsinhibierung auf Salz-haltigem Medium zeigte, ist die Identifikation und Untersuchung einer weiteren *raf36* knockout Linie erforderlich., um Aussagen über die Salz- Sensitivität treffen zu können.

Im Gegensatz zu *mkk2*-Pflanzen (Teige, 2004) wurde in *raf36* Pflanzen keine verminderte MPK4- und MPK6-Aktivierung nach Kälte Behandlung gefunden. Somit scheint Raf36 nicht in MKK2-vermittelte Signalwege involviert zu sein, die durch Kälte induziert werden.

raf36-Pflanzen stellten sich als hypersensitiv gegenüber *Alternaria brassicicola* Infektion heraus, genau wie MKK2EE Linien, die eine konstitutiv aktive Form von MKK2 (MKK2EE) überexprimieren (Brader, 2007). Jasmonsäure-Level waren nach *A.brassicicola* Infektion in *raf36*- and MKK2EE-Linien vermindert. Eine Transkriptom-Analyse nach *A.brassicicola* Infektion enthüllte deutliche Überlappungen in der Gruppe differentiell regulierter Gene in beiden Linien. Diese Ergebnisse legen eine Rolle von Raf36 zusammen mit MKK2 in *A.brassicicola* induzierten Stress Signalwegen nahe.

Zusammenfassend weisen die Ergebnisse auf eine Regulation von MKK2 durch Raf36 nach *A.brassicicola* Infektion hin. Raf36 scheint in diesem Kontext die Aktivität von MKK2 zu inhibieren. Der Mechanismus dieser Inhibition bleibt zu klären, genau wie die Rolle von Raf36 MKK2-vermittelten abiotischen Stress Signalwegen.

Abstract

The aim of the project was to elucidate the function of the MAPK (mitogen activated protein kinase) kinase kinase Raf36 in MKK2 mediated MAPK signalling in *Arabidopsis thaliana*. Raf36 belongs to the group of 60 Raf-like kinases in *A.thaliana* and was isolated as a putative MKK2 interactor in a *Yeast-two-Hybrid* (YTH) *Screen*. MKK2, one of the 10 *A.thaliana* MAPKKs, has functions in the abiotic as well as biotic stress signalling. MKK2 is involved in cold and salt stress signalling (Teige, 2004) and also in stress signalling after infection with the bacterial pathogen *Pseudomonas syringae* and the necrotrophic fungus *Alternaria brassicicola* (Brader, 2007).

The interaction between Raf36 and MKK2 was verified by a direct Y2H approach and additionally by *in vitro* pulldown assay. Using *in vitro* phosphorylation assays, it was demonstrated that the Raf36 is a functional kinase. The recombinant protein showed autophosphorylation, which was not observed with a mutated protein lacking kinase activity. Since MKK2 was not phosphorylated by Raf36 *in vitro*, the existence of an autoinhibitory domain at the amino-terminus was postulated. In this case a specific stimulus would be required for Raf36 activation. To circumvent this autoinhibition, several amino-terminal truncations of the Raf36 protein were generated. As expected these truncated proteins were able to phosphorylate MKK2 *in vitro.*

Two *knockout* lines, independent *raf36*-alleles named *raf36*-1 and *raf36*-2, from the SALK T-DNA insertion collection, were used as tools to elucidate Raf36 function *in planta*. The generation of Raf36 overexpressing lines failed, therefore *in planta* experiments were restricted to the *knockout* plants.

Germination assays revealed that the *raf36*-1 line, similar to *mkk2* plants, was inhibited in their germination on salt containing media as well as on media containing sorbitol and Methylviologen, an oxidative stress agent. Theses results pointed to an involvement of Raf36 together with MKK2 in osmotic / oxidative stress signalling. Since the second knockout line *raf36-2* did not show significant inhibition on salt, several T-DNA insertion lines are currently being analysed to identify a third *raf36* allele.

In contrast to *mkk2* plants (Teige, 2004), *raf36*- plants did not show any reduction in the MPK4 and MPK6 activity upon cold treatment. This result made a regulation of MKK2 by Raf36 after cold treatment improbable.

raf36-plants emerged to be hypersensitivity to *Alternaria brassicicola* infection, similar to MKK2EE lines overexpressing a constitutively active version of MKK2 (MKK2EE) (Brader,

2007). Jasmonic acids-levels were found to be reduced after fungal infection in both mutant lines. Transcriptome analysis of both lines revealed a significant overlap in differentially regulated genes in both lines after infection with the fungus. These results point to a role of Raf36 together with MKK2 in stress signalling upon *A.brassicicola* infection.

To summarise, Raf36 seems to regulate MKK2 in response to *A.brassicicola* infection. It is likely that Raf36 has an inhibitory effect on MKK2 in this context. The mechanism of this inhibitory effect remains to be elucidated, as well as a putative involvement of Raf36 in MKK2 mediated abiotic stress signalling.

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1.Introduction

1.1 Signal transduction in *Arabidopsis thaliana*- What role mitogen-activated protein kinases (MAPK) play

Plants as sessile organisms have to adapt to a broad range of environmental changes and cope with a multitude of adverse stresses. These stresses are commonly divided into abiotic and biotic stresses. Abiotic stresses comprise changes in the environment like cold, heat, drought, high salinity or toxins like heavy metals, while pathogens are the triggers of biotic stresses.
To adapt to these changing and often adverse conditions, plants are in need of an elaborate signalling network. Signalling is mainly performed by kinases, which account for approximately 5% of the *Arabidopsis thaliana* genes. Approximately 10% of all plant kinases are belonging to the group of MAPKs. MAPKs are serine/threonine kinases which are highly conserved in all eukaryotic organisms. The are not just involved in stress signalling but also in responses to hormones, in cell division and development (Colcombet and Hirt, 2008).
MAPK signalling works through three-tier cascades, built up by a MAP kinase (MPK), a MAP kinase kinase (MKK) and a MAP kinase kinase kinase (MAPKKK) (Fig.1.1). In *A.thaliana*, the complex MAPK signalling network is built up by 20 MPKs, 10 MKKs and 60 MAPKKKs (Group, 2002) .

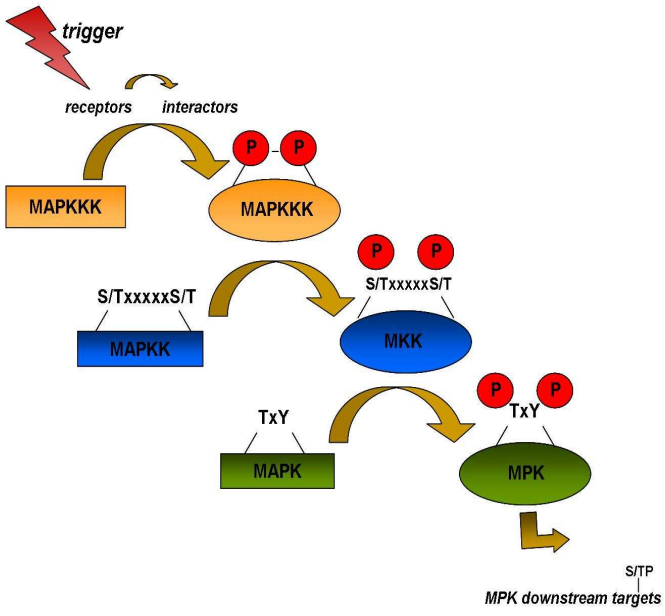


Fig1.1: Three-tier MAP kinase cascade
An appropriate trigger, external stress signal or internal developmental signal, is perceived by a receptor and transmitted by phosphorylation events, optionally via additional interactors, to a MAPKKK. The phosphorylated MAPKKK phosphorylates and thereby activates an MKK, the active MKK phosphorylates the last tier in the MAPK cascade, an MPK. The MPK phosphorylates downstream targets like transcription factors or other kinases, leading to the required adaptations of the cell.

1.2. Signalling events in a MAPK cascade

The phosphorylation events in a typical three-tier MAPK cascade are described from the bottom to the top: MPKs are phosphorylated by activated MKKs at two residues in the T-X-Y motif in their activation loop. Activated MPKs phosphorylate their substrates at serine or threonine residues in an SP/TP-motif, the minimal MPK target site (Songyang et al., 1996). As MKKs can phosphorylate threonine as well as tyrosine (Y)-residues, they are belonging to the so called dual-specificity kinases (Rudrabhatla et al., 2006). MKKs contain a MPK docking site, called D-site, for interaction with their MPK substrate. The core consensus motif of this D-site was defined as $(K/R)_{1-3}$-X_{1-6}-φ-X-φ, where φ stands for a hydrophobic residue. This D-site is also found in many MPK-substrates and was shown to contribute to the specificity of MAKK-MPK interaction (Bardwell, 2006). Other conserved motifs, binding different parts of the MPK protein, have been identified in mammalian MAPKs (Akella et al., 2008).

MKKs in turn are phosphorylated and thereby activated by MAPKKKs at their two target sites in the S/T-X_{3-5}-S/T motif in the activation loop (Colcombet and Hirt, 2008). MAP3Ks can be regulated by upstream regulators like MAP4Ks (MAPKKK kinases), which might directely interact with membrane-located receptors sensing outside stimuli.

When a MAPK cascade is triggered, the activated MPKs are the last chain link and forward the signal by phosphorylating downstream targets like other kinases or transcriptional regulators. The induced change in transcriptional activity enables the cell to adapt to the sensed stress.

To switch off the MAPK signal, MAPKs are dephosphorylated and thereby inactivated by different groups of protein phosphatases. The small group of so called MAP kinase phosphatases exclusively dephosphorylates MAPKs and fine tunes the MAPK triggered responses to biotic as well as abiotic stress (Liu et al., 2007; Ulm et al., 2002). PP2Cs (protein phosphatase 2C), a large group of phosphatases, are important regulators of many plant signalling pathways, among them MAPK signalling (Schweighofer et al., 2007).

1.3 Innate immune response

1.3.1 Co-evolution led to different levels of plant-pathogen interaction

Plants don't own adaptive immunity mechanisms like mammals. Confronted with pathogens they have to rely on their innate immune response. Different levels of immune responses are found in plants (de Wit, 2007) (Fig.1.2).

The first defence level in this innate immune response is triggered by pathogen-associated molecular patterns (PAMPs), and is therefore referred to as PAMP-triggered immunity (PTI) (Zipfel, 2008). PAMPs are aggressor-derived, small molecules conserved in large groups of related pathogens and are recognised by PRRs (PAMP recognition receptors), receptor-like kinases (RLKs) in the plant cell membrane. There are very few PAMP-PRRs identified up to now in *A.thaliana*. One of the best studied PAMPs is flg22, a 22- amino acid peptide from the eubacterial structural protein flagellin. Also the bacterial pathogen *Pseudomonas syringae* triggers plant defence responses by this PAMP. flg22 binds to the LRR-RLK FLS2 (flagellin-sensing 2), which functions in complex with BAK1 (Bri1-associated kinase1) (Chinchilla et al., 2006; Heese et al., 2007). The *Escherichia coli* protein EF-Tu (elongation factor thermo-unstable) binds to the membrane bound PAMP receptor EFR (EF-Tu receptor) (Zipfel et al., 2006), a LRR-RLK which might interact directly or indirectly with BAK1 as well. Also the chitin receptor CERK1 (chitin elicitor receptor kinase 1) was identified recently and shown to be involved in resistance to the fungus *Alternaria brassicicola* (Miya et al., 2007).

Stimulation of all mentioned RLKs seems to lead to the same downstream processes, which include MAPK activation, rapid ion fluxes across the membrane and production of antimicrobial substances such as reactive oxygen species (ROS) and phytoalexins and changes in gene expression. The transcriptional, metabolic and structural changes initiated in PTI enable the plant to defeat the invader. Missing PAMP recognition leads to enhanced susceptibility of the plant to the undetected pathogen.

During evolution, some plant pathogens developed strategies to circumvent PTI by injecting so-called effector or virulence proteins into the plant cell. Effector proteins suppress signalling events and thereby inhibit the plant defence mechanisms. This process, the second level of plant pathogen interactions, is called effector triggered susceptibility (ETS).

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 (coded by resistance- or *R*-genes). Upon specific recognition of

pathogen effector proteins, or in this case also named avirulence (Avr) factors, they are triggering a defence signalling cascade. 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 effector proteins. These different levels of plant pathogen interactions where converged into the 'Zig-Zag model' which illustrates the never ending competition between plants ad pathogens driven by co-evolution of new effector proteins and new plant *R* genes (Jones and Dangl, 2006).

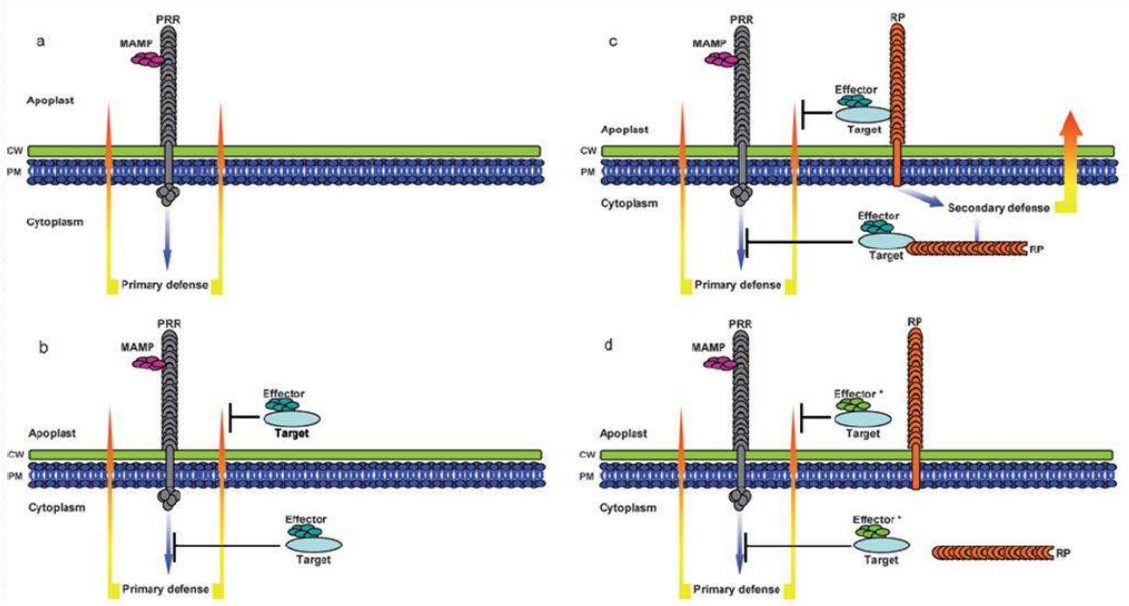


Fig.1.2:

Induction and suppression of innate immune responses in plants by microbial pathogens.

a) MAMP (microbe-associated molecular pattern) recognition by PRRs (pattern recognition receptors) in the cell membrane, triggering PTI (PAMP triggered immunity); the pathogen is avirulent

b) Secretion of an effector protein by a virulent pathogen which inhibits PTI, leading to ETS (effector triggered susceptibility); the pathogen is virulent

c) Attack by an avirulent pathogen; the plant expresses an RP (resistance protein, mostly LRR-NBS receptors) leading to specific recognition of a microbial effector protein and initiation of a secondary immune response, called ETI (effector triggered immunity); the pathogen is avirulent

d) The next evolutionary level of microbial effector proteins, which are not recognized by the plant RP protein anymore, allow infection; the pathogen is virulent

Plants feature another protection mechanism against pathogen, the systemic acquired resistance (SAR) (Grant and Lamb, 2006). SAR is unlike PTI or ETI not restricted to the infection site. It is induced systemically together with the ETI responses, and protects uninfected parts of the plant from future pathogen attacks.

Interestingly, plant microbial pathogens and symbiotic microbes induce the same set of defence responses (Zhao and Qi, 2008), also expressed in the fact that PAMPs are often referred to as 'microbe-associated molecular patterns' (MAMPS). The symbiotic microbes seem to have evolved mechanisms to overcome the basal plant defence. They do not trigger the development of disease symptoms and are able to establish a balance with their host. This has led to the idea to increase plant pathogen resistance by switching off signals that lead to the deteriorating disease symptoms.

1.3.2 Hormone signalling in *Arabidopsis thaliana* immune response

Hormones play a pivotal role in plant immune signalling. The main hormonal players in immune responses are jasmonic acid (JA), ethylene and salicylic acid (SA).

The basic idea of the function of theses different hormones in immune responses is an antagonistic effect of SA and JA/ethylene mediated defence signalling. (Koornneef et al., 2008) , cross-talk between them is well documented (Koornneef and Pieterse, 2008). SA mediated signalling is required for HR and SAR induction (see chapter 1.4.1) and is therefore active in response to biotrophic pathogens like *P. syringae*. As biotrophic pathogens need living tissue to feed on, HR induced cell death deprives them from food and water. The JA/ethylene pathway can restricts SA induced cell death spreading. In response to necrotrophic pathogens, which feed on dead tissue and can therefore not be defeated by cell death, JA/ethylene-mediated defence responses are required. Two branching points in this antagonistic regulation are, among others, the MPK4 downstream targets PAD4 and EDS1, which enhance SA signalling and suppresse some JA/ethylene mediated responses like *Pdf1.2* expression (Brodersen et al., 2006).

The presented model of the hormone interplay in immune response is of course simplified and leaves out some divergent aspects and exceptions. Although JA and ethylene signalling converge for example on induction of some defence response genes like *ERF1* and *HDA19* (Zhou et al., 2005), there are also examples for antagonistic effects of JA and ethylene signalling, like in the induction of some wound-induced genes (Norman-Setterblad et al., 2000).

Although SAR has long been considered as solely SA dependent, it is becoming more and more evident that JA might be involved as well, it might consitute the main long-distance messenger in this type of defence response (Truman et al., 2007).

Many pathogens are manipulating plant hormone signalling to promote disease. A prominent example is the *P. syringae* phytotoxin coronatin (COR) (Bender et al., 1999). Due to its structural similarity to JA derivatives, it mimics JA in regulating gene expression (Thilmony et al., 2006) and represses SA mediated defence responses. COR additionally mimics some auxin functions. Auxin is a main regulator of plant growth and development, and therefore a target for manipulation by a range of biotrophic pathogens. *Agrobacterium tumefaciens* induces auxin production to induce formation of 'crown galls', which provide the bacteria with all required nutrients. Manipulation of ABA signalling as a mechanism to weaken plant defence responses is also used by some pathogens such as *P. syringae* (de Torres-Zabala et al., 2007). The beneficial aspects of ABA induction for biotrophic pathogens are not completely examined yet. ABA mediated repression of callose and lignin deposition for cell wall reinforcement, as well as repressive effects on SA induced responses might play a role.

1.3.3 Two model organisms in plant- pathogen research: *Pseudomonas syringae* (*P.syringae*) and *Alternaria brassicicola (A.brassicicola)*

Two main groups of pathogen are distinguished, biotrophs which need a living host for feeding and reproduction, and necrotrophs which kill the host by inducing cell death and feed on the dead tissue. The well-studied bacterium *P. syringae* is one of the model organisms for biotrophic plant pathogens and the fungus *Alternaria brassicicola* is often used s a model for necrotrophic plant pathogens. As both organisms were used for infection assays in this work, they will be presented here in more detail.

P.syringae is a biotrophic bacterium which causes leaf spot disease in susceptible plant species. While infecting a plant, it uses a specialized type III secretion apparatus to inject a range of effector proteins into the plant cell, which help to defeat the flg22-induced PTI. Some of those Type III effectors are avirulence proteins triggering ETI and SAR, resulting in avirulence of the bacterial strain in the respective host. One of the *P. syringae* strains used in this work, a transgenic *P. syringae* pv.tomato DC3000 (*Pst*DC3000) strain, is avirulent in *A.thaliana Columbia* (*Col*-0) ecotype due to the avirulence gene *avrRptII* that had been introduced. The ETI inducing interaction between the bacterial Type III effector protein

AvrRptII, a protease, and the plant resistance protein RPS2 has been thoroughly examined (Coaker et al., 2005). Virulent *P. syringae* strains are not recognized by a plant *R*-gene. They produce a range of phytotoxins like coronatin (COR), which interferes with the plant hormonal pathogen-responses (see chapter 1.4.2) and thus induces systemic induced susceptibility (SIS). SIS is counteracting SAR.

Alternaria brassicicola is a necrotrophic fungus that causes dark leaf spots ('black spot disease') on all cultivated *Brassica* species, like broccoli or cabbage. The fungus produces a number of phytotoxins which induce cell death, and the necrotic lesions caused by the fungus can appear in all parts of the plant. Although *A. thaliana* is also belonging to the *Brassicaceae* family, most ecotypes exhibit resistance towards *A. brassicicola*, among them also the *A.thaliana Col*-0. JA responses are required for this resistance, as the JA signalling mutant *coi1* (coronatin-insensitive 1) is highly susceptible to *A. brassicicola.* Parallel to the JA-dependent resistance there was a JA-independent resistance mechanism identified, which requires production of the phytoalexin camalexin. Camalexin (Glawischnig, 2007) is induced upon a range of bacterial (Glazebrook et al., 1997) and fungal infections (Thomma et al., 1999). Camalexin biosynthesis mutants like *pad3* (Thomma et al., 1999) turned out to be highly susceptible for the fungus, although they did not feature any inhibition of JA signalling. SA and ethylene are most likely playing minor roles in the defence response, as several SA signalling mutants and the ethylene signalling mutant *ein2* did not exhibit increased *A. brassicicola* susceptibility (van Wees et al., 2003).

1.3.4 MAPK signalling in innate immune response

It is known that MPK3, MPK4 and MPK6 are activated upon a broad range of biotic stress stimuli, and although no link between MAPK signalling and ETI (or 'gene-for-gene-resistance') has been found, MAPK cascades are a central part of the PTI response. Several upstream components of MPK3, MPK4 and MPK6 in plant immune responses upon flg22 treatment have been identified. Transient expression experiments in protoplasts led to the model of a MEKK1-MKK4/5-MPK3/6 module triggering defence responses upon flg22 stimulation (Asai et al., 2002), regulating WRKY 22 and WRKY 29 as downstream targets. *In planta* experiments were not able to undoubtly confirm participation of MEKK1 in this cascade, as in *mekk1* plants flg22 still induces MPK3 and MPK6 activity, but not MPK4 activity. MPK3 transcription and MPK6 activity is even upregulated after flg22 treatment, pointing to an inhibitory effect of MEKK1 on MPK3/6 mediated defence responses

(Ichimura et al., 2006; Suarez-Rodriguez et al., 2007). A new model was proposing an alternative MAPKKK in MKK4/5 regulation, and a second MAPK pathway induced upon flg22 treatment, consisting of MEKK1 and MPK4. The predicted unknown MAPKKK upstream of MKK4/5 might be YODA, as a YODA-MKK4/5-MPK3/6 module is known to regulate stomatal development (Wang et al., 2007). A prove for YODA function in pathogen response in plants is still lacking. The missing link in the MEKK1-MPK4 cascade was just recently unrevealed by analysis of *mkk1/mkk2* plants (Gao et al., 2008; Qiu et al., 2008). The double mutants show the same phenotype like *mekk1* and *mpk4* plants. They are severly dwarfed, accumulate high SA and ROS levels, and constitutively overexpress a range of defence response genes like *PR1* and *PDF1.2* (Plant Defensin 2), accompanied with spontaneous cell death in the leaf tissue. Therefore the MEKK1-MKK1/2-MPK4 module is hypothesised to form a flg22-signalling pathway suppressing SA-induced defence responses and therefore working antagonistically to the MKK4/5-MPK3/6 module (Fig.1.3). Features of the MEKK1-MKK1/2-MPK4 cascade will be described more detailed in chapter 1.6. Already the possible involvement of MEKK1 in both of the pathways hinted to a tight cross-talk between the pathways, several findings like reduced MPK3/6 in *mkk1* plants upon flg22 treatment (Meszaros et al., 2006) or the existence of common regulators of MPK4 and 6, like the phosphatase AP2C1(Schweighofer et al., 2007), support this idea.

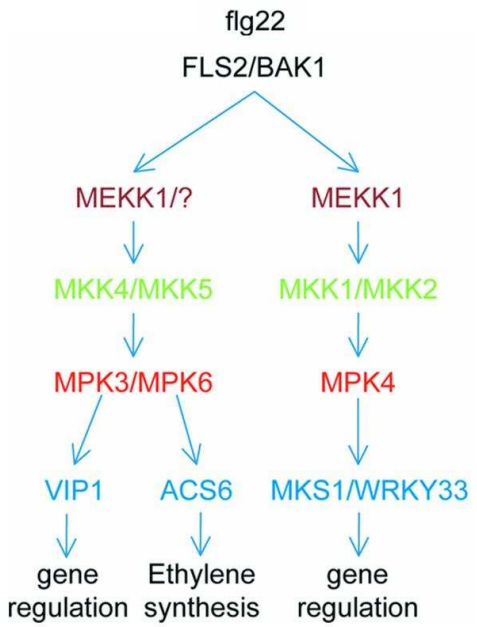


Fig.1.3:
Two antagonistic MAPK signalling cascades are induced upon flg22 treatment, the MKK4/5-MPK3/6 cascade regulated by MEKK1 or another (additional) MAPKKK, and the MEKK1-MKK1/2-MPK4 pathway repressing SA-mediated defence responses. The first one is inducing, the second one repressing SA mediated defence responses (picture adapted from Colcombet, Hirt (2008)).

PAMPs of bacterial, fungal or oomycetal origin seem to trigger the same set of defence responses in PTI, and several of them were shown to activate MPK3, MPK4 or MPK6.

Therefore the described pathways might not be flg22-specific, but form general signalling components in PTI.

And the picture is not complete yet, there are most likely a number of other MAPK modules cross talking with the two cascades described. Especially the MKKs MKK7, previously just connected to auxin signalling, and MKK9 turned out to act in pathogen signalling a well. Zhang et al. (2007) found evidence for a role of MKK7 in defence response, as the bud1 mutant, which overexpresses MKK7, accumulates SA and exhibits increased PR–gene expression and *P. syringae* resistance. An MKK9-MPK3/6 cascade under negative control of the MAPKKK CTR1 is contributing to the plant innate immune response by inducing ethylene and camalexin biosynthesis (Xu et al., 2008). Takahashi et al. (2007) suggested an inhibitory function of a MKK3-MPK6 module on JA signal transduction, indirectly influencing *Pdf1.2* gene expression. As the MPKs 1,2,7 and 14 were found to physically interact with MKK3 *in planta* and to be activated by MKK3 in transiently transformed protoplasts, they could form direct MKK3 downstream targets. PR-gene induction in MKK3 overexpressors implies additional MKK3 functions in SA pathway regulation (Doczi et al., 2007). Regulation of hormone biosynthesis turned out to be a major function for those MAPK modules in immune response (Schweighofer and Meskiene, 2008).

Some important downstream targets of MPK3 and MPK6 have been discovered, revealing the multiple functions of MAPK signalling in immune response. MPK6 has been shown to phosphorylate ACS6 [ACC (1-amino-cyclopropane-1-carboxylic acid) Synthase 6] (Liu and Zhang, 2004), linking the MPK to regulation of ethylene production. The transcription factor VIP1 (VirE1-interacting protein 1) (Djamei et al., 2007), regulating expression of PR1 and other defence genes, was identified as an MPK3 target. MPK3 was shown to phosphorylate the transcription factor VIP1, which is then translocated from the cytoplasm to the nucleus and leads to induction of defence genes like PR1 (pathogenesis–related 1). MKS1 (MAPK substrate 1) was identified as a MPK4 downstream target after flg22 stimulation (Andreasson et al., 2005).

1.4 MAPK signalling in abiotic stress signalling

Besides their role in biotic stress signalling, MAPKs are also enrolled in the response to a range of abiotic stresses (Fig.1.4). The best-studied MAPK cascade in abiotic stress signalling is the MEKK1-MKK2-MPK4/6 module, which is induced by salt and cold (Teige et al., 2004) and will be discussed in more detail in chapter 1.6. For most of the other

important abiotic environmental stresses, like drought, wounding, water stress (ABA mediated response), osmotic shock or ozone, the identity of most of the MAPK cascades involved still needs to be uncovered.

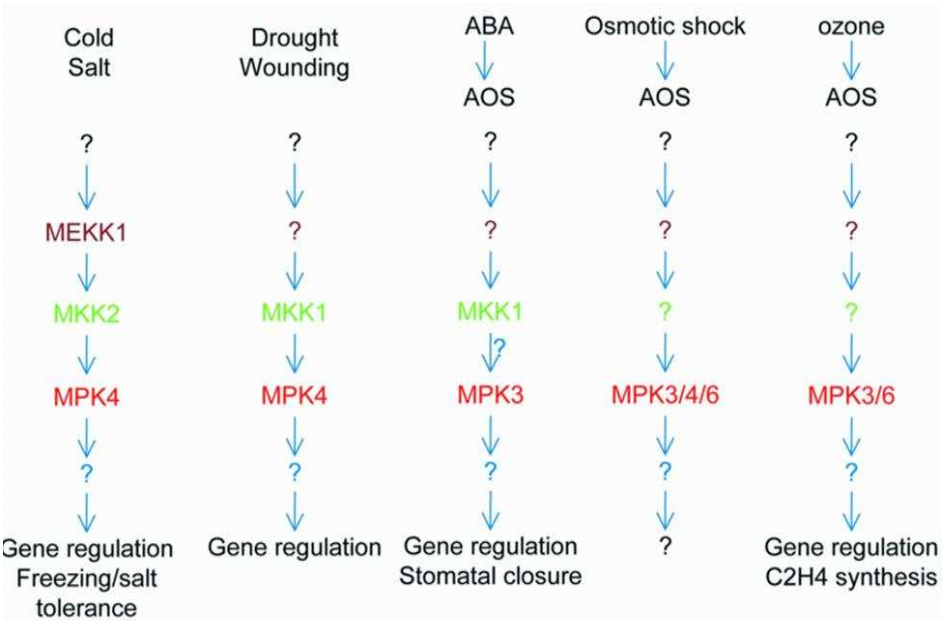


Fig.1.4:
MAPK signalling in abiotic stress responses: Cold/salt stress induces the MEKK1-MKK2-MPK4 cascade, the MKK1-MPK4 cascade is induced by drought and wounding, the MKK1-MPK3 cascade is induced after ABA (water stress), and MPK3, 4, 6 or MPK3 and 6 are induced upon osmotic shock or ozone treatment respectively (picture adapted from Colcombet, Hirt (2008)).

1.4.1 Ozone (O_3) triggered MAPK signalling

Ozone is a major air pollutant, high O_3 concentrations lead to large necrotic lesions in plant tissue and therefore have devastating effects in agriculture. But the effect of O_3 is not just economically interesting, ozone is a potent ROS generator and therefore used as a model stress for exploring ROS signalling effects. ROS production can be detected upon biotic (oxidative burst) and abiotic stresses (Fig.1.4) and ROS are important components of stress signalling. They induce MAPK and hormonal signalling and are involved in PCD (programmed cell death) induction, like observable in pathogen induced HR. The *rcd1* (radical cell death 1) mutant (Overmyer et al., 2005), which exhibits defects in JA, ethylene and ABA signalling, was identified due to its ozone-sensitivity. MPK3 and MPK6 have been identified as actors in O_3 triggered signalling. *mpk3* and *mpk6* plants are hypersensitive to

ozone (Miles et al., 2005), and both kinases are translocated to the cell nucleus after ozone treatment (Ahlfors et al., 2004). A MAPK phosphatase, MKP2, was identified as a common regulator of both MPKs upon ozone (Lee and Ellis, 2007). Most MAPK components involved in ozone induced signalling, or more broadly in ROS induced signalling, remain to be elucidated. In contrast to the ROS-induced activation of some MAPK signalling components, redox-regulatory proteins have been identified as potential MAPK targets (Feilner et al., 2005). This points to a complex cross regulation between MAPK pathways and ROS.

1.5 MKK2 in biotic and abiotic stress signalling

MKK2 was initially isolated from an *A.thaliana* cDNA library in a functional complementation assay of the osmosensitive yeast mutant pbs2Δ, which is lacking a MAPK kinase from the Hog1 pathway (Teige et al., 2004). In the yeast strain pbs2Δ hog1Δ, lacking Pbs2 and its downstream kinase Hog1, which had been transformed with MKK2, the *A.thaliana* MPKs MPK4 and MPK6, but not MPK3 were identified as MKK2 downstream targets. The closely related MKK1 was complementing the double knockout just together with MPK4, but not with MPK3 or 6. Cold and salt induced MKK2 activation by the MAPKKK MEKK1, and MPK4 activation by MKK2 could be shown in transiently transformed protoplasts. Interestingly, MKK2 was not activated by hydrogen peroxide (H_2O_2) and the pathogen elicitors flg22and ß-laminarin, while MKK1 was able to phosphorylate MPK4 upon those triggers, but not after salt and cold stress, pointing to at least partially divergent functions of theses closely related MKKs.

mkk2 plants are hypersensitive to cold and salt stress and in this mutant the MPK4 and 6 activation upon cold stress was impaired. Plants overexpressing a constitutively active MKK2 (MKK2EE) showed the opposite phenotypes, MPK4 and MPK6 are also constitutively active. The authors concluded that a MEKK1-MKK2-MPK4 cascade was mediating cold and salt stress responses.

The role of MKK2 in biotic stress was first adressed by Brader et al. (2007). The authors detected increased sensitivity and reduced JA and SA levels in MKK2EE plants after *P. syringae* infection and an increased susceptibility to *A.brassicicola*. *mkk2* plants did not show a pathogen response phenotype. Therefore a second MKK with partially redundant functions was postulated. Recently this question was answered by analysis of the double knockout

mutant *mkk1/2* which allowed positioning of MKK2 in a MEKK1-MKK1/2-MPK4 module (Fig.1.3) in innate immune response, like described in chapter 1.4 (Gao et al., 2008; Qiu et al., 2008)

1.6 MAPKKKs and Raf36

Arabidopsis thaliana MAPKKKs were identified based on their sequence homology to known MAPKKK from yeast and Mammals. More than 60 MAPKKKs in *A. thaliana* were identified and, according to their kinase domain sequence homology, divided into two main subgroups: the MEKK-type kinases (or group A kinases) and the Raf-like kinases. The Raf-like kinases are, based on their structural features, further divided in group B and C kinases. All group B members contain a considerably prolonged amino-terminal domain.

Most of the 12 members of the MEKK-type kinases are functional MAPKKKs, their involvement in MAPK signalling pathways has been proven. The first identified functional MAPKKK in plants was MEKK1 (Ichimura et al., 1998), which was found to regulate MKK2 after salt and cold stress and recently positioned upstream of MKK1/MKK2 in pathogen defence signalling (Gao et al., 2008; Qiu et al., 2008). YODA regulates stomatal development (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 et al., 2002) are belonging to the MEKK-like group of MAPKKKs. ANP1 can phosphorylate MPK3 and MPK6 in transient expression assays in protoplasts after H_2O_2 treatment, and the ANP1 orthologue NPK1 influences abiotic stress tolerance in *Nicotiana tabacum* (Kovtun et al., 2000).

The function of the Raf-like kinases in *A. thaliana* is predominantly unknown. In group B, CTR1 (constitutive triple response) could recently be positioned upstream of the MKK9-MPK3/6 module as a negative regulator in the ethylene pathway (Clark et al., 1998; Gao et al., 2003; Yoo et al., 2008), while the closely related MAPKKK EDR1 (enhanced disease resistance 1) negative regulates SA-mediated defence responses (Frye et al., 2001). Recently, another group B Raf-like kinase was reported to have inhibitory effects in salt induced stress signalling (Gao and Xiang, 2008)

In group C, ATN1(Tregear 1996) and AtMRK1 (Ichimura 1997) have been described, but none of them was shown to participate in MAPK signalling. Raf36 also belongs to group C of the Raf-like kinases. It is the only group C kinase that contains a serine-rich region in its N-terminus, and overall protein sequence similarity to the most closely related *Arabidopsis*

thaliana Raf-like kinase (At3g46930) is 56%, while similarity in the highly conserved kinase domain region is 69%. Except from the Serine-rich region and the kinase domain no other domain motifs could be identified in Raf36.

1.7 Regulation of mammalian Raf-kinases

In contrast to the Raf-like kinases in plants, the regulating mechanisms of the three mammalian Raf kinases A-Raf, B-Raf and C-Raf have been extensively studied. Theses kinases were isolated as homologues of retroviral oncogenes inducing tumor genesis, and B-Raf is mutated at a high frequency in human cancer cells. The Raf kinases are involved in essential cell activities like cell proliferation, survival, differentiation and apoptosis. Their regulation turned out to be highly complex and is still not completely elucidated. Although the regulation of the three Raf kinases differs, contributing to their different functions in the cell, they have some common regulatory mechanisms. As the best studied of the three Raf kinases is C-Raf, the regulation of this Raf kinase will be described in more detail. Like all three Raf kinases, it contains three conserved regions: the amino-terminal regions CR1 and CR2 domains and the carboxy-terminal region CR3 which contains the catalytic domain. The first level of regulation occurs by binding of small GTPases (like Ras) in the CR1 regio. The GTPases recruite C-Raf to the plasma membrane. The membrane located C-Raf is then phosphorylated from a number of different upstream kinases, such as p21 activated kinase (PAK) or Akt kinase, at numerous residues and render the kinase active. The variety of upstream kinases and the number of phosphorylated residues which were found in the amino-terminus as well as in the catalytic domain contribute to the tight regulation of C-Raf. The specific effects of phosphorylation of the different phosphorylation sites on the C-Raf activity are still under investigation and has just been clarified for a few cases (Chong et al., 2003). A number of inhibitory phosphorylation sites are phosphorylated in the unstimulated state, keeping Raf inactive. In this autoinhibitory state the amino-terminal domain was proposed to be in contact to the catalytic domain, thereby blocking the kinase activity. Dephosphorylation of inhibitory residues could be accomplished by the phosphatases PP1 and PP2A, which were shown to promote C-Raf activation. Further regulation of Raf36 activity and specificity occurs by binding of a variety of scaffolding proteins; which are promoting or inhibiting the interaction of Raf with its upstream or downstream partners by bringing them in close proximity or keeping them away from each other (Kolch, 2005).

An additional level of complexity of the mammalian Raf kinase regulation is added by the fact that C-Raf/B-Raf heterodimers are found in cells. They may be important for the regulation of distinct biological processes (Rushworth et al., 2006). In plants up to now not much is known about the regulation of Raf like kinases. Few examples for scaffolding proteins have been identified, but MAPKKKs might contain specific interaction domains in their elongated amino- or carboxy-terminal regions that exhibit scaffolding functions, like shown for the alfalfa MAPKKK OMTK1(Nakagami et al., 2004). One of the few Raf like kinases in *A.thaliana* who's regulatory mechanism had been investigated so far is CTR1 (Huang et al., 2003).

1.8 Aim of the project

The project described in this thesis started with the isolation of Raf36 in a yeast-2-hybrid screen, performed to identify unknown MKK2 interactors. The aim of the project was to elucidate the function of the Raf36-MKK2 interaction in MAPK stress signalling. The *in vitro* kinase activity of Raf36 was examined, and its ability to phosphorylate MKK2 was tested. *raf36* plants were tested for the cold and salt phenotypes that were observed in *mkk2* plants, and infection assays with the biotrophic pathogen *P. syringae* and the necrotrophic fungus *A. brassicicola* were used to uncover a possible role of Raf36 in defence signalling, like it had been shown for MKK2. After uncovering an increase in *A.brassicicola* sensitivity in *raf36* plants, we decided to access MKK2-connnected Raf36 function in defence responses by transcriptome analysis using CATMA arrays. To our knowledge, Raf36 is the first Raf-like kinase of the C-group that can be functionally linked to a known MAPK-signalling pathway.

2.Material and Methods

2.1 Bacteria strains and growth conditions

2.1.1. *Escherichia coli* (*E.coli*)

a) XL1-Blue endA1, hdsR17 (r_k^- m_k^+), supE44, thi, λ^-, recA1, gyrA96, relA1, lac^-, F'[proAB, $lacI^q$, lacZ, ΔM15, Tn10 (tet^R)] (Stratagene Inc., La Jolla, CA, USA)

b) BL21-Codon Plus (DE3)-RIL strain F-, ompT, hsdS (r_b^- m_b^-), dcm^+, Tet^R, galλ, endA, Hte [argU, ileY, leuW, Cam^R] (Stratagene Inc., La Jolla, CA, USA)

E.coli cultures were grown at 37°C in Luria-Bertani (LB) medium with appropriate antibiotics unless indicated otherwise.

Preparation of heat shock competent *E.coli* cells

Preparation of heat shock-competent *E. coli* was carried out according to the 'Inoue Method' (Inoue et al., 1990). An overnight culture grown in LB medium was inoculated 1:50 (OD_{600}=0.05-0.08) in 100 ml of 'SOB'-medium (and grown at 18°C to an OD_{600} of 0.6. After incubation on ice for 10min, the cells were spun at 2500g and 4°C for 10 min, and the pellet was resuspended in 80ml ice cold transformation buffer (TB). After another 10min on ice the cells were spun at 2500g and 4°C again and the pellet resuspended in 7ml ice cold TB-medium. After 10min on ice DMSO was added to a final concentration of 7%, after additional 10 min on ice the cells were aliquoted (40-50µl) in precooled tubes, quick-frozen in liquid nitrogen and stored at -80°C until further use.

Transformation of *E.coli* cells by heat shock

Heat shock competent cells were thawed on ice and incubated for 5min with 1µl vector DNA (ca. 0.5µg/µl). Cells were kept for 30sec at 42°C, then 300µl 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.

Preparation of electrocompetent *E. coli*

An overnight culture grown in LB medium was inoculated 1:100 in 250 ml of fresh LB media and grown at 37°C to an OD_{600} of 0.6-0.7. After incubation on ice for 30min, the cells were spun at 5000g and 4°C for 10 min, and the pellet was resuspended in 250ml 10% autoclaved and precooled glycerol solution in water. After spinning at 5000g and 4°C for 10min the pellet was resuspended in 125ml 10% glycerol solution and spun again. The washing was repeated once more with 10ml 10% Glycerol solution, followed by resuspension of the pellet in 0.5ml 10% glycerol solution and incubation of the cells for 30min on ice. The cells were aliquoted (40-50µl), quick-frozen in liquid nitrogen and stored at -80°C until further use.

Electro-transformation of *E. coli* cells

Electro competent cells were thawed on ice and incubated for 5min with 1µl vector DNA (ca. 0.5µg/µl). Cells were transferred to a precooled cuvette, and electroporated by an electric pulse of 12.5kV/cm (resistance 200Ω, capacity 25µF). 1ml LB medium was 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.

2.1.2 *Pseudomonas syringae*

P. syringae pv. *tomato* strain DC3000 (*Pst*DC3000) (Chen et al., 2000; Whalen et al., 1991)

a) *P. syringae* strains *Pst*DC3000 (*avrRpt2*) (kan^R, rif^R): carrying the avirulence gene avrRptII, a type III effector protein promoting resistance of Col-0 *wild type* plants to this strain
b) *Pst*DC3000 pVSP61 (kan^R, rif^R): lacking the avirulence gene

P. syringae cultures were grown at 30°C in NYG-medium with appropriate antibiotics.

P. syringae for infection assays were streaked from Glycerol stocks (12%) on a fresh NYGA plates with appropriate antibiotics and grown at 28°C for two days. Due to the light instability of rifampicin, plates were rapped in aluminium foil.

2.1.3 *Agrobacterium tumefaciens*

GV3110($rif^R gent^R$)/ pSOUP (tet^r) (Koncz and Horvath, 1996)

A tumefaciens cultures were grown at 28°C in LB medium with appropriate antibiotics.

Preparation of electrocompetent *A. tumefaciens*

Procedure was the same like for preparation of electrocompetent *E. coli*, but the overnight cultures was diluted 1:50 in fresh LB media, grown at 28°C, and centrifugation steps were done at 7000 g.

Transformation of electrocompetent *A. tumefaciens* cells

Procedure like for electrocompetent *E.coli,* but *A. tumefaciens* cells were grown at 28°C after transformation, and plates were wrapped in foil to protect them from light, as Rifampicin is unstable in the light.

2.2 Yeast

2.2.1 Yeast strain and growth conditions

Saccharomyces cerevisiae

L-40: MATα, hisΔ200, trp1-900, leu2-3, 112 ade2, LYS2:: $(lexA\ op)_4$HIS3 URA3:: $(lexA\ op)_8$ lacZ Gal4 gal80

S.cerevisiae cultures were grown at 28°C in YPD (complete) medium or SD (minimal medium and appropriate amino acids.

2.2.2 Yeast two-Hybrid (Y2H) assay

The YTH approach is a tool to test the capability of two proteins to interact. Therefore the protein of interest is fused to the DNA binding domain (BD) and the putative interaction partner(s) to the activation domain (AD) of a trancription factor. Both chimeric proteins, called bait protein (BD-fusion protein) and prey protein (AD-fusion protein), are co-expressed in yeast bearing a reporter gene under control of the trancription factor. If the bait protein (-BD) does not develop autoactivation, meaning it activates trancription of the reporter gene in absence of the prey protein (-AD), readout of the reporter can be used to detect protein interaction (Van Criekinge and Beyaert, 1999).

Here the lacZ gene encoding the enzyme ß-galactosidase was used as a reporter in the ß-galactosidase activity assay using the substrate artificial substrate ONPG (o-Nitrophenyl-β-D-galactopyranosid). ONPG is hydrolysed to galactose and o-nitrophenol, which causes a yellow colouring of the reaction. The colouring was quantified by measured the OD at wavelength 420 (OD_{420}) and so-called Units of ß-galactosidase activity were calculated. A second reportergene conferring HIS-auxotrophy enabled detection of protein interaction by growth assays on HIS-depleted selective media.

2.2.2.1 Y2H constructs

pAD-Raf36 : The Raf36 cDNA was amplified from pBluescript-Raf36 (RAFL 04-19-P16, received from RIKEN) with an additional SalI/PvuII restriction site and cloned into pGEM®-T Easy.From pGEM®-T Easy the Raf36 cDNA was cloned SalI/SmaI into a modified pAD vector with an additional SmaI site (created and kindly provided by Celine Forzani)
pBTM-MKK1-10: The MKK's were were cloned NcoI/NotI into the pBTM117 vector (constructs cloned and kindly provided by Iva Rajh).

2.2.2.2 Yeast transformation

An overnight culture was diluted to OD_{600}=0.2 in fresh YPD media and grown up to an OD_{600}=0.5-1.0. Cells were pelleted at 3500g for 5min at room temperature, resuspended in water and pelleted again at 3500g for 5min. The pellet was resuspended in 20ml TE/ LiAc mix.1ml aliquots were pelleted at 3500 g for 3min. The pellets were resuspended in 50µl TE/LiAc. 100µg single stranded DNA (10mg/ml, Sigma-Aldrich, Germany), autoclaved and boiled for 4min prior to use,1µg of vector DNA and 300µl of 40% PEG TE/LiAc mix were added. Cells were incubated for 30min at 30°C, followed by 15min at 42°C. To remove the PEG, 1ml of water was added, the cells were pelleted at 3500g for 3min and resuspended in 300µl l TE/LiAc. 100µl were spread on selective SD-plates.

2.2.2.3 ß-Galactosidase assay with substrate ONPG (Y2H interaction assay)

5ml overnight cultures grown at 30°C in SD medium with appropriate selection were vortexed to diperse clumps and 2ml were transferred to 8ml YPD medium and grown at 30°C for 3-5hrs. OD600 was measured, and three 1,5ml aliquots per sample were pelleted at 13.000rpm for 30sec. After removal of the supernatant the pellet was resuspended in 1,5ml Z-buffer, centrifuged again and resuspended in 300ml Z-buffer (concentration factor=5). 0,1ml

of the cell suspension was transfered to a fresh tube and quick-frozen in liquid nitrogen. The frozen tube was thawed in a 37°C water bath, the freeze-thaw-cycles were repeated twice and 700µl of Z-buffer+ ß-mercaptoethanol was added to the samples and the blank tube (100µl Z-buffer). 160µl of ONPG solution was added. As soon as the yellow staining became visible, the reaction stopped by adding 400µl 1 M Na_2CO_3. The exact incubation time was noted and the samples centrifuged for 10min at 13.000rpm to pellet cell debris. OD_{420} and OD_{600} of the supernatant was measured and the interaction strength was calculated in ß-Gal Units: U [ß-galactosidase activity hydrolysing 1µmol of ONPG/min*cell]=

$$\frac{OD_{420} \times 1000}{0{,}1\text{ml} \times \text{concentration fctor} \times \text{Time of reaction (min)} \times OD_{600}}.$$

2.3 *Alternaria brassicicola* strain and growth conditions

Alternaria brassicicola strain CBSnr 567.77 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands); kindly provided by Günther Brader

A. brassicicola was grown on PCA for two days at room temperature, then for at least 4 weeks (up to 12 weeks) stored at 4°C before use in infection experiments.

2.4 Plants

2.4.1 Plant lines

Raf36 T-DNA insertion lines were received from the SALK *Arabidopsis* T-DNA insertion collection or from SAIL (Syngenta *Arabidopsis* Insertion library) in Col-0 background (http://signal.salk.edu).

The homozygous *mkk2*-line (Teige et al., 2004) and *mkk1* ((Meszaros et al., 2006)was kindly provided by A.Djamei.

2.4.2 Growth conditions

Plants for proliferation, *Pseudomonas syringae* and *Alternaria brassicicola* infection assays were grown directly on soil. Sterilized and vernalized seeds (48 hrs at 4°C) were sawn on water-soaked soil (Blumenerde, Granuperl S3-6, # 50140050, *Knauf Perlite GmbH*, Vienna, Austria), covered with plastic to increase the humidity for better germination, and grown under the short day (8/16hrs day/night) or long day (16/8hrs day/night) conditions if not mentioned differently. The temperature was 22°C+/-5, the humidity 60% +/-20 and photon flux density of approximately 100 – 150 µmol m^{-2} s^{-1} .The plastic bag was opened after 2 days and removed after 5 days.

Plants for ozone treatments were grown on soil grown in growth chambers with 12/12hrs day/night, 22°C/16°C, 50/75% relative humidity and photon flux density 250µmol $m^{-2sec-1}$.

Seedlings for stress experiments and lines under selection were grown under sterile conditions. Sterilized seeds were sawn onto MS half strength agar plates with appropriate antibiotics or chemicals, vernalized on the plates, and grown under standard long day growth conditions. If needed, seedlings were transferred to soil after 10-12 days, covered for 2 days with plastic and grown under long day conditions like described before.

2.4.3 Seed sterilization

After harvest and sieving seeds were shaken for 10min in sterilization solution, then washed first twice with 70% ethanol, then twice with 96% ethanol and left in the sterile flow cabinet until dry. Sterile seeds were kept at 4°C, non sterile seeds under dry conditions at RT.

Sterilization solution:10mg/ml Baychlor (Bayrol, Planegg, Germany) in 96% ethanol

2.5 Plasmids

pAD-Gal4-2.1(LEU2, amp^R) (Stratagene, an Agilent Technologies division; USA): activation domain cloning vector

pBluescript II SK(-/+) (amp^R) (Stratagene, an Agilent Technologies division; USA): vectors for cloning and *in vitro* transcription, contain part of the *lacZ* gene which allows blue/white selection; +/- have different orientations of the multiple cloning site (MCS) within the *lacZ* gene

pBTM116: pGBT9 derivative containing the lexA binding-domain and TRP1 gene for selection in Trp$^-$ auxotrophic yeast strains (Bartel and Fields, 1995)

pBTM117: (created by Markus Teige): Modified pBTM116 with changed reading frame in the multiple cloning site and lacking an EcoRI restriction site

pCR2.1 TOPO (kan^R amp^R) (Invitrogen, Carlsbad, CA, USA): cloning vector

pET28a(amp^R, kan^R) :IPTG-inducible *E.Coli* expression vector for production of C-terminal HIS-tagged proteins (*Novagene*, Madison, WI, USA)

pER8 (spec^R, hyg$^{R)}$: estradiol inducible binary vector for transient and stable plant transformation (Zuo et al., 2000)

pGAD424 (LEU2, amp^R): activation domain cloning vector
(Clontech Laboratories, Inc., USA)

pGAD 425 (LEU2, amp^R): modified pGAD 424 with missing EcoRI site and frameshift in the MCS (created by Robert Doczi)

pGEM®-T Easy (amp^{R}Promega): cloning vector

pGEX-4T-1 (amp^R): IPTG-inducible *E.Coli* expression vector for production of N-terminal GST-fusion proteins *Amersham Biosciences*, Uppsala, Sweden

pGreenII 0029 (kan^R): binary plant expression vector for transient and stable transformation of plants (Hellens *et al.*, 2000; see also *www.pgreen.ac.uk*)

pGreenII GUSint (created by Robert Doczi): modified pGreenII 0029 with integrated GUS, cloned NcoI/KpnI and lacking two restriction sites (BamHI, XbaI) in the polyadenylation sequence

pRT100 (amp^R): for transient protein expression under control of the constitutively active 35S promoter (Topfer et al., 1987)

2.6 Media

Media were autoclaved for 20min at 120°C. All supplements (antibiotics, amino acids, plant hormones, chemicals) were sterile filtrated and added after autoclaving, except NaCl and sorbitol.

Antibiotics (1000x stocks):
ampicillin (amp): 50mg/ml in H_2O
chloramphenicol (CA):50mg/ml in e
gentamycin (gent): 50mg/ml in H_2O
hygromycin (hyg): 50mg/ml in H_2O (3000x stock)
tetracyclin (tet): 5mg/ml in ethanol
kanamycin (kan): 50mg/ml in H_2O
rifampicin (rif) : 20mg/ml in ethanol
spectinomycin (spec): 100mg/ml in H_2O

2.6.1 *E. coli* and *Agrobacterium tumefaciens* medium

Luria Bertani (LB) medium:
10g/l Bacto-peptone, 5g/l NaCl, 5g/l Bacto-yeast extract, 1g/l glucose; pH 7.2 with 5M NaOH; for solid medium 1% Bacto-agar was added before autoclaving

SOB medium (1l): 2% Bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$; pH 6,7-7,0 ($MgCl_2$, $MgSO_4$ sterile filtrated and added after autoclaving)

2.6.2 *Pseudomonas syringae* medium

NYG media:
5g/l Bacto-peptone, 3.3g/l Bacto-yeast extract, 20ml/l glycerol; ph 7.2 with 5M NaOH;
For solid medium (NYGA) 1% Bacto-agar was added before autoclaving.

2.6.3 *Saccharomyces cerevisiae* medium

YPD medium (complex medium): 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose
SD medium (minimal medium): 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose, respective amino acids:

AA	endconc.
lysine	30µg/ml
histidine	20µg/ml
uracile	20µg/ml
leucine	60µg/ml
tryptophane	40µg/ml
adenine	40µg/ml
methionine	20µg/ml

Amino acids were added from 100x stock solutions in H_2O, filter sterilized and stored at 4°C.
For solid medium 2% Bacto-agar was added before autoclaving.

2.6.4 *Alternaria brassicicola* medium

Potato carrot extract liquid medium (PCL): 300g potatoes (unpeeled, sliced) and 25g carrots (peeled, sliced) were added to 1l of boiling water and simmered for 20min. The water was filtered, adjusted to 1l by adding fresh water and autoclaved; for solid medium 15g of Bacto-agar was added before autoclaving
For solid medium (potato carrot agar; PCA) 1% of Bacto-agar was added before autoclaving.

2.6.5 *Arabidopsis thaliana* media

Arabidopsis germination medium (MS medium): 0.22% MS powder (Murashige-Scoog salt mixture with B5 vitamins, Duchefa, Netherlands), 1% sucrose (if not stated differently); pH 5.8 with 1M KOH; for agar plates 0,8% plant agar (Duchefa, Netherlands), for vertical plates for root growth experiments 1% plant agar was added before autoclaving. Antibiotics for selection were added after autoclaving.

2.6.6 *A.thaliana* cell suspension culture protoplasts media

Arabidopsis suspension medium: 100ml/l 10xMS macro elements (Duchefa, Netherlands) 1ml/l 1000xMS micro elements, 1ml 1000xmodified MS Fe-EDTA, 112mg/l B5 vitamins (Duchefa), 10g/l sucrose, 1mg/l 2,4-D; pH 5,7 with KOH

10 x macro elements: 1650mg/l NH_4NO_3, 1900mg/l KNO_3, 440mg/l $CaCl_2$ x $2H_2O$, 370 mg/l $MgSO_4$ x 7 H_2O, 170 mg/l KH_2PO_4

1000 x MS micro elements: 16.9mg/l $MnSO_4$ x H_2O, 8,6mg/l $ZnSo_4$x $7H_2O$, 6,2 mg/l H_3BO_3, 0.83 mg/l KI, 0,25 mg/l Na_2MoO_4 x 2 H_2O, 0.02 mg/l $CoCl_2$ x 6 H_2O, 0,025 mg/l $CuSO_4$ x 5 H_2O

1000 x modified MS Fe-EDTA: 11.15 mg/l $FeSO_4$ x $7H_2O$, 37,25mg/l Na_2-EDTA

B5-0.34M GM: 3,164g/l B5 powder (Duchefa), 0,34M glucose, 0,34M mannitol, 1mg/l 2.4D; pH 5.5 with KOH

B5-0.28MS: 3.165g/l B5 powder (Duchefa), 96g/l sucrose; pH 5.5 with KOH

2.7 Buffers and Solutions

Heat shock competent *E.coli*:

TB (transformation buffer): 10mM PIPES, 55mM $MnCl_2$, 15mM CaCl2, 250mm KCl; adjusted to pH 6,7 with 5M KOH before addition of $MnCl_2$

Yeast transformation:

TE/LiAc mix: 1xTE, 100mM LiAc

PEG TE/LiAc mix: 1x TE, 100mM LiAc, 40% PEG 3350 (from autoclaved 50% PEG stock solution in H_2O)

10xTE: 100mM Tris, 10mM EDTA pH 8.0

EDTA stock solution: 0.5M EDTA in H_2O; pH 8.0

ß-Galactosidase assay

Z-buffer: 60mM Na_2H2PO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 50mM ß-mercaptoethanol; pH 7,0

Z-buffer + ß-mercaptoethanol: 0,27ml of ß-mercaptoethanol added to 100ml of Z-buffer

ONPG solution: 4mg/ml in 0,1M Z- buffer, pH7

Mini preparation of plasmid DNA from *E.coli*

Buffer 1: 50mM Tris-HCL pH8, 10mM EDTA, 100µg RNase/ml

Buffer 2: 200mM NaOH, 1% SDS

Buffer 3: 1M potassium acetate (KAc) pH 5,5

10xTE: 100mM Tris-HCl pH 8,0, 10mM EDTA (pH 8.0)

Isolation of plant chromosomal DNA

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

SDS-PAGE

10 x SDS running buffer: 1,52g/l Tris, 72,1g/l glycine, 0,2g/l NaH_2PO_4

Western blotting

10x TB: 60.5g/l Tris, 30.9g/l boric acid in H_2O

10x PBS: 80g/l $NaCl_2$, 2g/l KCl, 11.5g/l $Na_2HPO4*7H2O$, 2g/l KH_2PO4

PBS-T: 1xPBS + 0,1% Tween

AP-buffer: 100mM Tris pH 9.5, 100mM $NaCl_2$, 100mM $MgCl_2$

Coomassie staining

staining solution: 0,1% Coomassie, Blue R-250, 40% ethanol, 10% acetic acid

destain solution: 40% ethanol, 10% acetic acid

Purification of GST-fusion proteins

1x PBS: 8g/l NaCl, 0,2 g/l KCl, 1,44 g Na2HPO4 · 12H2O, 0,24 g KH2PO4; pH to 7,4 with HCl

100x PMSF-stock: 100mM PMSF in isopropanol

wash buffer: 50mM Tris-HCl pH 8,0, 150mM NaCl

elution buffer: wash buffer + 20mM reduced glutathione (Sigma-Aldrich)

Purification of HIS-fusion proteins

lysis buffer: 50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole

wash buffer: 50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole

elution buffer: 50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole

In vitro co-immunoprecipitation

IVT-buffer: 50mM Tris-HCl pH 8, 150mM NaCl, 0,5% Nonidet P-40 (Sigma-Aldrich)

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 staining

Trypan clearing solution: 25g/10ml chloralhydrate in H_2O

Histochemical ß-glucuronidase (GUS) assay

X-gluc buffer: 50mM sodium phosphate buffer, pH 7,0, 10mM EDTA, 0,1% triton, 0,1% N-laurylsarcosylate

0.1M sodium phosphate buffer pH 7,0: circa 57,7ml 1M Na_2HPO_4 + 42.3 ml 1M NaH_2PO_4 per liter, adjust with pH-meter

Transformation of *Arabidosis thaliana* cell suspension culture protoplasts

PEG solution: 25% PEG 6000, 0,45M mannitol, 0.1M $Ca(NO_3)_2$ x 4 H_2O; pH 9.8 with KOH

A.thaliana suspension culture

Enzyme solution: 1% Cellulase (Serva), 0.2% macroenzyme (Serva) in B5-0.34M GM

2.8 Antibodies

2.8.1 Primary antibodies

Anti-c-Myc: Monoclonal antibody (9E10) from mouse, antigen in carboxyterminal domain of human c-Myc (kindly provided by Prof.E.Ogris); dilution for western blot: 1:5000

Anti-c-Myc (A-14, from Santa Cruz Biotechnology, USA): Polyclonal antibody from rabbit, antigen in carboxy terminal domain of human c-Myc; dilution for western blot: 1:5000

Anti-HA (12CA5): Monoclonal antibody from mouse, antigen hemagglutinine epitope, non-purified cell supernatant (kindly provided by Prof. Egon Ogris); dilution for western blot: 1:5000

Anti-HA (Y-11, from Santa Cruz Biotechnology, USA): Polyclonal antibody from rabbit , antigen hemagglutinine epitope; dilution for western blot 1:5000

Anti-AtMPK3: Rabbit, polyclonal, antigen MNTGGGQYTDFPAVD (C-terminus) (Davids Biotechnologie, Regensburg, Germany); affinity purified antibody used for western blot: dilution 1: 5000

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-MPK6 (Nuhse et al., 2000): rabbit, polyclonal, antigen FNPEYQQ (alfalfa MAP kinase MMK1 (Cardinale et al., 2000) (Davids Biotechnologie, Regensburg, Germany); crude serum was used for western blots: dilution 1:5000

Anti-Glutathion-S-transferase (GST 4H3, nanotools Antikörpertechnik,, Teningen, Germany): mouse, monoclonal; dilution for western blot: 1:2000

Anti-HIS (Santa Cruz Biotechnologies, USA): mouse, monoclonal; dilution for western blot: 1:2000

2.8.2 Secondary antibodies

Anti mouse IgG: Alkaline phosphatase conjugate from goat polyclonal (Sigma); dilution for western blot:1:5000

Anti-rabbit IgG: Alkaline phosphatase conjugate from goat, polyclonal (Santa Cruz Biotechology, USA); dilution for western blot: 1:5000

2.9 Primers

2.9.1 Primers for T-DNA insertion lines: genotyping and RT-PCR

SALK_044426 (*raf36-1*); annealing at 52°C, fragment length: genomic DNA 950bp, cDNA 650bp, with Lba1 450bp	At5g_for: 5'-GGCTACTGGACAATCTCCCGGGATA-3' At5g_re: 5'-GTTGCCGTGAAGCTTATCACTGTACCT-3'
SALK_014650 (*raf36-2*) annealing at 50°C ; fragment length: 900bp, with Lba1 950bp	C5_for_014650: 5'-TAACGTATTTTTCTCTAAATATTCCAAAAG-3' At5g_re_066501
SALK_014650 (*raf36-2*) ; RT-PCR annealing at 55°C, fragment length: genomic DNA 550bp, cDNA 450bp,	raf36-2_IIfor: 5'-ACTTGCGGTCTCCTTCAGGTC-3' raf36-2_IIre: 5'-CTCAGGCTTGTGCAAAGATCTT-3'
SALK_066501 annealing at 50°C fragment length genomic DNA 600bp, cDNA 500bp, with Lba1 500bp	At5g_for066501:5'-TCTTATGGTTTAGCTCTCAAATTTCTCC-3' At5g_re066501: 5'-CTCTGAGGTATAGCTTTTAATCCAGAA-3'
Garlic_51_H01.b.1a.Lb3Fa (SAIL) annealing at 52°C frgment length 200bp	MKK2-3'-for: 5'-ACATGGACCAGTGTTTTCGACTTGA-3' MKK2_3'_re: 5'-ACGTATCACAAAACATGAATTCCAGT-3'
T-DNA SALK	Lba1:5'-TGGTTCACGTAGTGGGCCATCG-3'
SAIL	LB1: 5'-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3'
	LB3 5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3'

Annealing temperature was 55°C for all primers. For amplifying DNA from the inserted T-DNA sequence, the standard primer Lba1 was used for SALK lines, the standard primers LB1 and LB3 were used for SAIL lines.

2.9.2 Primers for cloning constructs for protoplast/plant transformation

	forward primer
Raf36 in pAD annealing at 50°C	At5g-5': 5'- CAGTCGACAAATGGATGAAGAGGCTACTTCGTG-3' At5g-3': 5'- ACAGCTGAGCGAATTTAGGCTTCGGCAAGGC-3'
Raf36 in pGreenII 0029 (N-treminal Myc-/HA tag or C-terminal YFP-tag) annealing at 56°C	5gpGreen-5'Xho: 5'-ACTCGAGAATG GATGAAGAGGCTACTTCGT-3' 5gpGreen_3'PvuII: 5'-TCAGCTGAGCGATT TAGGCTTCGGCAAGGC-3'
Myc-Raf36 in pER8 annealing at 50°C	mycC5_inpER8: 5'- AACTCGAGATG GATGAAGAGGCTAC-3' C5_pER8re: 5'- AAACTAGTTCAAGCGAA TTTAGGCTTCGG-3'.
praf36::GUS in pGreenII GUSint annealing at 56°C	ProGUS_for912: 5'- GGATCCGCAATCATCATGGCCTATATCATAAAGTAAT-3' ProGUS_re: 5'- ACTCGAGCTCTAAGCTCGAAAACAAACTAAACAACCTT -3'

2.9.3 Primers for cloning Raf36-HIS fusion protein constructs

	forward primer
Raf36 in pET28 52°C	5gpGreen-5'Xho:5'- ACTCGAGAATGGATGAAGAGGCTACTTCGT-3' 5gpGreen_3'PvuII:5'-TCAGCTGAGCGATTTAGGCTTCGGCAAGGC-3'
ΔNRaf36A in pET28	A_fwd: 5'-CAATCTCGAGCCATGGCTATACCTCAGAGAT-3' ΔNre: 5'-CAATCTCGAGAGGTACCTCAGCGAATTTAGGCTT-3'
ΔNRaf36B in pET28	B_fwd: 5'-CAATCTCGAGCCATGG AAGCTGCTGATGTAT-3' ΔNre
ΔNRaf36C in pET28	C_fwd: 5'-CAATCTCGAGCCATGGCTGTTCCCGATGTGT-3' ΔNre
ΔNRaf36D in pET28	D_fwd: 5'-CAATCTCGAGCCATGGTGGAATCGGAAAAGG-3' ΔNre
ΔNRaf36E in pET28	E_fwd: 5'-CAATCTCGAGCCATGGGTCCTATCAGAGATC-3' ΔNre
Raf36K234M (site directed mutagenesis)	dead_for: 5'-GCTGTTGCCGTGATGCTTATCACTGTACCTGATG-3' dead_re: 5'-CATCAGGTACAGTGATAAGCATCACGGCAACAGC-3'

2.10 DNA methods

2.10.1 DNA isolation

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

2-3ml overnight grown culture in LB with appropriate antibiotics were pelleted, the pellet either stored at -20°C until further use or resuspended in 300µl buffer1. 300µl buffer2 were added for lysis of the cells and the samples were gently mixed. After 5 min on ice 300µl of buffer3 were added for neutralisation and the samples were centrifuged at 13.000rpm for 10min t room temperature The supernatant was transferred to a fresh tube and the DNA precipitated with 2 volumes of isopropanol + 0,1 volume of 5M sodium acetate (NaAc), pH 4,8 at -20°C for at least 20min. After centrifugation for 10min at 13.000rpm, the DNA pellet was washed first with 500µl of 70% ethanol followed by 500µl of 96% ethanol, air dried for 10min and resuspended in 50ul of H_2O or TE.
Plasmid DNA for sequencing was isolated with the Wizard® *Plus* SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions to avoid sequencing problems due to salt contaminations

2.10.1.2 Midi preparation of plasmid DNA from *E.coli*

To gain higher amounts of plasmid DNA, Midipreparations were carried out with the Jetstar Plasmid Purification Kit (Genomed, Löhne, Germany) according to the manufacturer's instructions.

2.10.1.3 Isolation of plant chromosomal DNA (for promoter cloning)

Arabidopsis seedlings (2 weeks old) were frozen in liquid nitrogen, grinded and the powder resuspended in 400µl EDM-buffer. After 5min on ice the samples were centrifuged at 4°C for 5min at 13.000rpm. The supernatant was transferred to a fresh tube and the DNA precipitated by addition of 1 Volume of isopropanol for 5min at room temperature, followed by a 5min centrifugation at 13.000rpm. The pellet was washed once with 70% ethanol, air dried for 5min and resolved in 50µl H_2O (overnight at 4°C). The genomic DNA was stored at 4°C.

2.10.1.4 Plasmid rescue from yeast for proliferation of plasmid-DNA (in *E.coli*)

2ml overnight cultures in SD medium with appropriate selection were pelleted at 13.000g for 5min at room temperature. The pellet was resuspended in 200µl breaking buffer. 0.3g of beads and 200µl phenol-chloroform were added, followed by vortexing for 2min and 5min centrifugation at 13.000g at room temperature. A second phenol chloroform extraction was performed with the supernatant. Precipitation was done with 2.5 volumes of 96% ethanol for 15min at -20°C, followed by 15min centrifugation at13.000g and 4°C. The pelleted DNA was washed with 70% ethanol and air dried for 1h before resuspension in 10µl water.
The DNA was transformed into *E.Coli* Xli Blue by heat shock.

2.10.2 Polymerase chain reaction (PCR)

PCR reactions were carried out with GoTaq® (Promega) for colony-PCR, Herculase® (Stratagene) for clonings which require a higher accuracy in the synthesis, and Pfu Ultra™ High Fidelity DNA Polymerase (Stratagene) for introducing point mutations because of here ability to amplify long targets.

2.10.2.1 GoTaq® PCR reactions

GoTaq® PCR reactions were performed in a volume of 20µl with 4µl 5x Go-Taq buffer, 0,4µl 10mM dNTPs (Fermentas), 0,3µl of each primer (10pmol/µl), 0,2ul GoTaq (5U/µl) and up to 1µl of template DNA:
denaturing at 94°C for 30 sec
annealing at the primer melting point for 1 min
elongation at 72°C for 1min/ 2kb template length.
After the last cycle reactions were kept at 72°C for 7 min (to allow finishing of any started synthesis) and then cooled to 4°C.

2.10.2.2 Herculase® PCR reactions

Herculase® PCR reactions were performed in a total volume of 50µl with 5µl 10x Herculase buffer, 1µl 10mM dNTPs (Fermentas), 1µl of each primer (10pmol/µl), 0,5µl Herculase® (5U/µl) and up to 1µl of template DNA.

After 2min at 95°C to denature the template DNA the following steps were carried out 10 times:
denaturing at 95°C for 30 sec,
annealing at the primer melting point for 1 min
elongation at 72°C for 1min/ 1kb template length
Then 20 cycles with the same conditions but 10sec prolongation of the synthesis step were performed.
Finally samples were kept at 72°C for 7min and then cooled to 4°C.

2.10.2.3 Pfu Ultra™ PCR reactions

Pfu Ultra™ PCR reactions were performed in a total volume of 50µl with 5µl 10x Pfu Ultra buffer, 1µl 10mM dNTPs (Fermentas), 1µl of each primer (10pmol/µl), 0,5µl Pfu Ultra™ (5U/µl) and up to 1µl of template DNA.
After 30sec at 95°C to denature the template DNA the following steps were carried out 22-25 times depending on the template:
denaturing at 95°C for 30 sec
annealing at the primer melting point for 1 min
elongation at 68°C for 2min/1kb template length
After the last cycle reactions were kept at 68°C for 7min and then cooled to 4°C.

2.10.2.4 RedExtract-N-Amp Plant PCR

RedExtract-N-Amp Plant PCR (Kit, Sigma-Aldrich) was performed for genotyping. For the PCR 1µl of the prepared DNA extract (stored at 4°C) and the appropriate primers were added to the REDExtract-N-Amp PCR ReadyMix and the PCR program was run according to the manufacturer's instructions.

2.10.3 Site directed mutagenesis

Site directed mutagenesis was performed to exchange a specific amino acid residue in Raf36 to turn the protein kinase inactive. As it was shown in the MKKK MEKK1 that mutation of a conserved lysine (K) residue in the ATP-binding loop of the protein to methionine (M) abolished the kinase activity (Asai et al., 2002), this residue was also mutated in Raf36 by an adenine to thymine exchange at position 234 of the Raf36 cDNA sequence.

This was achieved by a PCR reaction with *Pfu Ultra* DNA polymerase (QuickChange Site-directed Mutagenesis Kit, Stratagene, USA), primers containing the desired mutation and the target gene as a plasmid template (pBluescriptSK-Raf36). The PCR program was run according to the manufacturer's instructions. After agarose gel electrophoresis the plasmid DNA was purified by spinning the excised gel slice through a filter tip (5min, 13.000rpm) into a tube, transferring the liquid into a fresh tube to avoid contamination with agarose, and precipitation with isopropanol (for protocol see 'Minipreparation of Plasmid DNA from *E.Coli'*). The purified plasmid 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 B-buffer and 1 µl of DpnI (10U/ µl) and incubated at 37°C for 1-1,5h. Half of the digest was transformed by heat shock into *E.Coli* Xl1 Blue without any additional purification steps before. The mutated Raf36 was called Raf36K234M
From pBluescriptSK- Raf36K234M was cloned XhoI/NotI into pET28 (SalI/NotI) for protein production in *E.coli*.

2.10.4 Agarose gel electrophoresis

If not indicated otherwise 0,8-1% agarose gels were prepared by heating the appropriate amount of agarose in TAE-buffer (0,04M Tris-actetate, 0,001M EDTA) until dissolved. After cooling to 50-60°C ethidium bromide was added (100x stock 5mg/ml in H_2O) to visualise DNA under UV-light. The Gene Ruler 1kb DNA ladder (Fermentas) was used as a size standard. The Gels were run at approximately 5V/cm.

2.10.5 Gelelution

Gel elution of DNA fragments was carried out with the JET Gel Extraction Spin Kit (Genomed, Löhne, Germany) according to the manufacturers instructions.

2.10.6 Restriction digest

Restriction digests were carried out with restriction enzymes from Fermentas. 1-5µg of DNA was digested with 5U of restriction enzyme under appropriate buffer conditions and temperature for at least 1,5h. Loading dye was added to the digest and the samples as run on an agarose gel. For fragment isolation and cloning 2-3 digests were prepared, the required

fragment cut out of the agarose gel and eluted with the Jetstar Gel Extraction Spin Kit (Genomed, Löhne, Germany).

2.10.7 Dephosphorylation of fragments to inhibit blunt end religation

In a total volume of 40µl 4,6µl 10x Shrimp Alkaline Phosphatase (SAP) dephosphorylation buffer and 1,4µl SAP (1U/µl) (Roche Diagnostics) were added to the DNA to be phosphorylated and incubated at 37°C for 1h. The enzyme was inactivated by incubation at 72°C for 10min.

2.10.8 Ligation

Ligation of isolated DNA fragments was performed with T4 DNA Ligase (Promega) in a total volume of 15ul. 1,5µl 10x T4 DNA ligase buffer and 1µl of Enzyme (1-3U/µl) were incubated with varying ratios of vector- and Insert-DNA for 1h at room temperature or overnight at 16°C. 5-15µl of the ligation were transformed into heat shock competent *E. Coli* XLI Blue depending on the transformation efficiency.
Cloning into pCR2.1 TOPO (TOPO TA Cloning Kit, Invitrogen) or pGEM-T-easy was performed according to the manufacturer's instructions.

2.11 RNA methods

2.11.1 RNA isolation from plant material using TRI®-reagent (Sigma-Aldrich)

Approximately 100mg of plant material in a 1,5ml tube was frozen in liquid nitrogen. 200µl TRI®reagent was added and the material was grinded with a plastic pestle. After adding another 800µl of TRI®-reagent samples were incubated at 60°C for 5min and the plant debris was pelleted at 13.000rpm for 10min at 4°C. After transferring the supernatant to a new tube 200µl of Chloroform were added. Vortexing for 15sec was followed by incubation on ice for 3 min and centrifugation at 10.000rpm for 15min at 4°C. The supernatant (ca.500µl) was transferred to a fresh tube and the RNA was precipitated by adding 250µl of isopropanol and 250 µl of 0.8M sodiumcitrate/1.2M NaCl, gentle mixing and incubation for 10min at room temperature. The RNA was pelleted at 10.000rpm for 10min at 4°C, the supernatant removed and the pellet washed with 70% ethanol. The pellet as air dried for 5min and resuspended in 30-50 µl DEPC- H2O.

DEPC- H2O: 1l of distilled H2O was stirred with 1ml of 0.1% Diethylpyrocarbonate (DEPC) overnight and autoclaved to destroy the DEPC.

2.11.2 Deoxyribonuclease (DNAse) I treatment of RNA

To differentiate between amplification from contaminating DNA in a RNA isolation and the aimed amplification from the RNA template, RNA was treated with DNAse prior to use for RT-PCR. 2µg of RNA, 1µl of 10xDNAseI reaction buffer and 1µl DNAse (Fermentas, 1µg/µl, RNAse-free) in a total volume of 10 µl were incubated at 37°C for 30min. The enzyme was inactivated by addition of 1µl of 25mM EDTA and incubation at 65°C for 10min.

2.11.3 Reverse transcription (RT)

A mix of 1-2µg of RNA and 1µl Oligo-(dT) (0.5µg/µl) primers in a total volume of 15µl 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 5µl 5x M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase buffer, 1,25µl 10mM dNTP's, 0,5µl RNAse inhibitor (RNAse out, Invitrogen, 40U/µl)), and 1µl M-MLV Reverse Transcriptase (Promega, 200U/µl) were added to a total volume of 10µl. The samples were incubated at 42°C for 1h. The enzyme was inactivated by incubation at 70°C for 10min.

2.11.4 Preparation of RNA samples for microarray analysis

Leafs of 4 week old *A.thaliana* plants were shock frozen in liquid nitrogen and grinded with mortar and pestle. Per sample a total of 12-15 leafs from 4-5 plants were pooled. About 100mg per sample were used to isolate the RNA with the RNeasy plant mini kit (Quiagen) following the manufacturer's protocoll. RNA concentration was determined by measuring the absorbance at 260nm. Reverse transcription followed by reverse transcription of the cDNA to obtain amplified RNA (aRNA) was done with the Ambion Message Amp Amplification Kit according to the manual. The aRNA concentration was determined and 1 µg of aRNA run on agarose gel. To protect the RNA against degradation by RNAse, 10µl formamide were added to each sample before the gel run. If the aRNA synthesis was successful, a smear in the size range of 500-1000bp was visible on the gel. The aRNA was stored at -80°C. cDNA synthesis, hybridization and array scanning were performed as described in (Lurin et al., 2004) .

2.12 Protein methods

2.12.1 SDS polyacrylamide gel electrophoresis (PAGE)

Protein samples were separated on 10% or 15% polyacrylamide gels, 0.75 or 1.5mm thick, prepared according to Sambrook (2001). The PAGE Ruler prestained protein ladder (Fermentas) was used as a standard. Samples were mixed wit 4x SDS PAGE loading buffer and incubated at 95°C for 5min before loading on the gel. The gel was run in SDS running buffer.

For two gels:
Separating gel (10%, 0,75mm): 2,5ml 1,5M Tris-HCl (pH 8,8), 4,4ml 40% Polyacrylamid (National Diagnostics), 2,9ml H_2O, 0,1ml 10%SDS, 0,1ml 10% APS, 0,004ml TEMED

Stacking gel: 0,38ml 1M Tris-HCl (pH 6,8), 0,375ml 40% Polyacrylamid (National Diagnostics), 2.224ml H_2O, 0,03ml 10%SDS, 0,03ml 10% APS, 0,003ml TEMED

4x SDS PAGE loading buffer: 200mM Tris-HCl (pH 6,8), 8% SDS, 0,4% bromphenol blue, 40% glycerol, 400mM DTT

10 x SDS running buffer: 1,52g/l Tris, 72,1g/l glycine, 0,2g/l NaH_2PO_4

2.12.2 Western blotting

A PVDF membrane (Immobilion 0.2µm Transfer Membranes from Millipore, USA) was soaked for 10sec, in Methanol. The gel was placed on the membrane between two sheets of 3mm Whatman paper on both sides and the stack inserted into the wet blot device filled with transfer buffer (TB) with the membrane facing the +, the gel facing the – pole. The blot was run for 1-1,5h at 4°C with 60-70Volt. After blotting the membrane was incubated in blocking solution (PBS-T with 5% milk powder) at room temperature for at least one hour. The blocking solution was renewed and the first antibody was added. Incubation took place at 4°C overnight. To remove non-specifically bound antibody the membrane was washed 6x5min with blocking solution, then the secondary antibody was added for 1h at room temperature. After washing of the membrane 6x15min with blocking solution, the membrane was rinsed with AP-buffer. For detection the chemoluminescent alkaline phosphatase- (AP-) substrate CDP star (Amersham Biosciences) was pipetted on the membrane, incubated in the dark for one minute, excess liquid was removed, the membrane sealed in transparent plastic foil and exposed to a film for 10min up to several hours depending on the signal intensity.

2.12.3 Protein staining by Coomassie Blue R-250

For coomassie staining of proteins on a SDS-gel, the gel was incubated for 15-30min in coomassie staining solution, then put into destain solution for 45min or until protein bands were visible.

2.12.4 Protein staining by Ponceau S

On a PVDF membrane the proteins can be (reversibly) visualized by PonceauS staining. Therefore the membrane was shaken in PonceauS staining solutions (0,1% PonceauS in 5% acetic acid) for 30sec, then rinsed with water until the protein bands are clearly visible.

2.12.5 Production of GST-/ HIS-fusion proteins in *E.coli*

2.12.5.1 Constructs for production of Raf36-HIS fusion proteins

pET28-Raf36: The full length cDNA sequence of Raf36 was amplified with an additional XhoI-site at the 5'-end and an additional PvuII-site at the 3'-end and cloned into a modified pBluescript SK- with an additional SmaI and XhoI site. The fragment was cut XhoI/NotI and cloned into pET28, which leads to a C-terminal location of the HIS-tag.

pET28-ΔNRaf36A-E: The required parts of the Raf36-cDNA sequence were amplified with a NcoI-site, containing the ATG start codon, at the 5' end and a XhoI-site at the 3' end. Sequences of the primers used for amplification are given in table 1, together with the starting position of the truncated sequence in the full Raf36 cDNA sequence. The fragments were cloned NcoI/XhoI into pET28, which leads to a C-terminal location of the HIS-tag.

2.12.5.2 Induction test for production of HIS- and GST- fusion proteins

To determine the optimal conditions for induction of a fusion protein, an induction test with 3ml transformed BL21-cultures was carried out for every construct. The cultures were grown at different temperatures (16°C, 26°C, 30°C, 37°C) and samples were taken 4h after induction and after induction over night. Abundance of the GST-/HIS-tagged proteins was determined on a coomassie stained protein-gel after SDS-PAGE, or, in case of identification problems due to low abundance of the fusion protein, by western blot analysis with GST- and HIS-specific antibodies.

pET28-Raf36: 4hrs 28°C
pET28-Raf36 K234M: overnight 28°C
pET28-ΔNRaf36A-E: 4hrs 28°C
pGEX-MKK2: overnight 28°C
pGEX-MKK2EE: overnight 28°C
pGEX-MPK4KM: 16°C 4h

2.12.5.3 Purification of GST-fusion proteins

An overnight grown culture from a single transformed BL21 colony was diluted 1:50 in a total volume of 200ml LB with the appropriate antibiotics. The cells were grown to an OD_{600} of 0.6-1 (3-4hrs) at 37 °C and induced with 0,2mM isopropyl-β-D-thiogalactopyranosid in H_2O (IPTG, Sigma-Aldrich Corporation, St. Louis, MO, USA). Before adding the IPTG a sample of the non induced culture was taken as a negative control to identify the GST-fusion protein on the coomassie-stained gel. After induction of 4h up to overnight at 16-28°C (depending on the protein) cells were harvested by centrifugation (5000rpm, 10min, 4°C) and stored at -20°C or directly used for protein purification.
The pellet was resuspended in 10ml of 1xPBS buffer 1mM phenylmethylsulfonylfluorid (PMSF) as protease inhibitor and sonicated 3x15 sec at 200-300W on ice. After centrifugation at 10.000rpm for 15min at 4°C, 200µl glutathion beads (GE Healthcare, pre-washed 3x with 1xPBS) were added and the GST pull down was incubated for 1h at 4°C on a rotating wheel. The beads were spun down at 1000rpm for 1min at 4°C, washed with 4x 10ml of ice cold wash buffer and soaked dry with a syringe. Batch elution with 200-300µl of elution buffer was done four times after 15min incubation each on a shaker at 16°C, spinning them down and transferring the eluate to a fresh tube. Samples of each elution step as well as from the beads were run on a SDS-PAGE to determine the amount of GST fusion protein.

2.12.5.4 Purification of HIS-fusion proteins

An overnight grown culture from a single transformed BL21 colony was diluted 1:100 in a total volume of 400ml LB with the appropriate antibiotics. The cells were grown to an OD of 0.6-1 at 37 °C and induced with 1mM IPTG. Before adding IPTG, a sample of the culture was taken as negative control for SDS PAGE. After induction of 4h up to overnight (depending on the protein), cells were harvested by 10min centrifugation at 4°C and 3000rpm and resuspended in 10ml lysis buffer with 0,1% Triton, protease inhibitor (‘Complete, EDTA

free'(Sigma-Aldrich)) and 1mg/ml lysozyme (Sigma-Aldrich). After 30min of lysis on ice, sonication at 200-300W was performed for 3x15sec.To pellet the cell debris, cultures were centrifuged at 4°C, 10.000rpm for 20min, if necessary followed by a second centrifugation. Nickel beads (Ni-NTA-Sepharose, Quiagen) were in the meanwhile washed 3 times by adding 1ml of lysis buffer, centrifugation at 800rpm 4°C for 1min, and discarding the supermatant.500µl of washed nickel beads were added to the supernatant and incubated at 4°C on a rotating wheel for 1-2h.Beads were spun down at 4 °C for 1min at 1500rpm, washed 3x with 10ml of wash buffer and soaked dry with a syringe. Batch elution was performed with HIS-protein elution buffer, the procedure was like described for GST-protein purification.

2.12.6 Production of proteins by *in vitro* transcription translation (*in vitro* TT)

Protein production by *in vitro* transcription translation was done with the 'TnT coupled Reticuloside lysate system' (Promega) and radioactive labelling with ^{35}S-met according to the manufacturers manual. Template- DNA was cloned into the pGEMT-easy vector and transcribed from the T7 promoter. RNAse out (Invitrogen) was used as RNAse inhibitor.
Protein translation efficiency was tested by SDS-PAGE before using the proteins for *in vitro* co-immunoprecipitation experiments.

2.12.7 *In vitro* co-immunoprecipitation with [^{35}S]-Met labelled protein (*in vitro* TT) and GST fusion protein

Approximately 5µg of GST fusion protein and GST (negative control) were added to 10µl of *in vitro* TT reaction in IVB buffer (total volume of 100µl). After incubation on a rotating wheel at 4°C overnight, 25µl of washed glutathione beads were added to each sample and incubated on the rotating wheel at 4°C for another 4 hrs. After the pull down, beads were washed washed and resuspended in 25µl 4xSDS loading buffer. After SDS-PAGE, the gels were coomassie stained, dryed and exposed to a phosphoimager screen for two to five days.

Washing of glutathione beads:
1ml of IVT buffer was added to 25µl of beads. After centrifugation at 1000rm and 4°C for 1min, the buffer was removed and fresh buffer added. This was repeated three times, after the last step the beads were sucked dry with a syringe before adding the pull down reaction or the 4xSDS loading buffer, respectively**.**

2.12.8 Crude protein extracts of *Arabidopsis thaliana* plant material

Plant material (100-200mg) was grinded in 200µl laccus buffer and the samples were centrifuged for 15min at 4°C. The supernatant was transferred to a new vial. The fresh extract was used for pull down experiments or western blot analysis. After Bradford protein concentration measurement, the protein concentration of all samples were adjusted to 1µg/µl.

2.12.9 Crude protein extracts of *Arabidopsis thaliana* suspension cell culture protoplasts

The protoplasts were harvested in a swing-out centrifuge at 800 rpm for 7min at room temperature. The supernatant was removed with a vacuum pump and the pellet resuspended in 100µl laccus buffer. The fresh protein extract was used for pull down experiments or western blot analysis. After Bradford protein concentration measurement, the protein concentrations of all samples were adjusted to 1µg/µl.

2.12.10 Protein concentration measurements (Bradford assay)

The Bradford assay was carried out with the Bradford- protein assay reagent (BioRad, Germany). 2µl protein in ddH_2O (total volume 800µl) were mixed with 200µl of Bradford reagent. After 5min incubation at room temperature, the absorbance at 595nm wavelength was measured. A water control with Bradford reagent was included to subtract the background absorbance. The protein concentration was determined by the program, referring to the programmed values of a BSA calibration curve.

2.12.11 *In vitro* phosphorylation assays

2.12.11.1 Phosphorylation assays after immunoprecipitation (IP)

Phosphorylation assays after immunoprecipitation (IP) from crude plant or protoplast protein extracts were performed essentially according to (Cardinale et al., 2002). 200µg of total protein extract were incubated with 20µl Protein A beads (Amersham) and 2µl antibody for 4h. The beads were washed twice with SucI buffer, twice with kinase buffer, and sucked dry with a syringe. The kinase reactions were incubated for 30min at room temperature in a total volum of 16µl kinase buffer with 0,01µl 1M DTT (dithiothreitol), 0,15µl 10mM ATP, 0,1µl γ-^{32}P-ATP (1µCi) and, if required, 1,5µl myelin basic protein (MBP, 10mg/ml). SDS PAGE

4x loading buffer was added to stop the reaction. 8µl of the kinase reactions were used for SDS-PAGE on a 10-15% SDS-proteingel. The kinase activity was detected by autoradiography (the gel was exposed to a phosphoimager cassette).

2.12.11.2 Preparation of Protein A Sepharose beads (GE Healthcare)

The dried powder was suspended in ddH_2O overnight for swelling. The next day beads were washed with ddH_2O (200ml/g) on a sintered glass filter. The beads were washed once with SucI buffer and stored as 50% slurry in SucI buffer (total volume 30ml) at 4°C until use.

2.12.11.3 Phosphorylation assays with purified fusion proteins

Phosphorylation assays with purified fusion proteins were performed in a total volume of 20µl with 4µl 4xkinase buffer, 0,01µl 1M DTT, 0,15µl 10mM ATP, 0,1µl γ-^{32}P-ATP (1µCi) and 1,5µl myelin basic protein (MBP, 10mg/ml) if required. The reaction was incubated for 30min at room temperature and the samples treated like described above.

2.13 *Arabidopsis thaliana* plant protocols

2.13.1 Stress treatment of seedlings

2.13.1.1 Stress treatment for kinase assays

10-15 Seedlings (10-12 days old) grown on MS agar plates under sterile conditions were transferred to liquid MS medium in small petri-dishes and left at room temperature overnight to adjust.
Cold treatment was done on ice by removing the media carefully with a pipette and adding ice cold (4°C) media.
Salt treatment was done by adding up to 200mM NaCl to the MS media.

2.13.1.2 Continuous stress treatments

For germination assays sterilized seeds were sawn on MS plates containing 125mM /150mM NaCl, sorbitol or paraquat (Pq), vernalized for 48h and put into long day conditions.
For hormone treatments sterilized seeds were sawn onto ½ MS agar plates with the respective hormone, vernalized for 48h and grown under long day conditions for up to 12 days.

2.13.2 Infection assays on 4-week-old plants

2.13.2.1 *Alternaria brassicicola* infection

The infection was largely performed according to (Kariola et al., 2005) with minor modifications. *Arbidopsis* plants grown in soil were infected with 5µl of *Alternaria* spore suspension (ca. $5x10^5$ spores/ml, spores washed of from a fully grown PCA-plate, kept at 4°C to increase sporulation, with PCA liquid media) on 4-5 of the younger rosette leaves. The infected plants were kept under high humidity (circa 100%). Samples for microarray analysis, jasmonic acid (JA) and camalexin measurements, as well as Trypan staining of the fungal hyphae were taken after 1 and 2 and, respectively, 2 and 3 days post infection (dpi). Visual evaluation of the *Alternaria* sensitivity was carried out at 7dpi.

2.13.2.2 *Pseudomonas syringae* growth assay

Infection: 400ml NYG medium were inoculated with *Pseudomonas* from a NYGA plate and grown for 24h at 28°C. The culture was pelleted at 5000rpm for 5min at room temperature, the supernatant was removed and the bacteria were resuspended in 400ml of 10mM $MgCl_2$. TheOD_{600} of the suspension was adjusted to 0,1 (circa $1x\ 10^8$ colony forming units(cfu)/ml). The suspension was filled into a beaker and 0,02% of Vac-In-Stuff Silwet L-77 (Lehle Seeds, USA) was added. The plants used for infection were 5-7 week old, raised under short day conditions and grown through a mesh covering the soil. The potted plants were inverted into the solution and left immersed for 15 sec. The plants were covered with plastic foil and left at room temperature.
Growth assay: One hour after infection samples were taken to determine the amount of infecting bacteria, further samples were taken at day 1,2 and 4 after infection.
Samples for each time point were taken in triplicates. Per sample from two plants two leaf discs each were punched out and grinded in 200µl 10mM $MgCl_2$. 800µl 10mM $MgCl_2$ were

added, and depending on the time point different dilutions prepared for facilitate colony counting lateron. 100µl of each dilution was spread on NYGA plates and grown at 28°C. After two days the number of cfu's was counted and a growth curve was generated.
The dilutions used were: Day 0 - 1:2/ day 1- 10^{-1}, 10^{-2}/ day 2- 10^{-4}, 10^{-5}/ day3-10^{-4}, 10^{-5}

2.13.3 Sample preparation for Jasmonic acid (JA)/Camalexin measurements

Leafs of 4-5 plants per sample were quick-frozen in liquid nitrogen and grinded. 100mg of powder were transferred mixed with 300µl buffer (1-propanol: ddH_2O: HCl 2:1:0.002). 0.5µl dihydro-JA standard (100µg/µl) (Montesano M, 2005) were added to the samples for JA measurements. The samples were quick-frozen and kept at -80°C.
Measurements were done by Günther Brader with the vapour-phase extraction method (Schmelz et al., 2003) and GC-MS analysis.

2.13.4 *Trypan blue* stainings of fungal hyphae

2-3 small leafs were collected and incubated in a 1,5ml tube in 1ml of *Trypan Blue* solution (Sigma) for 2x5min under vacuum. The samples were incubated at 95°C for 2 min. After 1h at room temperature *Trypan* solution was removed and the leafs were incubated in clearing solution overnight until up to four days at room temperature. Cleared leafs were incubated in 96% Ethanol for 24hrs to remove the chlorophyll, and then kept in 76% Ethanol until use. Fungal hyphae were visualised under the stereo-microscope.

2.13.5 Stable *Arabidopsis thaliana* transformation by *Agrobacterium tumefaciens*- Floral dip method (Clough and Bent, 1998)

3ml overnight cultures of transformed Agrobacterium *tumefaciens* in LB with appropriate antibiotics were used to inoculate 50ml of fresh medium. These precultures were incubated overnight at 28°C. 300ml medium were inoculated with the precultures to OD_{600}=0.3 and grown until OD_{600}=0.8. The bacteria were pelleted (5000rpm, 15min, room temperature) and resuspended in 300ml freshly prepared 5% sucrose solution. 0.05% of Vac-In-Stuff Silwet L-77 (Lehle Seeds, USA) was added, the solution filled into a beaker and a plant in early flowering stage (developed flowers and siliques removed) was soaked in the bacterial solution for circa 10 sec. The plants were covered with plastic kept under low light conditions for 24 hrs. The plastic was removed and the plants were transferred to normal light conditions.

The seeds were harvested, sterilized and transformants were selected on MS-Agar plated with appropriate antibiotics. These T_1 –transformants were proliferated, and the seeds of the T2-generation were screened for homozygous transformant lines by Mendelian segregation analysis.

2.13.6 Generation of Raf36-overexpressing lines

2.13.6.1 Generation of transgenic lines with constitutive overexpression of Raf36

For generation of *Arabidosis* transgenic lines constitutively expressing the transgene, the vector pGreenII 0029 with the CaMV 35S promoter was used. The Raf36-full length ORF was amplified with a XhoI-site at the 5'-end and a PvuII-site at the 3'-end and cloned into a modified pGreen vector containing the different epitope-tags (aminoterminal Myc- or HA-tag or carboxyterminal YFP-tag). The epitope-containing vectors were kindly provided by Celine Forzani.The constructs were transformed into *Arabidopsis* using the floral dip method, transformants were selected by their kanamycin resistance. Expression of the transgene was tested by western blot with HA-/ Myc-specific antibodies or in case of the YFP tagged construct by microscopy.

2.13.6.2 Generation of transgenic lines with inducible overexpression

For generation of *Arabidosis* transgenic lines expressing the transgene after induction, the vector estradiol-inducible pER8 vector was used. The Raf36-full length ORF, together with a aminoterminal Myc-tag, was amplified with an XhoI-restriction site at the 5'-, and a SpeI-restriction site at the 3'-end. The Fragment was cloned into the pER8- vector.
The constructs were transformed into *Arabidopsis,* transformants were selected by their hygromycin resistance. Expression of the transgene was tested by western blot with Myc-specific antibodies.

2.13.7 Expression test with estradiol-inducible overexpressor-lines

Circa 10 14-day old seedlings from transgenic lines transformed with an estradiol-inducible pER8 construct were put in 5ml of H_2O with 40µM estradiol (1000x stock in Ethanol) + 0,01% Vac-In-Stuff Silwet L-77 (Lehle Seeds, USA) and incubated for 4h or overnight. The plant material was frozen in liquid nitrogen, grinded and resuspended in 4xSDS loading buffer. The samples were tested on a western blot for transgene expression.

For testing if expression of the transgene is induced, but the protein degraded quickly, the induction test was repeated with the proteasome inhibitors MG115 and MG132 (both 10uM, 1000x stock in DMSO). The peptides MG115 (Z-Leu-Leu-Nva-H) and MG132 (Z-Leu_leu_leu_CHO) are a specific inhibitors of the chymotrypsin-like activity of the proteasome.

2.13.8 Generation of transgenic *praf36::GUS* lines

A 912bp fragment of the sequence upstream of the Raf36-gene was amplified from genomic DNA with an additional BamHI-restriction site at the 5'-end and an additional XhoI-restriction site at the 3'-end. The fragment was cloned into the pGreenII 0029 GUSint vector (kindly provided by Robert Doczi) and transformed into *Arabidopsis thaliana Col*-0 wt. Transformants were selected using their kanamycin resistance.

2.13.9 Histochemical ß-glucuronidase (GUS) assay

Seedlings or plant parts from GUS reporter lines were incubated in X-gluc buffer in the dark at 37°C until the blue colour developed (2,5 hrs -ON). The plant material was destained in 70% ethanol overnight which also removed the chlorophyll, and analysed under the stereomicroscope.

2.14 *Arabidopsis thaliana* suspension culture protoplasts

All centrifugation in the following protocols were done without break.

2.14.1 Production of white *Arabidopsis thaliana* suspension culture protoplasts

Arabidopsis thaliana white cell suspension culture was grown in *Arabidopsis* suspension medium in the dark (150rpm, 22°C) and diluted 1:5 with fresh medium every 7 days. 50ml of 5 days old culture were pelleted (2000rpm, 1min, room temperature) and the pellet resuspendet in 25ml of enzyme solution to digest the cell wall. The volume was adjusted to 50ml with B5-0.34 M-GM medium and the mixture was shaken at 60rpm in the dark at room temperature for 1.5-4hrs, depending on when at least 50% of protoplastation was reached (checked with a light microscop). The cells were pelleted (1500rpm, 5min, room temperature) and resuspended in 45ml B5-0.34 GM medium. After centrifugation (1000rpm,

5min, room temperature) the floating cells were transferred to a 14ml tube and B5-0.28 MS medium was added. Cells were spun again (800rpm, 7min), the floating cells transferred to a fresh tube and protoplasts number and quality checked under the light microscope. The protoplasts were diluted to a concentration of $6x10^6$/ml and kept at 4°C up to use. Protoplasts were used for transformation for up to 24 hrs after protoplastation.

2.14.2 Transformation of *Arabidosis thaliana* cell suspension culture protoplasts

To 7-15µg of DNA in a total volume of 15µl (in ddH_2O) 50µl protoplast suspension (circa $2x10^5$ cells) was added and gently mixed. 150µl of PEG solution was added, gently mixed and incubated for 12-15 min in the dark at room temperature. Protoplasts were washed by adding 1ml of 0,275 M Ca $(NO_3)_2$, mixing gently by inverting and centrifugation (800rpm, 7min). The supernatant was removed and the cells resuspended in 0.5ml of B5-0.34 M GM and cultivated overnight in the dark. For stress experiments 3 repeats per sample were prepared and pooled after overnight cultivation. The pooled protoplasts were kept in the dark for 4 hrs before protein extraction (2.12.9) and immunoprecipitation of kinases (2.12.11.1).

2.15 Internet tools

Expasy (**Ex**pert **P**rotein **A**nalysis **Sy**stem): Analysis of protein sequences and structures as well as 2-D PAGE) (http://www.expasy.ch)

Genevestigator: Database for gene expression and analysis (https://www.genevestigator.ethz.ch/gv/index.jsp)

matDB: http://mips.gsf.de/proj/plant/jsf/athal/index.jsp

NASC (European Arabidopsis stock center): Ordering of mutant lines (http://arabidopsis.info)

NCBI BLAST (Basic **L**ocal **a**lignment **s**earch **t**ool**):** Used for DNA and protein sequence alignments (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

PubMed : Literature database (http://www.ncbi.nlm.nih.gov/pubmed)

SiGnaL (**S**alk **i**nstitute **g**enomic a**na**lyis **l**aboratory): Database of Arabidopsis mutant lines and available cDNA's (http://signal.salk.edu)

TAIR (**T**he **A**rabidopsis **I**nformation **R**esource) and **matDB**(**m**ips **A**rabidopsis **t**haliana **d**ata **b**ase) : Databases for genetic and molecular biology data from *Arabidopsis thaliana* (TAIR: http://www.arabidopsis.org)

2.16 Abbreviations

amp	ampicillin
arg	arginine
bp	base pair
ca	chloramphenicol
cDNA	complemetary DNA
cfu	colony forming units
dATP	desoxyadenosin-5'-triphosphate
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
ddH_2O	double-distilled water
DTT	1,4-dithiothreitol
EDTA	ethylene-diamine-tetra-acetate
EtOH	Ethanol
gent	gentamycin
his	histidine
HIS-	histamine
H_2O	water
H_2O_2	hydrogenperoxide
JA	jasmonic acid
kan	kanamycin
kb	kilo basepair
kDa	kilo Dalton
KCl	caliumchlorid
KOH	caliumhydroxid
LB	Luria Bertani medium

Leu	leucine
LiAc	lithium acetate
lys	lysine
met	methionine
NaCl	Sodium chlorid
MCS	multiple cloning site
NaOH	sodiumhydroxid
O_2^-	superoxide
ONPG	o-nitrophenyl-ß-galactoside
ORF	open reading frame
ROS	reactive ogygen species
RT	reverse transcription
rif	rifampicin
spec	spectinomycin
tet	tetracyclin
TRIS	tris (hydroxymethyl)-aminomethane
trp	tryptophane
ura	uracile
X-gluc	5-bromo-4-chloro- 3-indolyl- β-D-glucuronic acid
ß-gal	ß-galactosidase

3. Results

3.1 MKK2 interacts with Raf36 *in vitro*

A Y2H screen using MKK2 as bait against a Gal4-A fusion protein library from one- week-old *Arabidopsis thaliana* seedling was performed by Hybrigenics (www.hybrigenics.com), 150 positive clones were isolated. 8 putative MKK2 interaction partners were identified, among them the already known MKK2-interactor MEKK1 (Gao *et al.*, 2008; Qiu *et al.*, 2008) was also isolated in the screen.

Among the 150 positive clones, 30 represented Raf36 (At5g58950), a putative MAPKKK of the Raf-like MAPKKK group C with so far unknown function. The open reading frame (ORF) of the gene spans 1578 base pairs. The 59 kDa protein contains 525 amino acid residues. Except for the conserved kinase domain, which is located between position 621 and 1401 in the ORF sequence, no known conserved domain motifs were identified by bioinformatic analysis ('Expasy smart'). However, the amino-terminus contains a serine-rich region. This type of region has been shown to influence protein stability in phytochrome A, possibly indicating an increased rate of protein degradation upon domain phosphorylation (Kneissl, 2008; Trupkin *et al.*, 2007). Considering the smallest Raf36 clone isolated in the Y2H screen, the MKK2 docking site can be narrowed down to the region roughly 200 amino acids (nucleotide position 102 and 671) in the Raf36 open reading frame (ORF). The minimal region necessary for MKK2 and Raf36 interaction might therefore be largely found in the upstream regulatory region N-terminal to the Raf36 kinase domain (Fig.3.1).

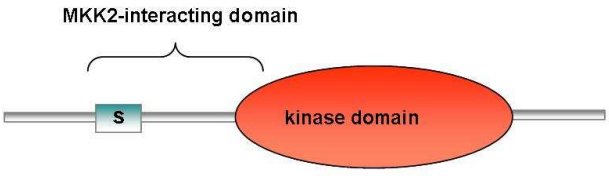


Fig.3.1:
Location of serine-rich region ('S'), kinase domain and MKK2-interacting domain in Raf36

Mainly based on sequence homology of the kinase domains, 48 kinases were ascribed to the group of Raf-like MAPKKKs in *A. thaliana*. A minority of these MAPKKKs, such as CTR1(Kieber *et al.*, 1993), has been studied so far and has been shown to be involved in MAPK signalling. Therefore Raf36 was a very interesting candidate for further investigation. Identifying a new upstream regulator of MKK2 and elucidating the function of this signalling module in the plant will broaden our picture of the MAPK signalling network.

3.1.1 Confirmation by *in vitro* pull down

The interaction between Raf36 and MKK2 was verified by *in vitro* pull down assays using recombinant, GST-tagged MKK2 to capture *in vitro* translated Raf36 protein. Raf36 was labelled with [^{35}S]-methionine ([^{35}S]-Met), produced by a T7-coupled transcription/translation system (Promega) and incubated with GST-MKK2 fusion protein or GST alone. Fig.3.1a shows that *in vitro* translated Raf36 interacts with the GST-MKK2 fusion protein but not with the GST protein alone. The input of [^{35}S]-Raf36 is shown in fig.3.2b, the input of GST-MKK2 fusion protein and GST-protein in Fig.3.2c.

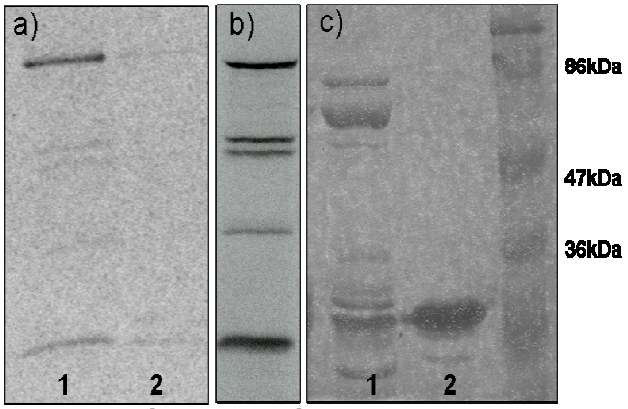


Fig.3.2:
In vitro pulldown of [^{35}S]methionine labelled Raf36-protein with MKK2-GST fusion protein
a) autoradiogram showing [^{35}S]-Raf36 pull-down with MKK2-GST (1), and GST (2)
b) autoradiogram showing the [^{35}S]-Raf36 input
c) Coomassie-stained gel showing the input of MKK2-GST (1) and GST (2)

3.1.2 Raf36-interaction with all 10 *Arabidopsis thaliana* MKKs was tested by a direct Yeast two-hybrid (Y2H) approach

To get information about the specificity of Raf36-MKK2 interaction, we decided to test the interaction of Raf36 with all 10 *A.thaliana* MKKs. We were especially interested in the Raf36 ability to interact with the closest MKK2 homologue MKK1 (Morris *et al.*, 1997).
In the Y2H approach, Raf36 was the prey protein, fused to the activation domain (AD) in the pAD vector. The 10 *A.thaliana* MKKs were used as bait proteins, fused to the binding domain (BD) in the pBTM117 vector.
Saccharomyces cerevisiae L40 were transformed with pAD-Raf36 and pBTM-MKK1-10 or with the empty pBTM. Positive interactions were detected by growth of the yeast transformants on selective medium lacking the amino acids leucine (selection for pAD vector), tryptophan (selection for pBTM vector) and histidine (reporter gene for protein interaction). To distinguish the yeast growth caused by auto-activation of the MKK-

constructs from growth caused by an interaction between Raf36 and the MKKs, yeast were in parallel transformed with the MKK-constructs and the empty pAD vector (Fig.3.3c,d).

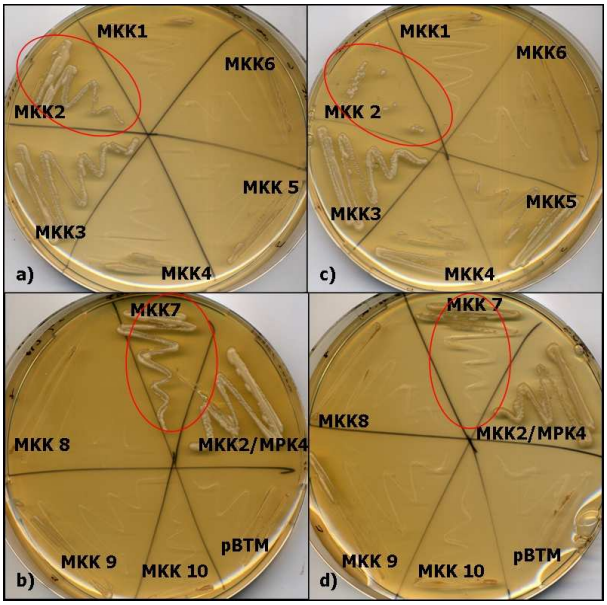


Fig.3.3.a:
Growth test on selective medium (SD -leu, -trp, -his) to test the interaction of Raf36 (-AD) with the 10 *Arabidopsis* MKKs (-BD) in a Y2H-approach (a+b). The MKKs were also transformed with the empty pAD vector to test for autoactivation of the MKK constructs in yeast (c+d).
MPK4 and MKK2 are known to show strong interaction and were therefore used as a positive control.

Interaction could be shown between Raf36 and MKK2 (Fig. 3.3a), and also between Raf36 and MKK7 (Fig. 3.3b). No conclusion could be drawn concerning Raf36 interaction with MKK3, as the MKK3-BD construct exhibited strong autoactivation (Fig. 3.3c). MKK4 and MKK5 as bait proteins also showed weak autoactivation, but no interaction with Raf36. Although MKK1 is the closest homologue of MKK2 and known to be partially redundant with MKK2 *in vivo*, MKK1 did not show any detectable interaction with Raf36 in the Y2H assay.

The *lacZ* gene was used as a second reporter gene. The ß-galactosidase activity resulting from lacZ reporter activation was measured in liquid assays, using o-Nitrophenyl-β-D-galactopyranosid (ONPG) as a substrate, to produce quantitative data (Fig.3.3e).

The ß-galactosidase activity also reflects the results seen on the plates, namely an interaction between Raf36 and MKK2 or MKK7. MKK3, 4 and 5 show ß-galactosidase activity with the empty prey. In this assay the autoactivation of the MKK4-BD construct is more pronounced than in the growth assay on selective plates. No conclusion can be drawn concerning their ability to interact with Raf36.

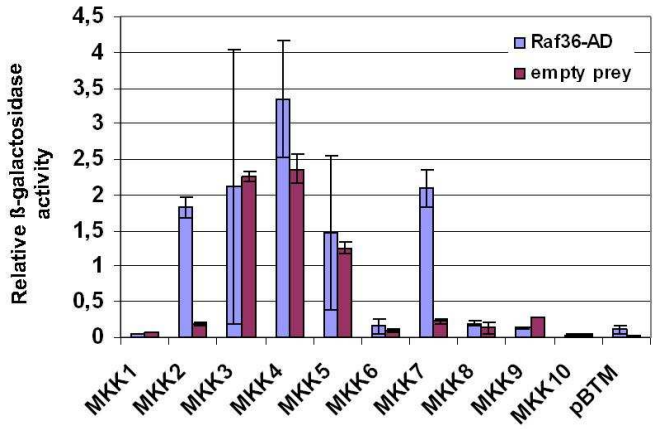


Fig.3.3.b:
Quantification of Raf36 interaction with all 10 *A.thaliana* MKKs by Y2H; values given were calculated after performing ß-galactosidase assays

3.1.3 In vitro kinase activity of Raf36

3.1.3.1 Raf36 shows weak autophosphorylation activity *in vitro* which is lost in a mutated, kinase inactive Raf36-version

To assess the function of Raf36, a histidine (HIS)-tagged fusion of the protein was produced in *E. coli* using the pET28 vector. The HIS-tag was fused to the amino-terminus of the protein. The kinase activity of the recombinant Raf36 protein was tested by *in vitro* phosphorylation assays (*in vitro* kinase assays) (Fig.3.4). In this assay HIS-Raf36 was incubated with ^{32}P- γ-ATP, alone or in combination with GST-MKK2 protein. When a protein exhibits kinase activity, it incorporates radiolabelled phosphate into the protein substrate. The labelled protein can be visualized after the reaction on an autoradiogram. The recombinant MKK2 protein was produced as fusion protein with an amino-terminal GST-tag, using the pGEX4T-1 vector.

A weak phosphorylation signal was detectable for Raf36 when incubated alone with the radioactive ^{32}P-γ-ATP (Fig.3.4a). To test if this weak phosphorylation signal is due to Raf36-autophosphorylation or just a background signal, a putative kinase inactive Raf36 protein was generated. To obtain this protein version, a conserved lysine (K) residue in the ATP-binding loop of the protein (Fig. 3.5, marked with '*') was mutated to methionine (M), as previously done for the MAPKKK MEKK1, resulting in a kinase inactive protein (Asai *et al.*, 2002). By site-directed mutagenesis a single base pair was exchanged (A to T at position 234 of the Raf36 cDNA sequence). The kinase activity of the mutant protein, named Raf36 K234M, was tested by *in vitro* kinase assay.

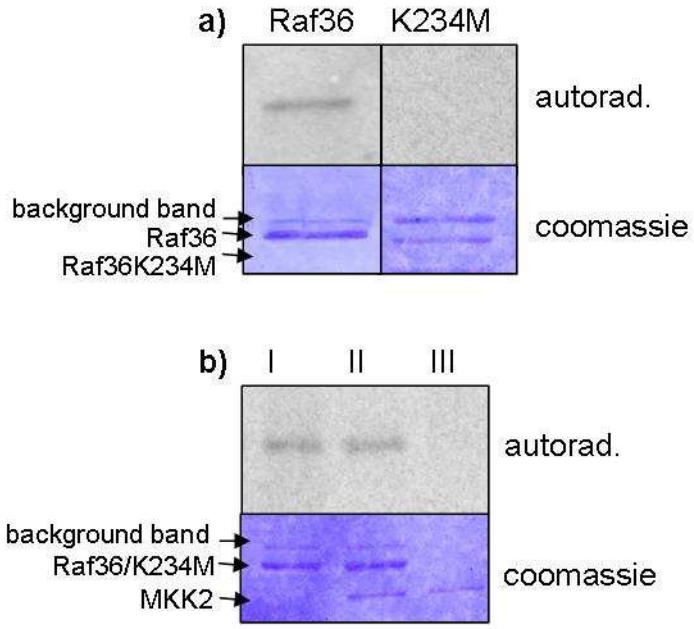


Fig.3.4:
In vitro phosphorylation activity of Raf36

a) autophosphorylation of Raf36 WT; abolished in the Raf36 K234M protein

b) Raf36 autophosphorylation in the absence (I) and in the presence of MKK2-GST (II), and absence of autophosphorylation for GST-MKK2 alone (III).

Raf36 K234M did not show any phosphorylation signal in the *in vitro* kinase assays (fig.3.4a). The introduced mutation in the ATP-binding loop resulted in a loss of the phosphorylation signal, which proves that the weak signal seen by *in vitro* kinase assays with the wild type Raf36 protein corresponds to autophosphorylation activity.
Next, the ability of Raf36 to phosphorylate MKK2 was tested (Fig.3.4b). Raf36 autophosphorylation was unchanged in the absence or presence of GST-MKK2 (lane I+II). GST-MKK2 itself did not show autophosphorylation (lane III). In this approach no phosphorylation of GST-MKK2 by Raf36 was detectable.

3.1.3.2 Recombinant amino-terminally truncated Raf36 can phosphorylate MKK2 *in vitro*

Raf36, compared to his closest homologues in *A.thaliana*, features an extended amino-terminal domain, containing a serine-rich region which might lower the stability of the protein. Alternatively, this amino-terminal region could contain an autoinhibitory domain which would require displacement to render Raf36 fully active and capable of phosphorylating its substrates. To test this hypothesis, 5 amino-terminally truncated Raf36-proteins fused to a HIS-tag (ΔNRaf36(-HIS) A-E) were generated (Fig.3.5). Table 1 lists the length and starting position of the truncated Raf36 sequence for each fragment. The ability of these proteins to autophosphorylate and to phosphorylate GST- MKK2 was tested by *in vitro* kinase assays (Fig.3.7).

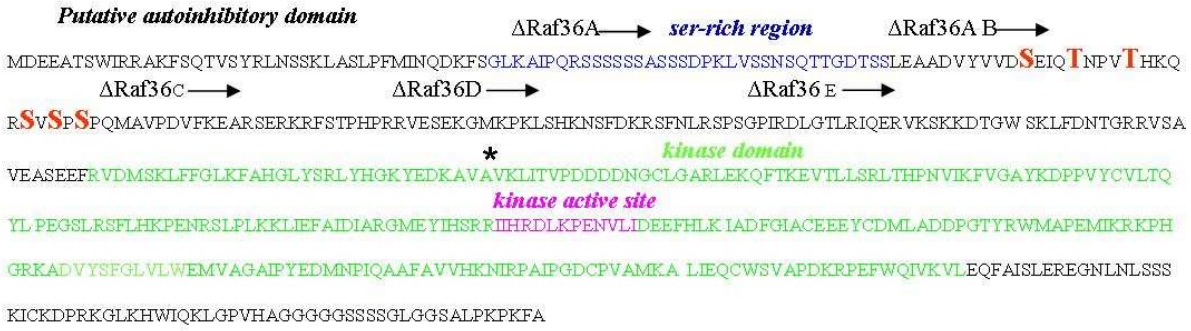


Fig 3.5:

Amino acid sequence of Raf36 with a serine rich region in the N-terminal part of the protein and the kinase active domain containing the highly conserved kinase active site. The start of the ΔRaf36A-E sequences is indicated.

The putative autophosphorylation sites are indicated in red.

The lysine (K) residue exchanged in the Raf36K234M mutant is marked with an asterisk (*).

	Length of the truncated protein (in amino acids)	Length of the missing region (in amino acids)	Starting position in Raf36 cDNA
ΔN Raf36A	483	42	130
ΔN Raf36B	450	75	229
ΔN Raf36C	420	105	319
ΔN Raf36D	397	128	338
ΔN Raf36E	369	156	472

Table 1: Length of the truncated Raf36 proteins, length of the missing protein region and starting position in the Raf36 cDNA

While the full length Raf36, as well as Raf36 K234M are produced in very low amounts as HIS-fusion proteins, all the truncated proteins were purified from *E.Coli* BL21 in approximately 10-fold higher concentrations (Fig.3.6). Additionally the full length fusion proteins were particularly unstable and sensitive to freezing and thawing. Therefore work with the truncations was considerably more convenient than with the full length versions Raf36 and Raf36 K234M.

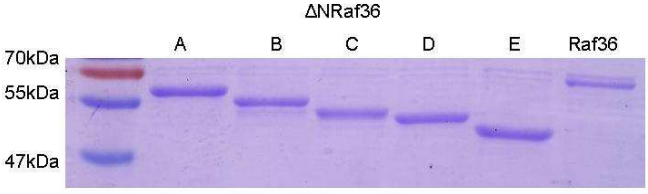


Fig.3.6:

Protein concentrations of ΔNRaf36 A-E and Raf36 produced and purified in parallel under same conditions. In case of Raf36 five times the volume of purified ΔNRaf36 A-E was loaded.

In vitro phosphorylation assays (Fig.3.7) revealed that the truncated versions ΔNRaf36A and B exhibited much stronger autophosphorylation activity than full length Raf36. ΔNRaf36C-E showed autophosphorylation levels similar to those of full length Raf36 protein. ΔNRaf36A, and the full length Raf36 protein did not phosphorylate GST-MKK2 *in vitro*. In contrast, all subsequent truncations (ΔNRaf36 B-E) could phosphorylate GST-MKK2, indicating that the N-terminal part including the serine-rich sequence function as an autoinhibitory domain. These results are summarised in Table 2.

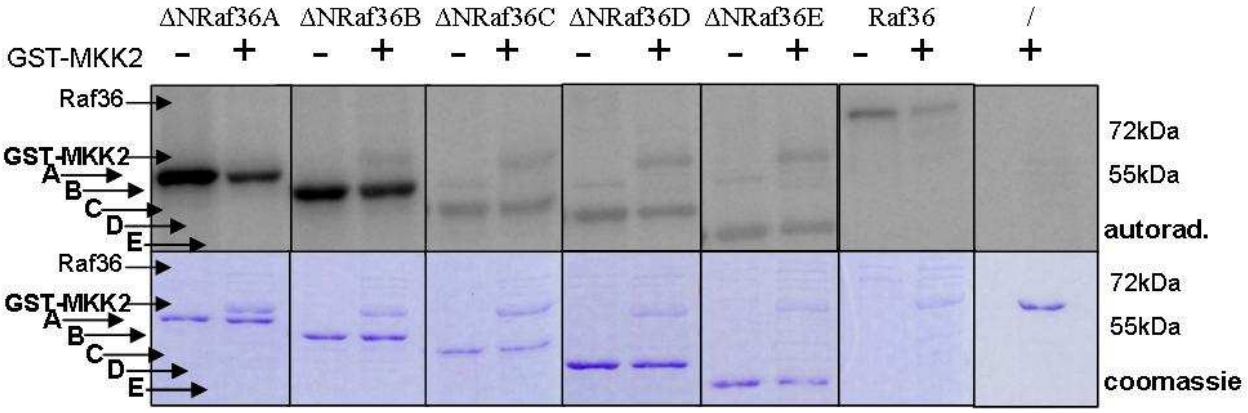


Fig 3.7:
Autophosphorylation and phosphorylation of MKK2-GST (*****) by the ΔNRaf36A-E and full length Raf36 in an *in vitro* kinase assay.

	autophosphorylation intensity	MKK2 phosphorylation intensity
ΔN Raf36A	++	-
ΔN Raf36B	++	+
ΔN Raf36C	+	++
ΔN Raf36D	+	++
ΔN Raf36E	+	++

Table 2:
Summary of ΔNRaf36A-E features: presence of the serine rich region, autophosphorylation activity and MKK2 phosphorylation *in vitro* .

3.1.3.3 N-terminal truncated Raf36 inhibits MKK2 activity *in vitro*

Next, it was tested whether the presence of ΔNRaf36D influences MKK2 activity *in vitro* (Fig.3.8). Kinase inactive MPK4 (MPK4KM), in which two conserved lysine residues are changed to methionine (Teige *et al.*, 2004), was used as a substrate for GST-MKK2.

Fig.3.8a shows that autophosphorylation activity was detectable for ΔNRaf36D, but not for GST-MKK2. MPK4KM as a kinase inactive protein did not show autophosphorylation and was not phosphorylated by ΔNRaf36D (Fig.3.8b), ΔNRaf36D did phosphorylate GST-MKK2 (Fig3.8c). In the presence of ΔNRaf36D, MKK2 and the known MKK2-substrate MPK4KM were all phosphorylated (Fig.3.8c). Phosphorylation of ΔNRaf36D can be attributed to autophosphorylation (Fig. 3.8a). ΔNRaf36D did not phosphorylate MPK4KM (Fig.3.8b). MKK2-GST strongly phosphorylated MPK4KM, but was clearly compromised in the presence of ΔNRaf36D, suggesting that ΔNRaf36D inhibits MKK2 kinase activity. The same results were found for ΔNRaf36C (23 amino acids longer).

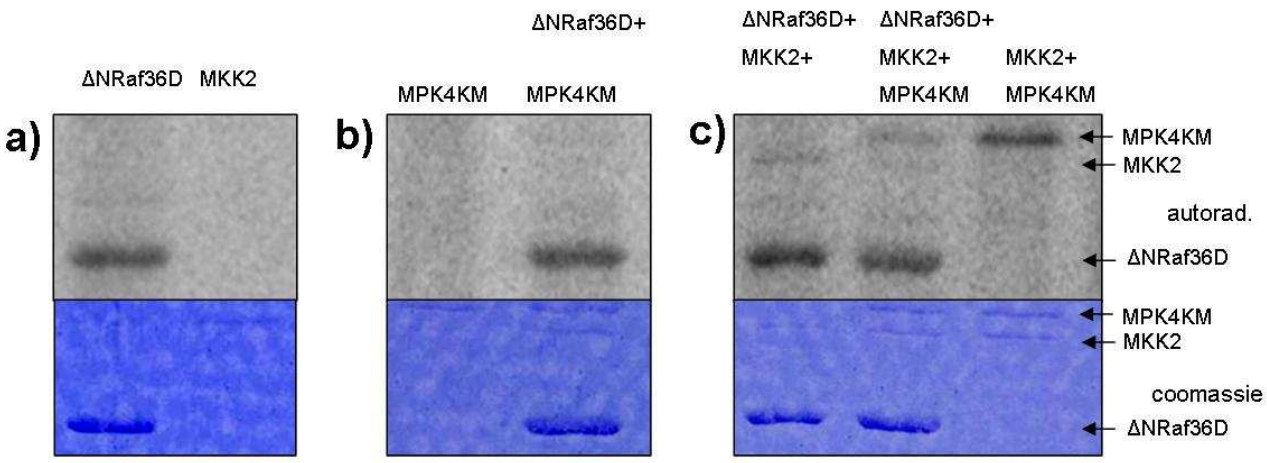


Fig.3.8:

In vitro phosphorylation assays with recombinant HIS-tagged ΔNRaf36D and GST-tagged MKK2 (63kDa) and MPK4KM (66kDa)

a) autophosphorylation of ΔNRaf36D, no autophosphorylation of MKK2

b) no autophosphorylation in MPK4KM and no phosphorylation by ΔNRaf36D alone
phosphorylation of MKK2 by ΔNRaf36D, phosphorylation of MPK4KM in the presence of MKK2 and ΔNRaf36D, phosphorylation of MPK4KM by MKK2

3.2 *In vivo* Data

3.2.1 Identification and analysis of Raf36 knockout lines

To assess the function of Raf36 *in vivo*, three SALK lines with T-DNA insertions within the gene locus At5g58950 were analysed. In two of the lines the T-DNA insertion was supposed to be located in the 5'untranslated region (5'-UTR), in the third line the T-DNA was suggested to lie in an exon within the open reading frame (ORF) (Fig. 3.9). To locate the T-DNA insertion and identify homozygous progenies, the received seeds were sown and the plants were genotyped. For genotyping, a primer in the left border sequence of the T-DNA was used together with a primer in the gene, about 500bp downstream or upstream of the T-DNA primer. PCR with genomic plant DNA leads to amplification in homozygous and heterozygous transgenic lines, but not in the wild type. A second primer pair was chosen at both sides of the T-DNA, leading to a PCR product in heterozygous lines and wild type lines, but not in homozygous knockouts. Genotyping led to isolation of homozygous transgenic plants for all three lines.

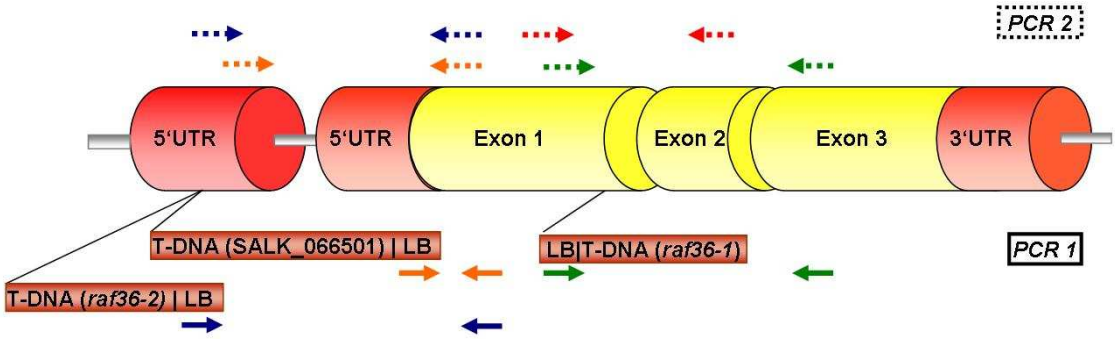


Fig.3.9:
Location of T-DNA insertions within the Raf36 genomic sequence. T-DNA and primer location (arrows) for the lines SALK_044426 (*raf36-1*) (green arrows), SALK_014650 (*raf36-2*) (blue arrows) and SALK_066501 (orange arrows) are indicated. Primer for PCR 1 are indicated as continuous arrows, primer for PCR 2 as dashed arrows. The red arrows indicate the primer location for *raf36-2* RT-PCR.

To test whether these three lines constitute real *raf36* knockouts, RNA was extracted from 14-day-old seedlings, and, after reverse transcription (RT), PCR was performed with two pairs of primers located in the Raf36 ORF (Fig.3.9, red dashed arrows: primer pair for *raf36-2* and SALK_066501 RT-PCR, green dashed arrows: primer pair for *raf36-1* RT-PCR).

The RT-PCR analysis revealed Raf36 transcription in homozygous plants of line SALK_066501, this line was therefore not a true *raf36* knockout line. In lines SALK_044426 and SALK_014650 no Raf36 transcript could be detected (Fig.3.10a). These two lines were named *raf36-1* (SALK_044426) and *raf36-2* (SALK_014650) and used for further experiments. *raf36-1* plants show under standard growth conditions a slightly dwarf phenotype, which is most striking up to four weeks. The plants do not show any other abnormalities. *raf36-2* plants are slightly smaller compared to WT plants, but the phenotype is not for all plants as obvious as shown in Fig. 3.10b.

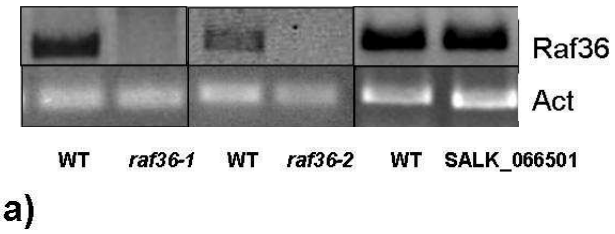


Fig.3.10:
Analysis of *raf36* lines
a) RT-PCR analysis of two-week old *raf36-1*, *raf36-2* and SALK_066501 and wild type (WT) seedlings; actin (Act) was used as a loading control.
b) phenotype of 3-week-old *raf36-1*, *raf36-2* and wild type (WT) plants grown on soil

3.2.2 Generation of Raf36-overexpressing lines

3.2.2.1 Expressing Raf36 under the control of the constitutively active 35S-promoter in transgenic lines leads to silencing

The Raf36-full length open reading frame was cloned into a modified pGreenII 0029. This vector contains the 35S promoter as well as three different epitope-tags, Myc-, HA- or YFP. To test the constructs, protoplasts were transiently transformed and transgene expression for 35S::*Myc-Raf36* and 35S::*HA-Raf36* was tested by Western blotting with Myc- or HA-specific antibodies. It revealed that with both Raf36-containing constructs expression was

very weak and in repetition not reliably reproducible. Fig. 3.11 shows the amount of Myc-Raf36 detected with Myc-specific antibody on a Western blot. The cells were co-transformed with a Myc-MKK2 construct to show the difference in expression levels. Raf36-YFP expression in protoplasts was tested by fluorescence microscopy. No fluorescence above background levels was detectable.

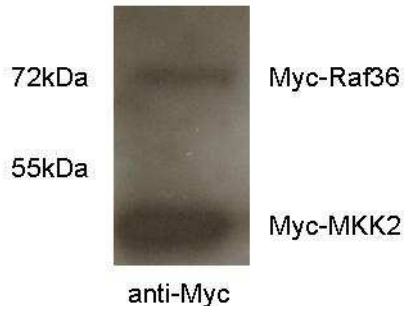


Fig.3.11:
Western blot analysis of Myc-Raf36 expression in transiently transformed protoplasts with Myc-specific antibody.

The constructs were also used for transformation of *Arabidopsis thaliana* plants (Col-0). Transgenic plants were isolated expressing: 35S::*Myc-Raf36*- 7 independent lines

35S::*HA-Raf36*- 4 lines

35S::*Raf36-YFP*- 2 lines

Seeds of the T2-generation were germinated under kanamycin selection to identify the number of T-DNA inserts by Mendel's laws: When the T-DNA segregates as a single locus, the relation of resistant to sensitive to seedlings is 3:1, in the case of two T-DNA insertions 15:1. The separation of the kanamycin resistance marker is shown in Table 3. In the majority of the lines ratios pointed to 1 or 2 T-DNA insertions. Just one of the 35S::Raf36-YFP lines gave a clear 3:1 (resistant to sensitive) ratio and therefore probably contains just one T-DNA insertion. In two lines more sensitive than resistant plants were detected. This can probably be attributed to silencing of the kanamycin resistance marker.

Western blot analysis revealed that none of the lines showed expression of the transgene, which could be due to silencing of Raf36, although expression of Myc-Raf36 and the resistance marker are not necessary linked. Loss of the kanamycin resistance in some of the T2 lines (and in the majority of T3 plants) also points to silencing of the inserted genes.

As overexpression of Raf36 might be harmful for plant development, we decided to generate inducible Raf36-overexpressing plants. In this way transgene expression can be induced after the plant reaches a certain developmental stage, in which deregulated Raf36 expression might be more tolerable.

	kan^R	kan^S	kan^R/kan^S ratio	T-DNA insertions
35S:*Myc-Raf*				
#1	43	10	4,3:1	1
#3	15	1	15:1	2
35S:*HA-Raf*				
#2	49	6	8,1:1	1-2
#3	100	2	50:1	3
#5	3	11	1:3,6	kan^R-silencing
#6	88	22	4:1	1
#7	73	11	6,6:1	1-2
#8	39	7	5,5:1	1
#9	88	20	4,4:1	1
35S:*Raf-YFP*				
#1	75	17	4,4:1	1
#2	44	4	11:1	1-2
#3	2	44	1:22	kan^R-silencing
#4	89	29	3:1	1

Tab. 3:
Segregation of kanamycin resistance marker in the T2-generation of independent transformant lines; numbers of T-DNA insertions according to the *x*"-test (P-value<0,95)

3.2.2.2 Generation of inducible Raf36-overexpressing lines

We used the pER8-vector containing the inducible XVE-promoter, which allows to switch on transgene overexpression by exposing the plant to estradiol. The Raf36 open reading frame fused to an N-terminal Myc-tag was cloned into pER8. The construct was transformed into *Arabidosis thaliana* and 14 independent transformant lines carrying the construct (selected with hygromycin) were obtained. After induction with estradiol none of these lines showed transgene expression on a Western blot with Myc-specific antibody (not shown). To exclude the possibility that the protein is expressed, but targeted by the poteasome for degradation, the induction was repeated in the presence of the proteasome inhibitors MG115 and MG132. Also in this experiment no Myc-Raf36 expression was detectable in western blot analysis. Therefore, the further *in vivo* experiments to elucidate the function of Raf36 were performed with the identified *knockout* lines.

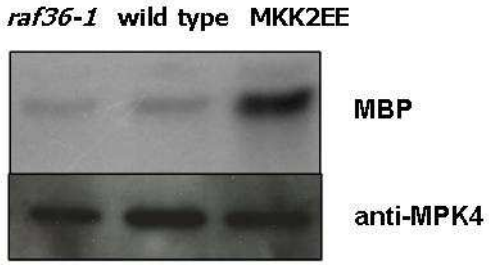
3.2.3 Role of Raf36 in MKK2 mediated abiotic stress signalling

Teige *et al.* (2004) showed that MKK2 activates MPK4 and MPK6 after cold and salt signalling. In *mkk2* plants MPK4 activation is abolished and MPK6 activity is reduced in response to stress. The opposite is observed in plants overexpressing constitutively active MKK2EE (Teige *et al.*, 2004), where MPK4 activity is constitutively activated (Brader *et al.*, 2007). As Raf36 is a putative MAPKKK, it would probably function upstream of MKK2 and modify its kinase activity in the plant. To test this hypothesis, the MPK4 activity in *raf36-1* plants was examined by *in vitro* kinase assays.

3.2.3.1 MPK4 activity in *raf36* is not elevated

The basal kinase activity of MPK4 was tested in *raf36-1* mutants and was compared to wild type as well as to MKK2EE overexpressors. Leaves were harvested from 4-week-old untreated plants, one leaf from 4-5 plants was pooled per sample and the experiment was done in triplicates. MPK4 and MPK6 were immunoprecipitated with specific antibodies from the leaf protein extracts, followed by *in vitro* kinase assays using MBP (myelin basic protein) as an MPK substrate. The protein level was detected on a western blot with an MPK4 specific antibody (Fig.3.12).

Fig.3.12:
Basal MPK4 activity in 4-week-old *raf36-1*, wild type and MKK2EE plants shown by *in vitro* kinase assay with MBP as a substrate. MPK4 protein levels were detected on a western blot with MPK4 specific antibody.



It revealed that *raf36-1* plants do not show a difference in the basal MPK4 activity compared with wild type plants. This result was not too surprising, because MKK2 basal activity is low, and it is upregulated after stimulation by an appropriate stress. An effect of Raf36 on MKK2 activity will probably be detectable only under stress conditions. Therefore, changes in MPK4 activity in *raf36* plants would also be expected only after stress, while in the MKK2EE line with a consitutively upregulated MKK2, increased MPK4 activity levels are detectable also without a stress stimulus. Therefore, MPK4 and MPK6 activities were tested in *raf36* seedlings after cold and salt treatment, under these conditions the activity of both kinases is impaired in *mkk2* plants.

3.2.3.2 MPK4 and MPK6 activity is not changed in *raf36* after cold stress

14-day-old *raf36*-, *mkk2*- and wild type seedlings were cold treated for 10, 20 or 30min. MPK4 and MPK6 were immunoprecipitated with specific antibodies from the protein extracts, followed by *in vitro* kinase assays using MBP (myelin basic protein) as an MPK substrate (fig.3.13). Protein levels were determined by western blot using MPK4-/MPK6-specific antibodies.

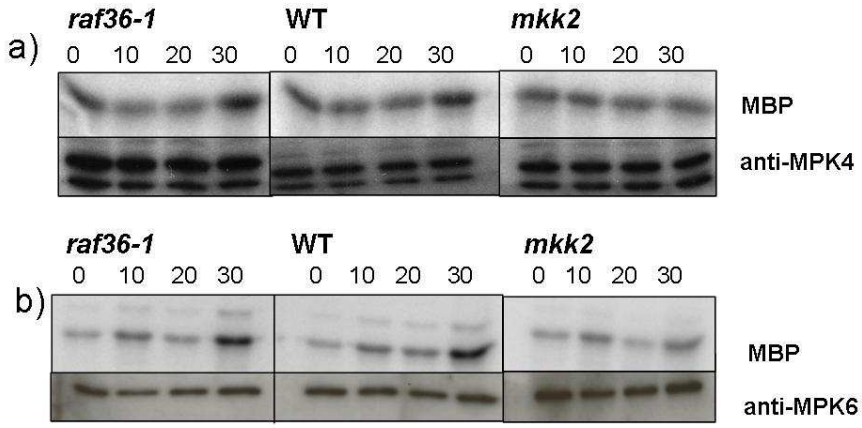


Fig.3.13:
In vitro kinase assays of MPK4 and MPK6 immuno-precipitated from cold treated *raf36*-, *mkk2*- and *wild type* seedlings.
a) MPK4 activity
b) MPK6-activity

While induction of MPK4-activity and MPK6-activity after cold treatment was abolished in *mkk2*-seedlings, in *raf36-1* seedlings MPK4 and MPK6 were equally activated as in wild type plants. These results indicate that Raf36 is not involved in MKK2-mediated cold stress signalling via MPK4 and MPK6.

3.2.3.3 Germination of raf36-1 is inhibited by salt stress

14-day old *raf36-1*, *mkk2* and wild type seedlings were treated with salt stress. MPK4 and MPK6 were immunoprecipitated with specific antibodies from the protein extracts, followed by *in vitro* kinase assays using MBP (myelin basic protein) as an MPK substrate. Protein levels were determined by Western blots using MPK4- and MPK6-specific antibodies.
The *in vitro* kinase assays did not give reproducible results. In none of the lines a reproducible kinase induction could be achieved even with a salt concentration of 250mM. The putative involvement of Raf36 in salt stress signalling was therefore assessed by germination assays. Teige *et. al* (2004) showed, that *mkk2*-germination is strongly inhibited under NaCl stress. For this reason, *raf36* germination was compared to *mkk2* and wild type germination on salt. For each experiment, about one hundred seeds of the *raf36*-1, *mkk2* and wild type plants were sown on the same MS (Murashige and Skoog medium) plate containing

125mM NaCl. The number of seedlings which had developed green cotyledons was counted at day 4 as a percentage of seeds sown (Fig.3.14a). The values were divided by the amount of seedlings with green cotyledons on MS plates, the control is in this case set to 1.

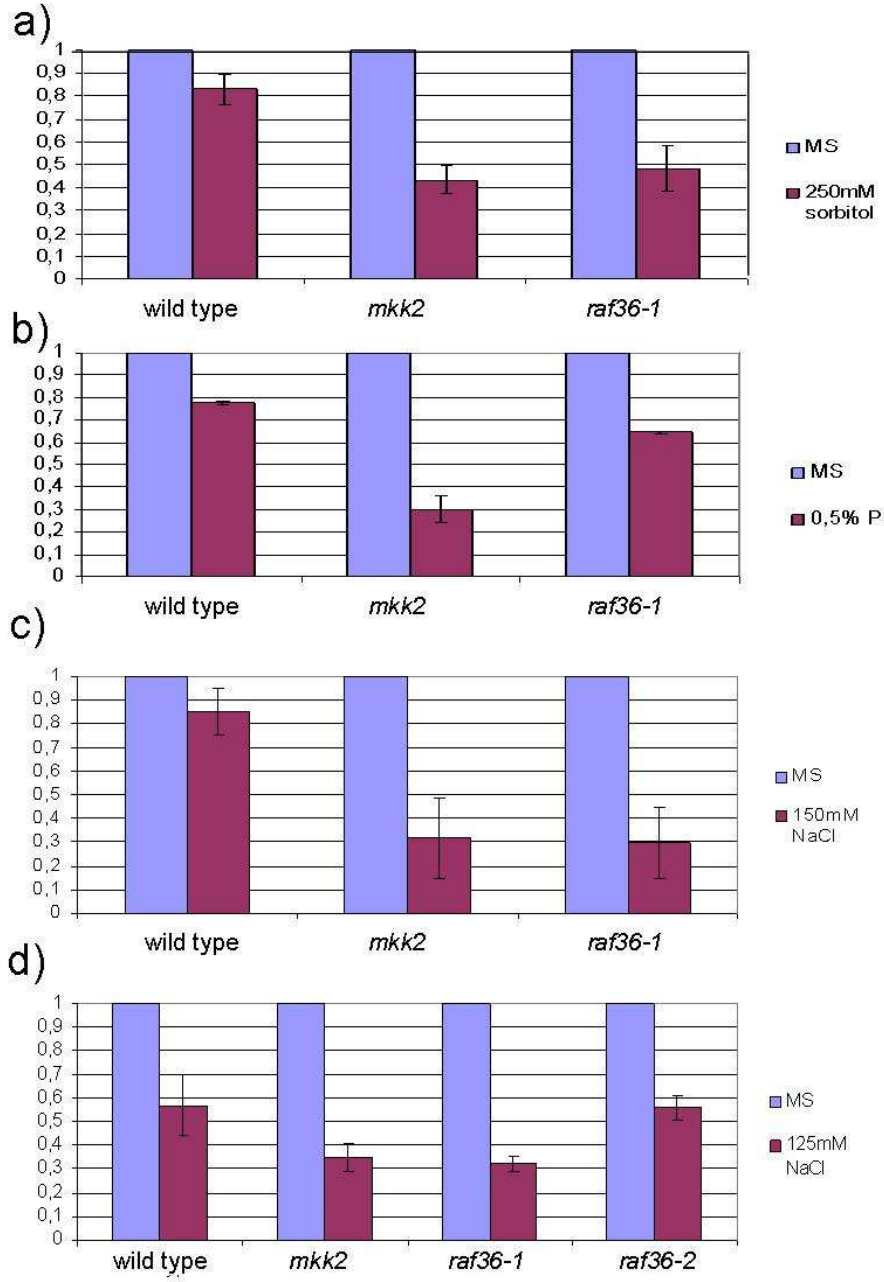


Fig. 3.14:
Relative amount of seedlings with green cotyledons after 4 days on MS and MS containing 150mM NaCl. Values given (+/-SD) are relative levels referring to the percentage on MS media (set to 1).
a) 150mM NaCl.
b) 250mM sorbitol
c) 0,5µM paraquat (Pq)
d) percentage of *raf36-2* seedlings with green cotyledons compared to *raf36-1*-, wild type- and *mkk2*- seedlings on 125mM NaCl..

Such as *mkk2*, seeds of *raf36-1* are inhibited in germinating under salt stress compared to wild type seeds (Fig. 3.14a). Salt stress is inducing osmotic and oxidative stress in plants. To determine whether inhibition of germination in *raf36-1* and *mkk2* on salt was due to osmotic stress, the germination of *raf36-1* seeds was tested on 250mM sorbitol (Fig. 3.14b). In addition, the effect of paraquat (methylviologen) was tested on *raf36-1* germination as a trigger for oxidative stress conditions (Fig. 3.14c). Paraquat is a well known herbicide that is

able to catalyse chloroplastic formation of reactive oxygen species (ROS) such as superoxide (O_2^-). Sorbitol and Pq inhibited *raf36-1* germination, but to a lesser extent than observed on NaCl.

These results suggest a role for Raf36 and MKK2 in several abiotic signalling pathways, since sensitivity to salt and paraquat was increased in the knockouts. The *raf36-1* line displayed a weaker abiotic stress sensitivity compared to the *mkk2* line in theses assays. The second knockout allele *raf36-2* showed no significant difference in germination on salt containing media compared to wild type plants (Fig. 3.14d).

3.2.3.4 *raf36-1, mkk1 and mkk2* plants do not show changes in sensitivity towards ozone

Plant exposure to ozone leads to accumulation of reactive oxygen species (ROS), the so called oxidative burst, which can induce cell death. Reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) are triggering stress responses not just after abiotic stresses, but also in the pathogen induced hypersensitive response (HR) (Laloi *et al.*, 2004). For this reason ozone treatments can be used as a model system for ROS mediated stress responses in the plant.

To test whether *raf36-1* and *mkk2* are changed in their ROS-sensitivity compared to *wild type*, ozone treatments with 4-week old *raf36-1* and *mkk2* plants were performed.

mkk1-plants were included in the experiment, as MKK1 is the closest *A. thaliana* homologue and was found to be partially redundant to MKK2, e.g. in plant innate immunity (Gao *et al.*, 2008; Qiu *et al.*, 2008). The plants were exposed to 8hrs of 350ppm of ozone (O_3). Due to the ozone-induced cell death, ions are released in the surrounding media. Therefore the sensitivity of plants towards ozone can be quantified by ion leakage measurements (Fig. 3.15).The ozone exposed plants were placed in 5ml of H_2O at room temperature, and the conductivity was measured after 1hr. The total ion leakage was determined after freezing and thawing the plants in the same surrounding water. Values shown in Fig. 3.15 represent ion leakage as a percentage of the total ion leakage in the measured plant.

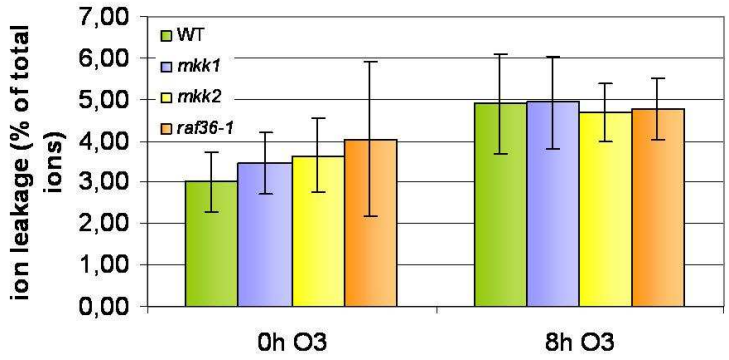


Fig.3.15:
Ion leakage (%+/-SD) in 3-week-old wild type, *raf36-1*, *mkk1* and *mkk2* plants after 8hr of 350ppm ozone. The measurements were done in triplicates, and the experiment was repeated 4 times. Shown is a summary of the results with the ion leakage before and after 8hrs of ozone treatments as a percentage of the total ion leakage.

While typical ozone-sensitive mutants like *rcd1* (Overmyer *et al.*, 2000) have ion leakage values of 20% or more, the tested lines displayed values lower then 5%. Comparison to wild type plants shows that ozone sensitivity is unchanged in *mkk1*, *mkk2* and *raf36-1* lines.
The experiments were done at the University of Helsinki, in collaboration with Prof. J. Kangasjärvi, Department of Biological and Environmental studies.

3.2.4 Role of Raf36 in biotic stress signalling

To test a possible involvement of Raf36 in MKK2 mediated biotic signalling pathways, biotrophic and necrotrophic responses of 4-week old *raf36* plants were examined. As MKK2 together with MKK1 had in a genetic approach shown to be involved in SA mediated defence signalling, and *mkk1 mkk2* plants are resistent to *Pseudomonas syringae (P.syringae)* (Gao *et al.*, 2008; Qiu *et al.*, 2008), we chose this biotic pathogen to gain information about a possible involvement of Raf36 in SA-mediated defence signalling. Additionally, plants overexpressing constitutively active MKK2 (MKK2EE) had been shown to be more susceptible to the necrotrophic fungus *Alternaria brassicicola* (Brader *et al.*, 2007). *A. brassicicola* infection of *raf36* was therefore performed to gain information about a possible involvement of Raf36 in JA mediated defence signalling. In MKK2EE, two conserved threonines (T) are mutated to glutamic acid residues (E), thereby mimicing the phosphorylated state and rendering the kinase constitutively active.

3.2.4.1 *raf36-1* plants do not show increased resistance upon *Pseudomonas syringae* infection

Two strains of *Pseudomonas syringae* pv.*tomato* DC3000 have been tested, *P. syringae* PsDc3000 pVSP61, being virulent in Col-0 plants and *Ps. syringae* PsDc3000 pV288, carrying the avirulence gene avrRptII and therefore being avirulent for Col-0 plants. Plants react to the virulent strain with PTI (PAMP-triggered immunity), and to the avirulent strain with ETI (effector-triggered immunity). Both defence responses are involving SA signalling. 4-week-old plants were infected with *P. syringae* by dipping the whole plant into a bacterial solution. To quantify bacterial growth in the plant, leaf discs were punched out at 1 hr, 1 day, 2 days and 3 days after infection and grinded in liquid. Several dilutions of this extract were spread on selective medium. After two days *P. syringae* colony forming units (cfu) on the plates were counted (Fig.3.16). *P. syringae* infection with both strains led to similar bacterial growth curves in *raf36-1* and wild type plants. The experiment was repeated 3 times, results of one representative experiment are shown in Fig.3.16.

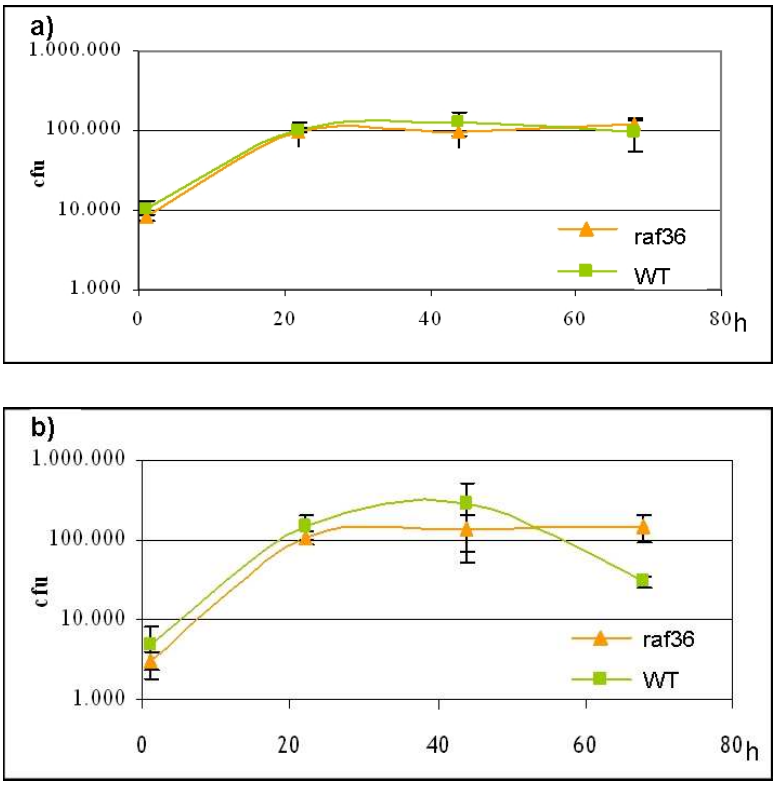


Fig.3.16:
Pseudomonas syringae growth in infected plant leafs up to 3days after infection quantified by counting of the colony forming units (cfu) +/-SD

a) *P.syringae* PsDc3000 pV288 (avrRptII)

b) *P.syringae* PsDc3000 pVSP618(virulent)

3.2.4.2 *raf36* mutants show increased sensitivity to *Alternaria brassicicola* infection

For the infection of plants with the necrotrophic fungus *Alternaria brassicicola*, a drop of spore suspension was placed on the leaf blade. In Col-0 plants, which are *A. brassicicola* resistant, this treatment leads to the appearance of small necrotic spots at the infection site (Fig.3.17a). The *A. brassicicola* hypersensitive *pad3* mutant (Zhou *et al.*, 1999), which cannot synthesis camalexin, a phytoalexin necessary for *Alternaria* resistance, develops large necrotic lesions around the infection site and was used as a control for successful infection. To quantify the level of damage, the size of the necrotic area around every infection site was estimated 7 days post infection (dpi). Three different categories of damage where distinguished based on the evaluation system published by Brader et.al (2007):

the necrotic area around the infection side covers less then 25% of the leaf area, found at most infection sites of *Col*-0 leaves

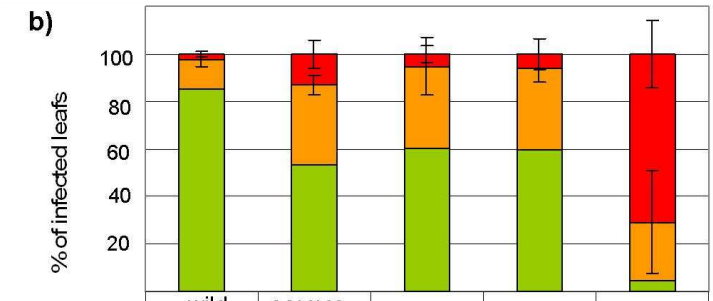
the necrotic area around the infection side covers between 25% and 50% of the leaf area

the necrotic area around the infection side covers more then 50% of the leaf area, found at most of the *pad3* infection sites

Fig.3.17:
Leaves of MKK2EE-, *raf36-1* and wild tye (WT) plants 7 days post *Alternaria brassicicola* infection

a) predominant levels of necrosis

b) quantification of damage by evaluation of the necrotic area size (% of total leaf size+/-SD). Shown is a summary of the results of 6 independent experiments. Line *raf36*-2 was included in only two of the 6 experiments.



	wild type	MKK2-EE	raf36-1	raf36-2	pad3
>50%	2,3	13,2	5,3	6,0	81,9
25-50%	12,8	33,9	34,6	34,0	28,7
<25%	84,9	53,0	60,1	60,0	5,2

Both *raf-36* lines display an increased sensitivity towards *A. brassicicola* compared to wild type (Fig.3.18). The *raf36-1* displays a stronger phenotype then *raf36-2*, MKK2EE-plants are even more sensitive then the *raf36* -lines.

To achieve a closer look at the fungal growth in the different lines, leaves were detached from the plant 2 dpi and stained with Trypan Blue to visualize the fungal hyphae (Fig.3.18). Corresponding to the previous findings, wild type leaves displayed hardly any hyphal growth, while in MKK2EE, *raf36-1* and *raf36-2*, fungal hyphae were growing out from the site of infection. In leaves of the hypersensitive *pad3* mutant, spreading of fungal hyphae was even stronger.

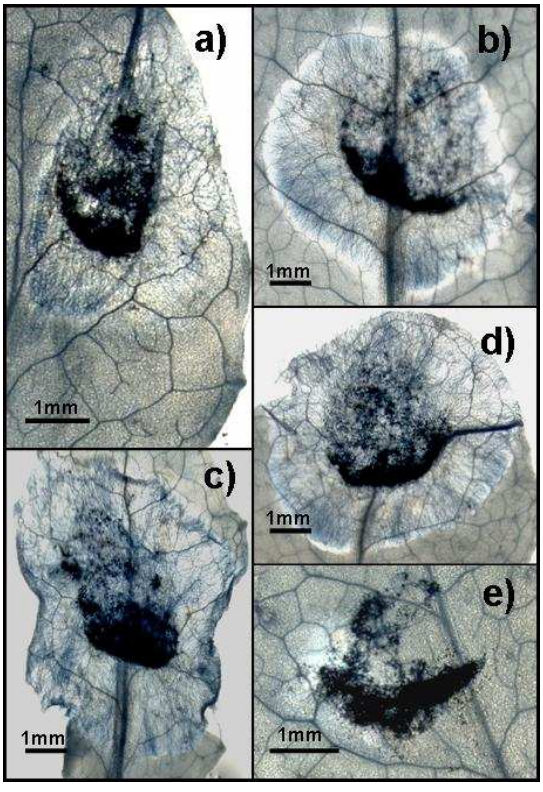


Fig.3.18:
Trypan blue staining of *A.brassicicola* hyphae around an infection site 2 dpi in
a) *raf36-1*
b) *raf36-2*
c) *pad3*
d) MKK2EE
e) wild type

3.2.4.3 Jasmonic acid levels are changed in 4-week-old *raf36-1* and MKK2EE plants after *A. brassicicola* infection, camalexin levels are unchanged

Resistance to *A.brassicicola* was shown to be jasmonic acid dependent, as coi1 mutants defective in JA signalling (Feys *et al.*, 1994), show increased sensitivity against the fungal pathogen (Thomma *et al.*, 1998; Vijayan *et al.*, 1998). Additionally to this JA-mediated resistance pathway, Thomma *et al.* (1999) uncovered the existence of a JA- independent resistance mechanism, based on the presence of the phytoalexin camalexin. In the *pad3* mutant, which has been used as an infection control for the *A.brassicicola* infection assays, biosynthesis of camalexin is abolished. Although JA signalling is not affected in theses plants, they are highly sensitive to the necrotrophic fungus.

To answer the question, whether *A. brassicicola* sensitivity in theses lines is connected to changes in JA levels or camalexin levels, we decided to determine the amounts of JA and camalexin in 4-week-old *raf36-1* and MKK2EE plants 1day and 2days after *A.brassicicola* infection. The measurements were done in triplicates. For each sample 12-16 leaves from 3-4 plants were pooled. The experiment was repeated 3 times, results of one representative experiment are shown in Fig. 3.19.

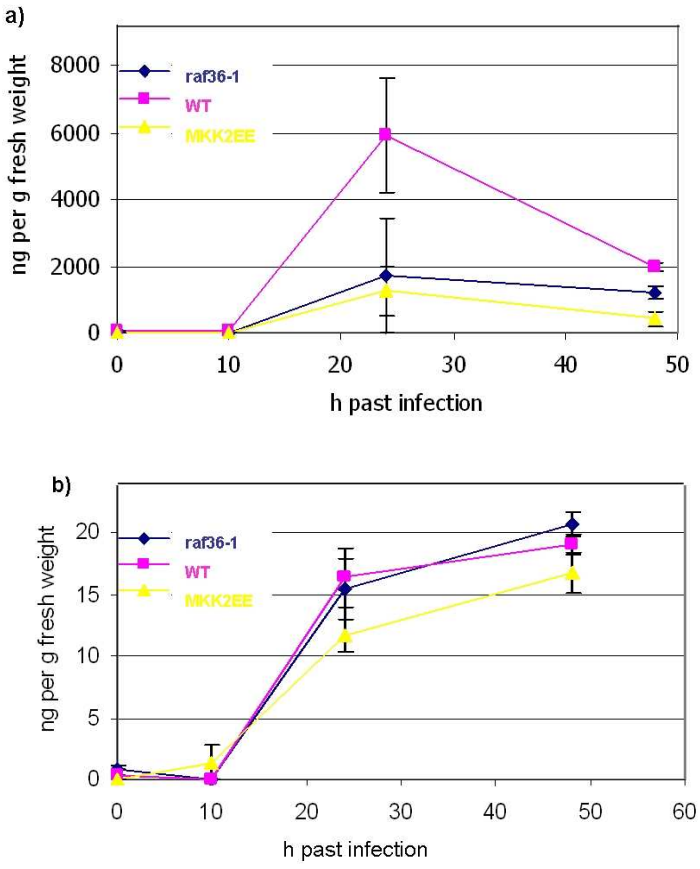


Fig.3.19:
Quantification of :
a) jasmonic acid
b) camalexin
(ng/g fresh weight+/-SE) measured in MKK2EE- ,*raf36-1* and wild type plants 10hrs, 24hrs and 48hrs after *Alternaria brassicicola* infection. Three replicates, each containing a pool of 12-16 leaves collected from 4 plants were measured. Measurements were done by Günther Brader.

Despite large biological variances between the samples in the total JA and camalexin amounts, the tendency of reduced JA induction in MKK2EE plants and *raf36-1* plants compared to wild type was visible in all experiments 1 day after infection. Camalexin levels in both mutant lines were comparable to those in wild type. The inhibited JA induction can explain the increased *A. brassicicola* sensitivity of *raf36*-lines and MKK2EE plants.

3.2.4.4 *raf36 mkk2* double knockouts are sensitive to *Alternaria brassicicola*

The *in vitro* phosphorylation assays using MPK4 as a substrate (Fig.3.8) showed that Raf36 decreases MKK2 activity. *raf36-1* plants and MKK2EE plants have an *A. brassicicola* sensitive phenotype (Fig.3.17b), while *mkk2* and wild type plants are resistant against the fungus. This results hints to a negative regulatory effect of Raf36 on MKK2 activity. Therefore, r*af36 mkk2* double knockout plants sensitivity against *A. brassicicola* would be expected to be rescued. To test this hypothesis, *raf36-1* and *mkk2* plants were crossed, and plants homozygous for both mutations were identified by genotyping. The *raf36 mkk2* plants were slightly dwarfed when compared to the *raf36-1* single knockout line, which means that *mkk2* deficiency did not rescue the slightly dwarfish phenotype (Fig. 3.23a). In addition, *raf36 mkk2* plants were also not rescued of the *Alternaria brassicicola* sensitivity, as scoring the damage 7 dpi led to the same results as for *raf36-1* single knockouts (Fig.3.23b).

a)

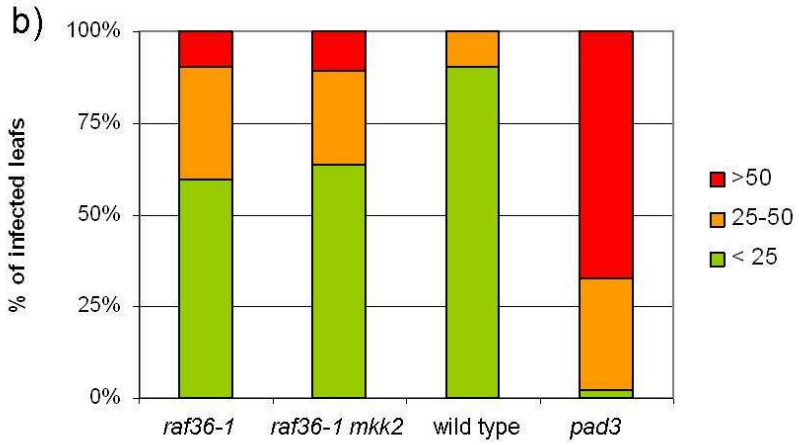


Fig.3.23:
Homozygous *raf36 x mkk2* double knockout plants:

a) growth phenotype of *raf36-1 mkk2* and *raf36-1* compared to wild type and *mkk2*

b) quantification of damage by evaluation of the necrosis size (% of total leaf size)

3.2.4.5 Transcriptome analysis of untreated *raf36-1* and MKK2EE plants and after *Alternaria* infection

To support our hypothesis that Raf36 is an upstream regulator of MKK2, and that both kinases are involved in an *Alternaria brassicicola* induced signalling pathway, we also decided to perform a transcriptome analysis of untreated *raf36-1* and MKK2EE plants and of the same plant lines 1 day after fungal infection. The cDNA was hybridized to a CATMA array, which contains 24576 gene specific tags (GSTs) corresponding to 22089 genes. GSTs are amplicons specific for each gene. Information related to the GSTs are available through http://urgv.evry.inra.fr/projects/FLAGdb++/HTML/index.shtml. Two biological repeats were used for every compared sample pair (always mutant against wild type), and a dye swop ruled out dye bias of the results. Therefore 4 chips were used per sample pair. Sample labelling, hybridization and statistical evaluation was performed by Sandra Pelletier (URGV, France). For the following analysis all the significantly up- or downregulated genes from two biological repeats were considered. Significance was determined as described under http://www-urgv.versailles.inra.fr/pub/ADT-19_Statistical_Analysis.pdf. The full list of differentially regulated genes in both lines is given in the Appendix.

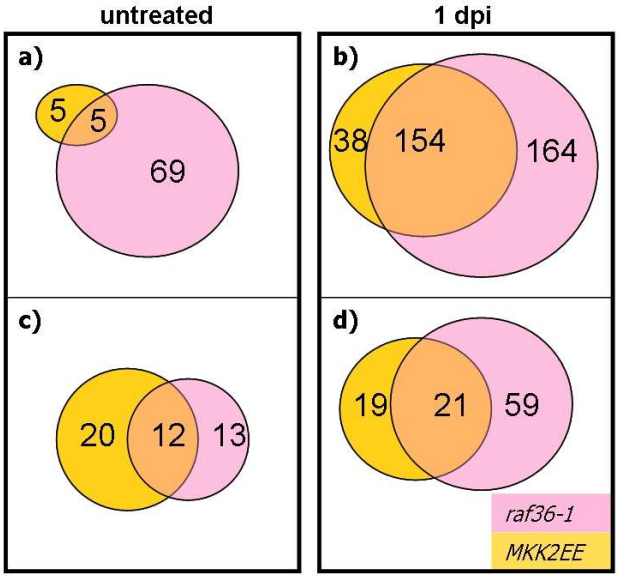


Fig.3.20:
Venn diagram showing overlapping gene expression between untreated *raf36-1*- and MKK2EE-plants and plants 1 day after *A.brassicicola* infection (1 dpi) as measured by the :
a ,b: upregulated genes
c ,d: downregulated genes

First the expression profiles of the untreated raf36-1 and MKK2E plants were compared (Fig. 3.20a,c). In untreated raf36-1 plants, 74 genes were upregulated compared to wild type. In MKK2EE plants 10 genes were upregulated (Fig. 3.20 a). 5 of those genes were upregulated in both lines, among them 3 transcription factors and two unknown proteins (Table 4). The transcription factors were RAV2 and WRKY53, which are both chitin-induced (Libault *et al.*, 2007) as well as ATBT2, a transcription factor involved in gametophyte development. WRKY53 plays an important role in basal resistance and is negatively regulated by JA (Murray *et al.*, 2007).

AT1G68840	RAV2 (transcription factor)
AT4G23800	WRKY53 (transcription factor)
AT3G48360	ATBT2 (transcription factor)
AT3G15450	unknown protein unknown protein
AT2G25735	unknown protein

Table 4:
Overlapping upregulated gene expression in untreated *raf36-1* and MKK2EE plants

The overlap of genes concordantly downregulated in both untreated lines was 50% for *raf36-1* (12 out of 25) and 38% for MKK2EE (12 out of 32). The 12 genes are shown in Table 5. 8. Several of these genes are involved in biotic and abiotic stress signalling, like oxidative stress, salt and cold stress. TAT3, which is JA- and wound-induced, was also downregulated in both lines (Table 5).

biotic stress	
AT2G24850	TAT3 (tyrosine aminotransferase 3)
AT1G66100	thionine, putative
oxidative stress	
AT4G33870	Peroxidase
AT1G62180	thioredoxin
AT2G38240	oxidoreductase, 2OG-Fe(II) oxygenase family protein
Abiotic stress	
AT4G17090	beta-amylase (CT-BMY)
AT5G49480	sodium-responsive calcium-binding protein (ACP1)
AT3G61890	ATHB-12; transcription factor, homeobox-leucine zipper protein 12
AT3G50970	low-temperature-induced protein LTI30 (LTI30)
Unknown function	
AT5G54470	transcription factor, zinc finger (B-box type) family protein
AT2G31040	ATP synthase protein I -related protein
AT3G44450	unknown protein

Table 5: Overlapping downregulated gene expression in *raf36-1* and MKk2E plants

Next, the expression profiles 1 day after *A.brassicicola* infection were compared. Overlaps in the group of upregulated genes in both lines compared to wild type were striking (Fig.3.20b). 48% of the genes differentially upregulatd in raf36-1 (154 out of 318) and 80% of the genes upregulated in MKK2EE (154 out of 192) were overlapping 1 day after fungal infection. 45 of those genes are involved in biotic and abiotic stress signalling (Table 6), among them 12 genes known to be involved in JA-mediated signalling, but also genes involved in ethylene signalling (ASA1 and EIL1), or SA-mediated signalling (ATHSPRO2 or RCA) were differentially regulated in raf36-1 and MKK2EE.

Biotic stress		
AT1G19660	wound-responsive family protein	wound response
AT1G59870	PEN3 (penetration 3)	defence response to fungus, systemic acquired resistance
AT5G43940	ADH2 (alcohol dehydrogenase)	wound response
AT4G23100	PAD2 (phytoalexin deficient 2; glutamate-cysteine ligase)	wound and defence response to fungus, heat, ozone response
AT5G05730	ASA1 (anthranilate synthase alpha subunit 1)	wound response, response to bacterium, response to ethylene
AT3G19260	LOH2 (LAG1 homolog 2)	defence response to fungus
AT5G05170	CEV1 constitutive expression of VSP1;cellulose synthase)	defence response
AT1G20840	TMT1 (tonoplast monosaccharide transporter 1)	response to nematode
AT1G58360	amino acid permease 1 (AAP1)	response to nematode
AT1G69850	ATNRT 1:2 (*Arabidopsis thaliana* nitrate transporter 1:2)	response to nematode
AT5G13740	ZIF1 (zinc induced facilitator 1)	response to nematode
AT1G37130	NR2 (nitrate reductase 2)	response to symbiotic fungus
AT1G76180	ERD14 (early response to dehydration)	defence response, ABA response, cold and drought response
AT2G40000	ATHSPRO2	defense response to bacterium, SA response, oxidative stress
Contd. biotic stress		
AT2G39730	RCA (Rubisco activase)	defense response to bacterium, cold and light response
AT4G01050	glycoprotein family protein	defense response to bacterium,
AT1G20020	ATLFNR2 (ferredoxin-NADP(H)-oxidoreductase)	defence response to bacterium,
AT2G27050	EIL1 (ethylene-insensitive3-like1)	ethylene response
AT2G37220	RNA binding protein	innate immune response, cold response
AT5G60410	small ubiquitin-like modifier (SUMO) E3 ligase	defense response,

		cold and drought response
Abiotic stress		
AT2G16500	ADC1(arginine decarboxylase 1)	oxidative stress response, cold and salt response
AT1G49670	unknown protein	oxidative stress response
AT2G15560	unknown protein	oxidative stress response
AT3G57290	ATEIF3E-1 (eukaryotic translation initiation factor 3E)	salt stress response
AT4G32410	CESA1 (cellulose synthase 1)	salt stress response
AT5G49720	ATGH9A1 (*Arabidopsis thaliana* glycosyl hydrolase 9A1)	salt stress response
AT5G35630	ATGSL1 (glutmine synthase 2)	salt stress response
AT4G16260	BG1 (beta-1,3-glucanase 1)	salt stress response
AT3G60750	transketolase, putative	salt stress response, response to cadmium ion
AT3G01470	ATHB-1 (*Arabidopsis thaliana* homeobox 1).	response to blue light, response to salt stress
AT4G13770	CYP83 A1 (cytochrome P450 83A1)	response to UV
AT4G36220	F5H (ferulate 5-hydroxylase)	response to UV
AT5G04590	SIR (sulfite reductase)	salt and cold stress response
AT3G23700	S1 RNA-binding domain-containing protein	cold response
AT1G16880	uridylyltransferase-related	response to cold,
AT1G13260	RAV1 transcription factor	response to low temperature
AT2G16280	KCS9 (ketoacyl-CoA synthase 9)	cold response
AT3G48750	CDC2 (cell division control 2).	cold response
AT1G42970	GAPB (glyceraldehyde-3-phosphate dehydrogenase B)	response to cold, response to light stimulus, response to sucrose stimulus
AT5G63980	HOS2 (high expression of osmotically responsive genes 2)	cold and drought response
AT3G46970	ATPHS2	drought and heavy metal stress response
AT4G36990	ATHSF4 (*Arabidopsis thaliana* heat shock factor 4)	heat stress response
AT3G09440	HSC70-3 (heat shock cognate 70 kDa protein 3)	heat stress response
AT5G42020	BIP (luminal binding protein)	heat stress response, response to ER

		stress
AT1G54990	AXR4 (auxin resistant 4)	response to auxin stimulus, response to mechanical stimulus

Table 6:

Assortment of genes involved in biotic and abiotic stress signalling , which are significantly upregulated in *raf36-1* and MKK2EE plants 1 day after *A.brassicicola* infection

The 21 genes which are concordantly downregulated in raf36-1 and MKK2EE 1 day after A. brassicicola infection (Fig.3.20 d) contain 11 genes involved in biotic and abiotic stress signalling and 5 genes of unknown function (Table 7).

biotic stress		
AT1G17420	Lox3 (lipoxygenase 3)	wound response, response to fungus
AT1G72520	lipoxygenase, putative	wound and defence response
AT2G24850	TAT3 (tyrosine aminotransferase 3)	wound response
AT1G65390	ATPP2-A5 (*Arabidopsis thaliana* phloem protein 2-like A5)	defence response
AT2G46400	WRKY 46	response to chitin
AT2G44840	ATERF13 (*Arabidopis thaliana* ethylene responsive element binding factor 13)	ethylene mediated signalling,response to chitin
AT3G23230	ERF/AP transcription factor family protein	ethylene mediated signaling pathway, response to chitin
abiotic stress		
AT2G28190	CSD2 (copper/zinc dismutase 2)	oxidative stress response
AT3G51920	CAM9 (calmodulin 9)	salt and drought stress response, response to ABA
AT2G42530	COR15B (cold regulated 15B)	cold stress response
AT3G22840	ELIP1 (early light inducible protein)	cold and light response

Tab.7:

Assortment of genes involved in biotic and abiotic stress signalling, which are significantly downregulated in *raf36-1* and MKK2EE plants 1 day after *A.brassicicola* infection

MKK2EE and *raf36-1* showed an evident overlap in genes that were differentially expressed compared to the wild type 1 day after *Alternaria brassicicola* infection. This group of genes contains crucial genes for biotic stress responses, as is expected if Raf36 and MKK2 are involved in the same biotic stress signalling pathways. The abundance of abiotic stress induced genes that are differentially regulated in both lines is a further hint for the involvement of Raf36 in MKK2-mediated signalling also under abiotic stress conditions.

3.2.4.6 Localisation studies with the Raf36 promoter show overlap in the expression pattern of Raf36 and MKK2

To study the tissue-specific activity of the Raf36 promoter, a 912bp fragment of the sequence upstream of the Raf36 ORF (Fig.3.21) was amplified from genomic DNA. The fragment was cloned into a pGreen vector upstream of a ß-glucuronidase (GUS) reporter gene and transformed into *A. thaliana*.

ProGUS_for

ctggttactatcaagcaatcatcatggcctatatcataaagtaatttgggccagttttattattaatgggccgaa
gtctatggcctttagaatttatttgactaaaaaagggaacctaaattaaatttctctttcagacagaataattag
agcatatcagagatgctgacagacagattaatacgctgtcgttttaacgtatttttctaaatattccaaaagaat
ttatttattttccacattttatctgtgaaaaaaaaagaaaaaaaaaatccatctaaaaaggcgtaagaggtctgg
attcgtcaaaaaggtctttgagaatccaaaaacgtcttatggtttagctctcaaatttctccttcgattccatct
tcctccggcttcaagtcgccggatcttcttgatttccgattcacttggctttcctcttctcttccttttaacaca
cgacgacgcttcatagtttttgttgtgattcaggtaaacaaatatcgaaaactagtttcctaaatttgttgatt
cagatcgatcttttaaccctcattatcgttttcaatagcttctatggattcgattttctgagtattgttgcttga
ttttgtaggaagaggtatgtaattcaggattttccggtgattttggtgggaacgtttggagaaatttcgagctta
gtagtattcgtaggaaagttttcttcttttatgatttgtagttttgtggattaatggttgaaggtgagaaatgg
ggcttcgatggatttgaaaagattttgtatcttcgtggaaatgagatcgttggagattgcagcaagtcatctcat
tcgtattcacgggtttgcttaggtactttggagatattgagagttttggttgtttagtctttgataaaaaggttg
Tttagtttgttttcgagcttagag(atg)

ProGUS_re start→

Fig.3.21:
Sequence of the Raf36-promoter fragment cloned into the GUS reporter construct.
The sequence of the promoter fragment is marked in red, location of the primers used for fragment amplification are indicated.

Seven independent, GUS-expressing lines were obtained and the histochemical staining of 2-week-old seedlings and adult plants was performed. The staining intensity was varying in the

different lines, but they all showed the same pattern of promoter activity. Pictures from one representative line are shown (Fig.3.22).

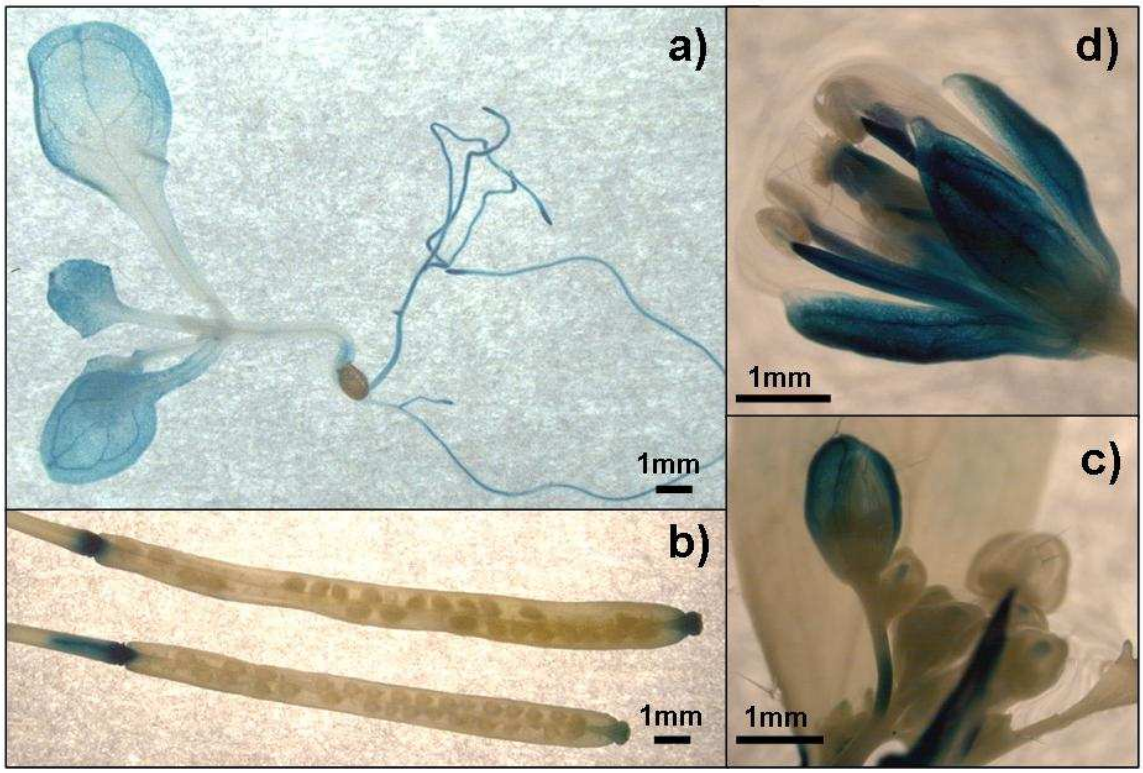


Fig.3.22:
GUS expression in *Arabidopsis thaliana* transformed with the *praf36*:*GUS* construct. Shown is a 2-week-old seedling (a), mature siliques (b), flowerbuds (c) and a flower (d).

In 2-week-old seedlings, a strong GUS activity was visible in the entire roots, which was most intensive in the root tip. The cotyledons as well as the first true leaves exhibited a diffuse pattern of GUS staining, which was often more dense close to the leaf edge (Fig. 3.22a). Regarding the siliques of 6-8-week-old plants, staining was detectable in the abscission zone and in the stigma of siliques, but not in seeds (Fig. 3.22b). In flower buds, GUS activity was visible in the sepals and further increased during development of the flower (Fig. 3.22c). GUS activity was additionally detectable in the flower, precisely in the style and in the filaments of the stamina (Fig. 3.22d). The rosette leaves and cauline leaves in flowering plants also showed a diffuse promoter activity with varying strength; the strongest expression was also in this stage seen at the leaf edges.

The expression pattern of *pMKK2::GUS* in plants has been shown by Armin Djamei (PhD thesis, 2007). Promoter activity was visible in the roots, mainly in the root tip, as well as in the leaves of seedlings and leaves of mature plants. In these plants, like in the *praf36*::*GUS*

transformants, the GUS activity in the leaves was diffuse and staining was the strongest close to the leaf edges. Staining was also detectable in the pistil and the sepals of unopened flower buds and flowers (weakly), like shown here for *praf36::GUS* (Fig. 3.22c and d).
As the localisation pattern of promoter activities is widely overlapping between the *praf36*::*GUS* and the *pMKK2::GUS* lines, which shows that Raf36 and MKK2 are both expressed in the same tissues, it can be concluded that an interaction between the two proteins *in vivo* is probable.

4. Discussion

A.thaliana contains more then 60 MAPKKKs. This classification was exclusively based on sequence similarity to known MAPKKKs, such as the yeast and mammalian Raf-kinases. For most of those *A.thaliana* kinases, a function has not yet been shown. Therefore the isolation of Raf36 as a putative MKK2 interactor in a yeast two-hybrid screen encouraged us to study the function of this MAPKKK. This work represents the first study on a member of group C MAPKKKs showing that Raf36 is indeed an upstream regulator of MKK2.

4.1 Raf36 can interact with MKK2 and MKK7, but not with MKK1 *in vitro*

In the first part of this work, Raf36 interaction with MKK2 was verified by *in vitro* pull down assays. The ability of Raf36 to interact with all 10 MKKs was tested in a directed Y2H approach. In this approach Raf36 could interact with MKK2, but not with the closest MKK2 homologue MKK1, which functions redundantly to MKK2 in biotic stress (Gao *et al.*, 2008; Qiu *et al.*, 2008). Instead of MKK1, MKK7 was identified as a second putative Raf36 interactor. MKK7, besides being a negative regulator of polar auxin transport, has been shown to play role in immune response pathways (Zhang *et al.*, 2007). These results open up the possibility that Raf36 is involved in biotic stress signalling by regulating more than one MKK.

4.2 *In vitro* results identify Raf36 as an active kinase

The kinase activity of a recombinant HIS-tagged Raf36 protein was tested by *in vitro* kinase assays. Weak autophosphorylation of the protein could be detected. The signal was not detectable any more in a mutated HIS-Raf36 protein, which carried a single base pair mutation. This mutation changed a highly conserved lysine residue in the ATP-binding loop to a methionine residue. The same mutation was also shown to abolish the kinase activity of the MAPKKK MEKK1 (Asai *et al.*, 2002). Raf36 can therefore be considered to encode an active kinase.

4.3 Raf36 can phosphorylate MKK2 upon deletion of amino-terminal parts, suggesting the existence of an autoinhibitory domain in the amino-terminus

The recombinant HIS-Raf36 protein was not able to phosphorylate the artificial substrate MBP (myelin basic protein) or the MKK2 protein. Due to the weak autophosphorylation of Raf36, an autoinhibition of the kinase activity was postulated. Raf36 features a prolonged amino-terminal domain, which contains no conserved domains except for a serine-rich region. The postulated autoinhibitory domain might be localised within this region, covering and thereby sterically blocking the autophosphorylation sites and the kinase domain. Interaction with a binding partner or phosphorylation by an unknown upstream regulator could induce a structural change in this domain, setting free the autophosphorylation sites and activating the kinase. The human MAPKKK MTK1 has an autoinhibitory domain in its carboxy-terminus which binds to the kinase domain and thereby blocks interaction with the substrate. Binding of an activator releases the autoinhibition and renders the MTK1 kinase active (Mita et al., 2002) . Autoinhibition by binding of an N-terminal region to the catalytic domain was also hypothesised for the mammalian Raf kinases (Rushworth et al., 2006) . To prove the existence of an inhibitory domain in Raf36, truncated HIS-Raf36 proteins were generated, which carried deletions of different lengths at the amino-terminus. The two longest Raf36 truncations, called Raf36ΔNA and Raf36ΔNB, with deletions of 42 and 75 amino acid residues from the amino terminus, showed a clear increase in autophosphorylation. In addition, Raf36ΔNB showed phosphorylation of the substrate MKK2. Since the smaller truncated proteins ΔNRaf36C-D showed a decreased autophosphorylation signal, one or several phosphorylation sites could be expected within the truncated region between amino acid 75 (the start of ΔNRaf36B) and amino acid 105 (the start of ΔNRaf36C). There are six potential phosphorylation sites situated within this region, four serine and two threonine residues (marked in bold in Fig.3.4). Phosphorylation of Raf36 at one or several of theses N-terminal sites *in vivo* by an unknown upstream regulator might release the autoinhibition, and the activated Raf36 might then be able to phosphorylate sites in this area itself. Truncation of the N-terminal regions in Raf36ΔNA and B might lead to conformational changes relieving autoinhibition. This again might render the Raf36 able to phoshorylate (some of) these N-terminal phosphorylation sites and maybe thereby gain its full activity and the capability to phosphorylate the substrate MKK2. This would explain the increase in the phosphorylation signal in Raf36ΔNA and B compared to the

autophosphorylation of Raf36. The loss of the phosphorylation sites in the truncated region would explain the decrease of phosphorylation in Raf36ΔN C-D. It also is feasible that Raf36 is active as a homodimer or *in vivo* as a heterodimer with another Raf-like kinase, like it had been shown for the mammalian C-Raf and B-Raf kinases (Rushworth et al., 2006). As autophosphorylation sites are in general found in the kinase domain , the phosphorylation of the amino-terminal sites might happen by trans-phosphorylation between the dimer partners.
Regarding that truncation of the first 42 amino acids was enough to increase autophosphorylation, but truncation of the first 72 amino acid residues was required for enabling Raf36 to phosphorylate the substrate MKK2, the inhibitory domain would be expected within the first 72 amino acids of the full length Raf36 protein. To summarise these results, elimination of a putative autoinhibitory domain together probably together with phosphorylation events in the N-terminus, displayed by the changes in autophosphorylation, render Raf36 active, and the active kinase was able to phosphorylate MKK2 *in vitro*.
Certainly it would be more convincing to find the right trigger to render the full length Raf36 active. An elegant approach to identify an appropriate stimulus would have been to transiently express Raf36 in the protoplast system and search for suitable conditions to render Raf36 active. This approach has been successfully undertaken by Teige *et al.* (2004), who showed an activation of MKK2 in protoplasts after salt and cold, but not after H_2O_2, heat or flg22 treatment. However, the very low expression of the recombinant Myc- or HA-tagged Raf36 in protoplasts made this approach unsuitable.

4.4 Raf36 reduces MKK2 activity *in vitro* and might act as a negative upstream regulator of MKK2

In case Raf36 constitutes an upstream regulator of MKK2, the phosphorylation of MKK2 by Raf36 should alter MKK2 activity. This question was addressed by using *in vitro* kinase assays to determine the MKK2 activity in the presence of ΔNRaf36, and the kinase inactive version of MPK4 was used as an MKK2 substrate. In this way, the MPK4 phosphorylation would be attributed to either the ΔNRaf36 or either the MKK2 kinase activity. The results showed a reproducible decrease in MPK4 phosphorylation in the presence of MKK2 and ΔNRaf36 compared to MPK4 phosphorylation by MKK2 in the absence of ΔNRaf36. Therefore it can be concluded, that ΔNRaf36 has an inhibitory effect on MKK2 *in vitro*.

Although ΔNRaf36 alone did not phosphorylate MPK4 *in vitro*, it is possible that in a complex with MKK2, ΔNRaf36 would be in close proximity to MPK4 and could (weakly) phosphorylate MPK4. To answer the question, whether MPK4 phosphorylation was due to MKK2 or to ΔNRaf36 activity, the experiment would have to be repeated with a kinase inactive version of MKK2.

There are two ways to explain the inhibitory effect of Raf36 on MKK2 activity. First, Raf36 could bind MKK2 and thereby sterically inhibit the kinase. Second, the inhibition could be a secondary effect of MKK2 phosphorylation by Raf36. In the first case, MKK2 phosphorylation by Raf36 would not modify its activity, and the kinase inactive Raf36 protein should have the same inhibitory effect on MKK2 activity *in vitro*. It is thereby assumed, that the kinase inactive Raf36 protein can still bind its substrate. In the second case, Raf36 phosphorylation of MKK2 would be necessary to suppress MKK2 activity. This aspect is interesting, since phosphorylation of MKK2 was so far just known to increase the kinase activity of the protein. Raf36 might use phosphorylation sites in MKK2, which are different from the ones targeted by MKK2 activators. Phosphorylation of these target sites would lead to the observed inhibition of MKK2. A first approach to investigate the existence of such divergent phosphorylation sites would be to test if Raf36 can phosphorylate the MKK2EE protein (Teige *et al.*, 2004). In this mutated MKK2 version, two conserved threonine residues are changed to glutamate, which mimics the phosphorylated state. The resulting, constitutively and highly active kinase cannot be phosphorylated at these two positions any more. Therefore, if Raf36 could still phosphorylate MKK2EE *in vitro* would suggest the existence of divergent phosphorylation sites targeted by Raf36. Exact determination of these target sites could be done by deletion studies or mass spectrometry.

4.5 The role of Raf36 in MKK2-mediated salt and cold stress signalling is unclear

MKK2 has been shown to act in abiotic stress signalling (Teige *et al.*, 2004). *mkk2* plants feature increased cold and salt sensitivity, and the induction of MPK4 activity as well as the MPK6 activity was reduced after cold treatment. The opposite was observed in plants overexpressing MKK2EE. These plants were more cold and salt resistant, and their basal MPK4 and MPK6 activities were enhanced.

MPK4 basal activity was therefore tested in *raf36* knockout plants. It revealed that basal MPK4 activity in *raf36* plants was similar to wild type levels. We assumed that a change in MKK2 activity and hence in MPK4 activity would require a trigger to become detectable in *raf36* plants. Therefore we examined MPK4 und MPK6 activity after cold and salt treatment. It turned out that after cold treatment, MPK4 as well as MPK6 activity in *raf36* plants reached wild type levels, while induction of both MPKs was diminished in *mkk2* knockout plants (Teige *et al.*, 2004). Therefore, we concluded that Raf36 is not involved in cold stress signalling. Since no reproducible results could be achieved in the salt experiments, the role of Raf36 in salt stress signalling was investigated by determining the germination rate of *raf36* seeds and *mkk2* seeds on media containing salt or several other agents. It appeared that seeds of both lines were inhibited in their germination on salt, sorbitol and paraquat compared to wild type seeds. This suggests, that Raf36 and MKK2 could be involved in several abiotic signalling pathways. In the salt germination experiments, two *raf36* alleles were tested. However, after salt stress the second knockout allele *raf36-2* showed no difference in its germination compared to wild type plants. In addition, *raf36-2* plants showed a weaker dwarf phenotype then *raf36-1* plants. Therefore, *raf36-2* might be a weaker allele, exhibiting a low level of *Raf36* transcription. The fact that no *Raf36* transcript was detected in the RT-PCR performed from *raf36-2* seedlings could be attributed to a low abundance of the transcript which stayed below detection levels due to an insufficient number of amplification cycles. A second possibility for the difference between *raf36-1* and *raf36-2* germination behaviour on salt is that inhibition of *raf36-1* germination on salt is Raf36-independent and caused by another mutation in this T-DNA insertion line. Although the line was backcrossed once, there might still be additional T-DNA insertions present in the plant genome. To answer the question whether the observed salt inhibition on *raf36-1* germination is due to the lack of the Raf36 protein, additional T-DNA insertion lines are being analysed to identify a third *raf36* allele.

4.6 *raf36* plants do not show increased sensitivity to ozone

The ozone treatment is often used as a model system for ROS induced plant responses. ROS have been shown to be involved in basically any kind of plant responses to abiotic as well as biotic stresses (Laloi *et al.*, 2004). The complex crosstalk between MAPKs and ROS largely remains to be elucidated (Pitzschke and Hirt, 2009). The read-out of the ozone treatments is

ROS induced cell death, measured by ion leakage of the treated plant tissue. ROS induced cell death is regulated by a complex interplay between cell death promoted by ethylene and salicylic acid (SA), and jasmonic acid (JA), which is needed for lesion containment (Overmyer *et al.*, 2000). Therefore ozone phenotypes can point to defects in ROS signalling and cell death regulation, but also to defects in the regulation of stomatal opening, as closed stomata are the first level of protection against ozone (Vahisalu *et al.*, 2008). Testing the sensitivity of *raf36* plants and *mkk2* plants to ozone revealed no change in sensitivity compared to wild type plants, making defects in ROS signalling or cell death regulation in response to ozone treatment improbable.

4.7 In contrast to MKK2EE, *raf36* plants do not show increased resistance against the biotrophic pathogen *Pseudomonas syringae*

MKK2EE plants have been shown to exhibit increased resistance towards the biotrophic bacterial pathogen *Pseudomonas syringae*. Therefore, *raf36* plants were tested for resistance against this pathogen. Two different strains were applied, a virulent and an avirulent strain. Both strains are inducing SA-dependent defence responses, including an oxidative burst, SA accumulation and the induction of SA regulated PR genes. But the two strains are triggering different defence responses in the plant: PTI (PAMP triggered immunity) and ETI (effector triggered immunity, also gene-for-gene resistance or R-gene mediated resistance). Testing both strains would therefore allow us to distinguish between a putative role of Raf36 in PTI, activated by the virulent strain, or in ETI, activated by the avirulent strain. Bacterial growth of both *P.syringae* strains was found to be similar in *raf36* plants and in wild type plants, which argued against a participation of Raf36 in signalling upon infection with *P.syringae*. On the other hand the growth and *Alternaria brassicicola* sensitivity phenotype was milder in *raf36* plants then in MKK2EE plants. This might be due to a redundancy of Raf36 with another MAPKKK, as the *A.thaliana* genome encodes more then 60 MAPKKKs most of which the function is not yet known. In this case it would not be surprising if the difference in resistance to *P.syringae* compared to wild type plants is too small to be detected in the bacterial growth assay.

It has to be mentioned, that increased *P.syringae* resistance has also been reported for *mkk1 mkk2* double knockouts, caused by the accumulation of high SA levels and a constitutive defence response (Gao *et al.*, 2008; Qiu *et al.*, 2008), phenotypes which were also found in

mekk1 and *mpk4* plants. Therefore MKK2 could be positioned in a MEKK1-MKK1/2-MPK4 pathway negatively regulating SA defence responses. *mkk2* plants were reported to exhibit a wild type like *P.syringae* response, which can be explained by the functional redundancy with MKK1. In contrast to these results, Brader *et al.* (2007) reported a higher *P.syringae* resistance of *mkk2* plants. Considering these results, the increase in *P.syringae* resistance of MKK2EE plants is surprising. Finding *mkk1mkk2* plants more resistant to the pathogen due to increased SA levels, MKK2EE plants would be expected to be more sensitive and to show reduced SA levels. Although some reduction in SA levels was found in MKK2EE plants after *P.syringae* infection, JA levels were dramatically reduced (Brader *et al.*, 2007). In immunity responses to biotrophic pathogens, SA and JA signalling often work antagonistically, whereby SA suppresses JA-mediated responses and vice versa. MKK2EE plants were shown to be more resistant against *P.syringae*, despite the reduced SA levels. However, this resistance might be caused by major effect on JA levels.

4.8 *raf36* plants and MKK2EE plants are more sensitive to *Alternaria brassicicola*

Plants mainly use SA defence responses against the biotrophic pathogen *P.syringae*, although JA induction also occurs (Brader *et al.*, 2007). Resistance against the necrotrophic fungus *Alternaria brassicicola* seems to rely exclusively on JA responses (Glazebrook, 2005; Thomma *et al.*, 1998), as well as on the phytoalexin camalexin (Glawischnig, 2007). Therefore, mutants with defects in JA production or signalling such as *coi1* (Feys *et al.*, 1994) or in camalexin production, like the *pad3* mutant (Thomma *et al.*, 1999), revealed to be highly sensitive towards *A. brassicicola*. In contrast, *A. thaliana Col*-0 plants are resistant to the fungus. MKK2EE plants showed an increased sensitivity against the fungus (Brader *et al.*, 2007). In infection assays with *raf36* plants, we found an increased sensitivity also in theses plants to *A. brassicicola*. The sensitivity of both plant lines was not caused by a lack in camalexin production, since no aberrant camalexin levels could be detected after fungal infection. For this reason, the decreased JA induction observed in both lines after *A. brassicicola* infection probably accounts for the higher sensitivity to the fungus. The sensitivity of *raf36* plants was less severe than that observed for MKK2EE plants. The less pronounced sensitivity of *raf36* plants *to A. brassicicola* infection could be caused by putative functional redundancy of Raf36 with other MAPKKKs.

In contrast to these results, it was shown that *mpk4* plants were highly susceptible to *A. brassicicola* and impaired in JA induction. This JA suppression was also observed in *mpk4* plants expressing the SA hydrolase NahG, in which the high SA levels were abolished by the the SA metabolizing enzyme. Therefore, the repression of the JA response was not a secondary effect of the high SA accumulation in *mpk4* plants *(Brodersen et al., 2006).* Impaired JA induction was also detected in MKK2EE plants, which featured constitutively high MPK4 activity (Brader *et al.*, 2007). In case that MPK4 was an MKK2 downstream target in *Alternaria brassicicola* induced defence responses, elevated JA levels would have been expected in *mpk4* plants. The hypothesis of a Raf36-MKK2-MPK4 module in *A.brassicicola* defence response seems therefore improbable. Another MPK would be expected downstream of the *raf36*-MKK2 module. A good candidate would be MPK6, which is a downstream target of MKK2 in abiotic stress signalling. In addition, *mpk6* plants showed an increased *P. syringae* sensitivity (Menke *et al.*, 2004). To be able to position Raf36 upstream of MKK2 and MPK6, it would be necessary to assess MPK6 activity in *raf36* and MKK2EE plants after infection with the fungus, but i*n vitro* kinase assays did not deliver reproducible results. Assuming that the variability detected in the MPK6 activity was due to the variability in the infection process by the fungus, this problem might be solved by using fungal elicitors for stimulating the plant defence response. The drawback of a synthetic fungal elicitor is, however, that it might not initiate exactly the same responses as the fungus itself. Moreover, the plant defence response seems to differ for different necrotrophic pathogens. Up to now, *Alternaria brassicicola* resistance had just been connected to JA signalling and camalexin production, *Botrytis cinerea (B.cinerea)* resistance additionally involves ethylene signalling (Glazebrook, 2005), which has not been assessed for its significance in RAF36 signaling.

4.9 Transcriptome analysis supports common Raf36 and MKK2 functions

Transcriptome data were obtained from untreated *raf36* and MKK2EE plants, as well as from both plant lines 1day after *Alternaria brassicicola* infection. Comparison of these data sets revealed an overlap of differentially regulated genes in both untreated plant lines. Five genes were found commonly upregulated in the untreated *raf36* and MKK2 plants. Two of these five genes were coding for chitin responsive transcription factors, RAV2 (Libault *et al.*, 2007) and WRKY53. WRKY53 gene expression is known to be downregulated by JA and

ethylene and induced by SA. Moreover, WRKY53 is involved in the response to *P.syringae* infection, together with the nematode resistance protein-like AtHSPRO2 (Murray *et al.*, 2007). The AtHSPRO2 gene, whose expression is also downregulated by JA, was found to be upregulated in both mutant lines 1day after *A.brassicicola* infection. Among the 12 commonly downregulated genes in untreated *raf36* and MKK2EE plants were the JA-responsive TAT3 gene, an aminotransferase (Chico *et al.*, 2008), and genes that were classified as abiotic stress responsive genes, such as the drought and salt responsive transcription factor ATHB-12 (Shin *et al.*, 2004) and the salt responsive ATCP-1, a calcium-binding protein. These findings would suggest a role for Raf36 not just in MKK2-mediated biotic stress responses, but also in abiotic stress responses. The overlap in the set of genes with altered expression in *raf36* and MKK2EE plants became even more obvious after *Alternaria brassicicola* treatment. 154 genes were differentially upregulated in both plant lines, which corresponded to nearly 50% of the differentially regulated genes in the *raf36* line and up to 80% of the differentially regulated genes in the MKK2EE line. Biotic and abiotic stress functions could be ascribed to a third of theses genes. PAD2, which is an enzyme involved in camalexin synthesis in response to pathogen attack, as well as some other fungus responsive genes were overexpressed. This seems to contradict the fungal sensitive phenotype of both plant lines but might hint to a general deregulation of important components of the fungal defence.

The group of commonly downregulated genes after *A. brassicicola* infection contained, besides TAT3, the JA responsive Lox3 lipoxygenase and the WRKY46 transcription factor, which is chitin responsive. Two ethylene responsive transcription factors were found in that group, pointing to a downregulated ethylene response. JA and ethylene are exhibiting synergistic effects in response to a range of pathogens (Glazebrook, 2005). Considering the impaired JA induction upon *A. brassicicola* infection in both plant lines it would therefore not be surprising to detect also an impaired ethylene response in the mutants.

To summarize, the overlap in the differentially regulated genes in *raf36* and MKK2EE plants strongly supports a connection between Raf36 and MKK2 functions in *Alternaria brassicicola* induced defence signalling.

4.10 No rescue of the *raf36* growth phenotype and the *A.brassicicola* sensitivity in *raf36 mkk2* double mutants

Assuming that Raf36 is an upstream regulator of MKK2 activity, *raf36 mkk2* double mutants would be expected to exhibit the same phenotype as *mkk2* mutants, a wild type like growth and resistance to *Alternaria brassicicola.*

However, the generated homozygous *raf36 mkk2* double mutants were morphologically similar to *raf36* single knockouts. Examination of the *Alternaria brassicicola* sensitivity revealed no change in the sensitivity compared to *raf36.*

To explain this unexpected double mutant phenotype, two scenarios are probable. Either another MKK is overtaking the MKK2 tasks in the *raf36 mkk2* mutant, or the Raf36 effect on *A. brassicicola* resistance is mediated by another MKK than MKK2. The most probable candidate for redundancy with MKK2 would be the closest homologue MKK1. MKK1 was already shown to be partially redundant to MKK2 in SA-mediated defence responses (Gao *et al.*, 2008; Qiu *et al.*, 2008). Assuming that MKK1 would be able to functionally substitute for MKK2 during *A.brassicicola* infection, in *raf36 mkk2* double mutant plants a higher MKK1 activity would be expected since Raf36 is suggested to act as a negative upstream regulator of MKK2. However this possibility requires the ability of Raf36 to interact with MKK1, which was not detected in Y2H assays.

In contrast, MKK7 was found to interact with Raf36. MKK7 was first identified as a negative regulator of polar auxin transport (Dai *et al.*, 2006). MKK7 overexpression in plants led to accumulation of SA and PR gene induction, while reduction of MKK7 mRNA levels by antisense expression was compromising SA mediated resistance to *P.syringae* (Zhang *et al.*, 2007). Therefore MKK7 seems to be a positive regulator of the SA pathway in immune responses. Up to now, no information about an involvement of MKK7 in JA signalling is available. Due to the antagonistic effects of SA and JA under biotic stress conditions, a decreased JA induction after pathogen infection could be expected in MKK7 overexpressing plants. In addition, the transcriptome profile of *raf36* plants showed that a few auxin-induced genes are differentially regulated in the *raf36* knockouts (Table 4.1). Interestingly, none of theses genes except from ATAPP1 was found to be differentially regulated in the transcriptome data of the MKK2EE plants, which supports the idea of an MKK2 independent Raf36 function. To test whether MKK7 is also a Raf36 downstream target in *A. brassicicola* induced stress signalling, it would be necessary to generate *raf36 mkk7* as well as *raf36 mkk2 mkk7* plants and examine their *A. brassicicola* sensitivity.

raf36 plants untreated		Rat
AT5G54490	PBP1 (PINOID-BINDING PROTEIN 1)	0,72
AT1G56150	unknown protein_ auxin-responsive family protein	0,71
raf36 plants 1 day after *A.brassicicola* infection		
AT3G04730	IAA16; indoleacetic acid-induced protein 16	0,95
AT1G23080	PIN7 (PIN-FORMED 7)	0,82
AT3G07390	AIR12; auxin-induced protein	0,96
AT5G62000	ARF2 (AUXIN RESPONSE FACTOR 2)	0,80
AT4G36760	ATAPP1_ aminopeptidase P	0,77

Table 4.1:
Auxin-reponsive genes upregulated in *raf36* plants; the 'Rat' (ratio) = Log2 (signal intensity *in raf36-1*) - Log2 (signal intensity in *wild type*)

4.11 Conclusions

Raf36 was identified as an active kinase with the capacity to interact with MKK2 *in vitro*. Furthermore it was shown that the amino-terminal truncations of Raf36 were able to phosphorylate MKK2 *in vitro* and reduced the MKK2 activity on the substrate MPK4. These *in vitro* results imply that Raf36 can regulate the MKK2 activity.

In the second part of this work, Raf36 function was examined *in planta*, using *raf36* knockout plants. MKK2 had been connected to salt and cold stress signalling (Teige *et al.*, 2004), and its signalling upon pathogen infection (Brader *et al.*, 2007; Gao *et al.*, 2008; Qiu *et al.*, 2008). Therefore, at first the MPK4 and MPK6 activity in *raf36* plants was tested after cold treatment. The activities of both MPKs were unchanged in the *raf36* plants, which led to the conclusion that Raf36 is not part of the MKK2-mediated cold signalling pathway. Assessment of a role of Raf36 in salt stress signalling led to inconclusive results, since one of the two *raf36* knockout alleles exhibited no salt sensitivity such as *mkk2*. The role of Raf36 in biotic stress signalling was analysed in pathogen infection assays with *raf36* plants. Two types of pathogens were applied, the biotrophic bacterium *P. syringae* and the necrotrophic fungus *Alternaria brassicicola*. No changes in sensitivity towards the biotrophic pathogen *P.syringae* could be detected in *raf36* plants.

However *raf36* plants as well as MKK2EE overexpressing plants exhibited enhanced sensitivity towards *Alternaria brassicicola*. These findings fit well to the *in vitro* data showing inhibition of MKK2 activity by Raf36. Assuming that Raf36 acts as an inhibitor of MKK2 activity also *in vivo*, *raf36* plants should have enhanced MKK2 activity and show the same phenotype as MKK2EE plants. Knowing that *mpk4* plants exhibit an increased *A. brassicicola* sensitivity as well, it is probable that another MPK, maybe MPK6, is acting downstream of MKK2 in the fungal induced signalling pathway.

No differences in the production of camalexin were measured between *raf36*, MKK2EE and wild type plants. On the other hand, JA induction in both lines was reduced which points to a defect in JA signalling in both mutants. Transcriptome analysis supports the theory that Raf36 acts upstream of MKK2 in *A. brassicicola* triggered defence responses by revealing a large overlap in differentially regulated genes in both lines after infection with the fungus. Among the commonly up- or downregulated genes are found a number of biotic stress and JA induced genes. In addition, a variety of abiotic stress genes were differentially regulated in both mutant lines, suggesting a role for Raf36 also in MKK2-mediated responses to salt and cold stress.

The analysis of *raf36 mkk2* mutants, which were phenotypically similar to *raf36* single mutants, led to the conclusion that another MKK could be redundant to MKK2. In the emerging model, *raf36* would act as a negative regulator of MKK2 upon *A. brassicicola* infection. In parallel, Raf36 would also be regulating the activity of another MKK, maybe MKK7, which was found to interact with Raf36 in Y2H assays. As mentioned earlier, MKK7 was shown to have a positive regulatory function in SA signalling (Zhang *et al.*, 2007). In contrast, MKK2 was shown to act in a pathway negatively regulating SA (Gao *et al.*, 2008; Qiu *et al.*, 2008), together with MPK4. Since the *A. thaliana* genome encodes more then 60 MAPKKKs and only 10 MKKs, it seems likely that a single MKK could be regulated by more than one MAPKKK. Therefore, both MEKK1 and Raf36 could alter MKK2 activity and be able to trigger divergent immune responses upon pathogen infection. The interplay between these different signalling modules would then shape the specificity of the *A. brassicicola* infection response.

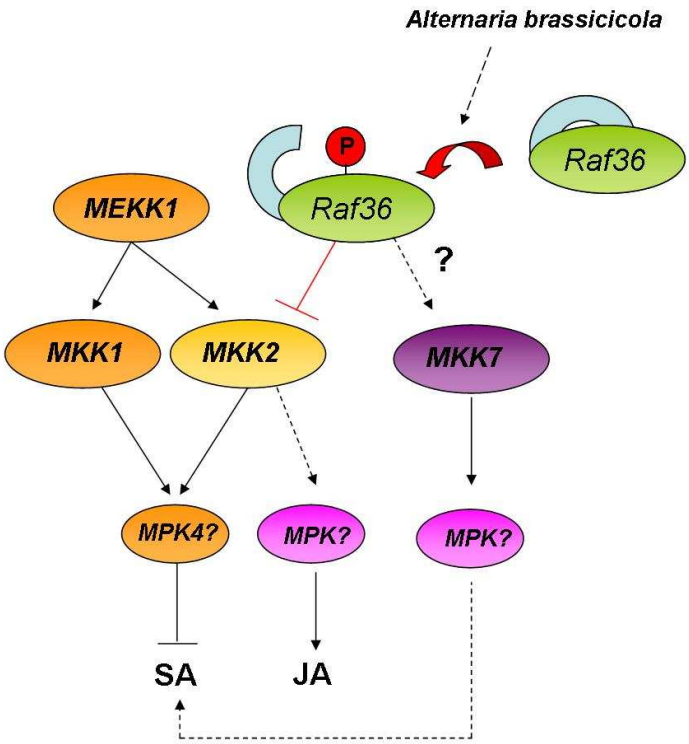


Fig.4.1:

Working model for the role of the MAPKKK Raf36 in *Alternaria brassicicola* response. Raf36 was shown to interact with MKK2 *in vitro*. Upon deletion of a putative inhibitory domain in its amino-terminus Raf36 could phosphorylate MKK2 *in vitro* and inhibit its activity. Interaction of Raf36 with MKK7 was also found in the Y2H approach. MKK7 was found to be a positive regulator of SA signalling, however the downstream MPK is unknown, as well as the MPK downstream from MKK2 in *Alternaria brassicicola* induced stress signalling. The MEKK1-MKK1/2-MPK4 pathway is negatively regulating SA responses.

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Appendix

Transcriptome analysis /(CATMA arrays) from *raf36-1* and MKK2EE plants 1 day after *A.brassicicola* infection

Expression levels are quantified as log2 values of the measured intensity. The 'Rat' (ratio) given in Table A1 is the difference between expression levels in raf36-1 or MKK2EE and expression levels in the WT, calculated as Log2 (intensity *in raf36-1*/ MKK2EE) - Log2(intensity in *wild type*)

Table A.1: Overlap in upregulated gene expression patterns

Gene	function	Rat *raf36-1*	Rat MKK2EE
AT4G04610	APR1 (PAPS REDUCTASE HOMOLOG 19)	2,74	1,77
AT3G02470	SAMDC (S-ADENOSYLMETHIONINE DECARBOXYLASE)	2,71	1,93
AT3G24503	ALDH2C4 (REDUCED EPIDERMAL FLUORESCENCE1); aldehyde dehydrogenase (ALDH1a)	2,5	1,9
AT4G35630	PSAT; phosphoserine transaminase	2,32	1,48
AT5G42020	BIP; luminal binding protein 2 (BiP-2)	2,14	1,66
AT5G05730	ASA1 (ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1)	2,12	0,93
AT2G31460	unknown protein	2,03	1,51
AT3G18290	EMB2454; ubiquitin-protein ligase	1,94	1,34
AT4G36220	FAH1 (FERULATE-5-HYDROXYLASE 1); cytochrome P450 84A1 (CYP84A1)	1,87	1,22
AT2G16500	ADC1_ arginine decarboxylase 1 (SPE1) (ARGDC)	1,86	1,38
AT3G09440	ATP binding_ heat shock cognate 70 kDa protein 3 (HSC70-3) (HSP70-3)	1,79	1,09
AT5G04590	SIR; sulfite reductase (ferredoxin)	1,76	1,11
AT1G50480	THFS (10-FORMYLTETRAHYDROFOLATE SYNTHETASE)	1,73	1,22
AT4G15530	kinase	1,73	0,97
AT2G24180	CYP71B6 (CYTOCHROME P450 71B6)	1,71	0,84
AT4G01050	unknown protein_ hydroxyproline-rich glycoprotein family protein	1,61	1,34
AT1G15125	S-adenosylmethionine-dependent methyltransferase	1,53	0,83
AT4G36990	HSF4 (HEAT SHOCK FACTOR 4)	1,5	0,65
AT5G13750	tetracycline:hydrogen antiporter/ transporter_ transporter-related	1,45	1,01
AT2G27050	EIL1 (ETHYLENE-INSENSITIVE3-LIKE 1)	1,45	1,14
AT1G59870	ATPase_ ABC transporter family protein	1,44	1,31
AT3G27770	unknown protein	1,44	1,17
AT5G19220	ADG2 (ADPG PYROPHOSPHORYLASE 2); glucose-1-phosphate adenylyltransferase large subunit 1 (APL1) / ADP-glucose pyrophosphorylase (ADG2)	1,44	1,21
AT2G45220	pectinesterase	1,43	0,84
AT2G16660	unknown protein_ nodulin family protein	1,42	1,16
AT3G02090	MPPBETA; metalloendopeptidase	1,4	0,82
AT3G18060	nucleotide binding_ transducin family protein / WD-40 repeat family protein	1,36	0,96
AT4G29900	ACA10; calcium-transporting ATPase	1,35	1,21

AT2G29670	unknown protein	1,35	1,06
AT5G13750	tetracycline:hydrogen antiporter	1,35	0,7
AT5G17860	calcium:sodium antiporter (CAX7)	1,34	0,72
AT5G26360	chaperonin, putative	1,34	0,81
AT5G48300	ADG1 (ADP GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1); glucose-1-phosphate adenylyltransferase small subunit 1 (APS1) / ADP-glucose pyrophosphorylase (ADG1)	1,32	0,75
AT1G08650	PPCK1 (PHOSPHOENOLPYRUVATE CARBOXYLASE KINASE)	1,32	0,91
AT2G16280	acyltransferase	1,31	0,9
AT1G37130	NIA2 (NITRATE REDUCTASE 2)	1,29	1,17
AT3G60750	transketolase	1,29	1,14
AT1G19660	unknown protein_ wound-responsive family protein	1,29	0,85
AT1G30090	unknown protein	1,28	0,78
AT1G24100	UDP-glycosyltransferase	1,28	0,91
AT3G49990	unknown protein	1,27	0,96
AT5G64820	unknown protein	1,26	1,1
AT1G74960	FAB1 (FATTY ACID BIOSYNTHESIS 1)	1,25	0,97
AT5G61160	AACT1; transferase	1,24	0,73
AT4G32020	unknown protein	1,23	0,75
AT2G29630	thiamine biosynthesis family protein	1,23	0,8
AT3G01470	ATHB-1; homeobox-leucine zipper protein 5 (HAT5) / HD-ZIP protein (HB-1)	1,23	0,85
AT5G42090	unknown protein	1,22	0,95
AT1G26580	unknown protein	1,22	1,15
AT5G40450	unknown protein	1,22	1,29
AT1G31970	ATP-dependent helicase	1,22	0,77
AT2G40000	unknown protein	1,21	1,03
AT2G29980	FAD3 (FATTY ACID DESATURASE 3)	1,21	0,81
AT3G19260	LAG1 HOMOLOG 2 (LONGEVITY ASSURANCE GENE1 HOMOLOG 2)	1,2	0,87
AT3G48000	ALDH2B4 (ALDEHYDE DEHYDROGENASE 2); aldehyde dehydrogenase (ALDH2)	1,19	0,95
AT1G74100	sulfotransferase	1,19	0,83
AT5G53450	ORG1; kinase	1,19	0,78
AT2G42100	structural constituent of cytoskeleton_ actin, putative	1,18	0,81
AT4G04640	ATPC1_ ATP synthase gamma chain 1	1,18	1,06
AT5G08530	FMN binding / NADH-ubiquinone oxidoreductase 51 kDa subunit	1,17	0,88
AT4G23100	RML1 (ROOT MERISTEMLESS 1)/ gamma-glutamylcysteine synthetase (GSH1)	1,17	0,74
AT2G30440	serine-type peptidase	1,17	0,86
AT5G15640	mitochondrial substrate carrier family protein	1,17	0,82
AT5G49720	KOR1 (KORRIGAN); hydrolase)	1,16	0,75
AT1G76180	ERD14 (EARLY RESPONSE TO DEHYDRATION 14)	1,15	0,98
AT3G26670	unknown protein	1,15	0,83
AT3G46970	ATPHS2; phosphorylase	1,15	0,97
AT1G18070	GTP binding / translation release factor	1,13	0,93
AT5G60410	ATSIZ1; DNA-binding family protein	1,12	1,03

AT2G39730	RCA (RUBISCO ACTIVASE)	1,12	1,1
AT3G10060	FK506 binding / peptidyl-prolyl cis-trans isomerase	1,11	0,79
AT1G31660	unknown protein_ bystin family	1,1	0,78
AT5G63980	SAL1; 3prim(2prim),5prim-bisphosphate nucleotidase / FIERY1 protein (FRY1)	1,1	0,67
AT5G19990	ATSUG1; ATPase_ 26S proteasome AAA-ATPase subunit (RPT6a)	1,1	0,79
AT1G03740	protein kinase	1,1	0,78
AT1G13260	RAV1; transcription factor	1,1	0,76
AT4G21790	TOM1 (TOBAMOVIRUS MULTIPLICATION 1)	1,09	0,68
AT5G13740	carbohydrate transporter	1,08	0,71
AT3G33002	unknown protein_ pseudogene, ribosomal protein S2p family	1,08	0,93
AT2G24820	oxidoreductase_ Rieske (2Fe-2S) domain-containing protein	1,07	0,75
AT5G08650	GTP-binding protein LepA, putative	1,07	1,08
AT3G19553	cationic amino acid transporter	1,05	0,82
AT4G34290	unknown protein_ SWIB complex BAF60b domain-containing protein	1,05	0,76
AT3G13460	unknown protein	1,05	0,79
AT1G78680	gamma-glutamyl hydrolase (GGH1)	1,05	0,75
AT5G43940	formaldehyde dehydrogenase (glutathione)_ alcohol dehydrogenase class III / GSH-FDH (ADHIII)	1,05	0,73
AT1G21630	calcium ion binding	1,04	0,97
AT3G56050	protein kinase	1,04	0,95
AT2G36380	ATPase _ ABC transporter family protein	1,03	0,7
AT1G12800	RNA binding_ S1 RNA-binding domain-containing protein	1,03	0,93
AT1G14170	nucleic acid binding_ KH domain-containing protein	1,03	0,87
AT1G24510	T-complex protein 1 epsilon subunit, putative	1,03	0,79
AT5G35630	GS2 (GLUTAMINE SYNTHETASE 2)	1,02	0,95
AT1G54990	unknown protein	1,02	0,66
AT5G03900	unknown protein	1,01	0,78
AT1G72150	PATL1 (PATELLIN 1; SEC14 cytosolic factor family protein	1,01	0,8
AT2G32240	unknown protein	1	0,98
AT3G07810	heterogeneous nuclear ribonucleoprotein, putative / hnRNP, putative	0,99	0,67
AT1G58360	AAP1; amino acid permease	0,99	0,67
AT1G80380	phosphoribulokinase	0,99	0,8
AT2G40330	unknown protein_ Bet v I allergen family protein	0,99	1,1
AT1G65960	GAD2 (GLUTAMATE DECARBOXYLASE 2	0,98	0,96
AT5G14120	unknown protein_ nodulin family protein	0,98	0,89
AT4G12290	copper amine oxidase, putative	0,98	0,69
AT5G57630	CIPK21 (CBL-interacting protein kinase 21)	0,97	0,87
AT4G00370	ANTR2; organic anion transporter	0,97	0,75
AT1G20840	carbohydrate transporter	0,97	0,67
AT2G14910	unknown protein	0,97	0,76
AT4G32410	CESA1 (CELLULASE SYNTHASE 1)	0,97	0,66
AT2G40800	unknown protein	0,94	0,74
AT2G31410	unknown protein	0,94	0,74

AT5G05170	CESA3 (CELLULASE SYNTHASE 3)	0,93	1,02
AT2G37220	RNA-binding protein cp29, putative	0,93	0,74
AT1G49670	oxidoreductase	0,93	0,72
AT5G58330	malate dehydrogenase, putative	0,92	0,66
AT5G21160	unknown protein	0,92	0,77
AT3G09020	alpha 1,4-glycosyltransferase family protein	0,92	0,86
AT1G22570	proton-dependent oligopeptide transport (POT) family protein	0,91	0,84
AT5G42870	lipin family protein	0,91	0,74
AT5G17910	unknown protein	0,91	0,87
AT4G11790	unknown protein	0,9	0,65
AT2G35050	protein kinase	0,9	0,7
AT1G16880	unknown protein_ uridylyltransferase-related	0,88	0,73
AT3G57290	EIF3E_ eukaryotic translation initiation factor 3E / eIF3e (TIF3E1)	0,87	0,74
AT2G03890	inositol or phosphatidylinositol kinase	0,87	0,66
AT2G23390	unknown protein	0,86	0,82
AT1G08930	ERD6; early-responsive to dehydration stress protein/ sugar transporter	0,86	0,66
AT3G07180	unknown protein_ GPI transamidase component PIG-S-related	0,85	0,66
AT1G20020	oxidoreductase	0,84	0,87
AT1G27980	carboxy-lyase	0,84	0,65
AT1G68550	AP2 domain-containing transcription factor, putative	0,82	0,68
AT2G37480	unknown protein	0,82	0,67
AT1G42970	GAPB (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE B SUBUNIT)	0,81	0,8
AT1G09620	tRNA synthetase class I (I, L, M and V) family protein	0,81	0,94
AT2G45290	transketolase	0,81	0,75
AT3G16000	MFP1 (MAR BINDING FILAMENT-LIKE PROTEIN 1)	0,81	0,81
AT3G23700	S1 RNA-binding domain-containing protein	0,8	0,73
AT1G07280	unknown protein	0,79	0,69
AT3G52200	LTA3; acyltransferase	0,78	0,66
AT4G36760	ATAPP1_ aminopeptidase P	0,77	0,87
AT4G13770	CYP83A1 (CYTOCHROME P450 83A1)	0,77	0,8
AT3G14620	CYP72A8; heme binding monooxygenase	0,77	0,75
AT2G15560	unknown protein	0,76	0,67
AT5G17630	glucose transporter	0,76	0,66
AT5G61010	exocyst subunit EXO70 family protein	0,76	0,78
AT1G52780	unknown protein	0,74	0,65
AT3G06550	unknown protein_ O-acetyltransferase-related	0,74	0,67
AT5G55630	KCO1; calcium-activated potassium channel	0,73	0,68
AT1G52870	unknown protein_ peroxisomal membrane protein-related	0,73	0,65
AT4G39780	AP2 domain-containing transcription factor, putative	0,72	0,75
AT4G08593	unknown protein	0,71	0,64

Table A.2: Overlap in downregulated gene expression patterns

gene	function	Rat raf36-1	Rat MKK2EE
AT5G63160	transcription regulator_ speckle-type POZ protein-related	-1,29	-0,82
AT3G51920	CAM9 (CALMODULIN 9)	-1,18	-0,65
AT2G28190	CSD2 (COPPER/ZINC SUPEROXIDE DISMUTASE 2)	-1,00	-0,67
AT3G23230	ethylene-responsive factor, putative	-0,95	-0,79
AT5G03210	unknown protein	-0,93	-0,86
AT5G10695	unknown protein unknown protein	-0,93	-0,74
AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3)	-0,90	-0,92
AT1G65390	ATPP2-A5; disease resistance protein (TIR class), putative	-0,87	-0,72
AT2G46400	WRKY46; transcription factor	-0,86	-0,66
AT4G32480	unknown protein	-0,86	-0,77
AT1G72520	lipoxygenase, putative	-0,85	-0,95
AT2G44840	ATERF13; ethylene-responsive element-binding protein	-0,83	-0,89
AT1G07135	unknown protein	-0,82	-0,77
AT3G22840	ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN9	-0,82	-0,82
AT3G23920	beta-amylase	-0,80	-0,94
AT3G19030	unknown protein	-0,79	-0,66
AT2G42530	cold-regulated protein (cor15b)	-0,78	-1,04
AT1G05670	UDP-glucoronosyl/ transferase	-0,76	-0,80
AT1G17420	LOX3; lipoxygenase	-0,76	-0,82
AT3G15520	peptidyl-prolyl cis-trans isomerase	-0,75	-0,70
AT2G22870	EMB2001	-0,73	-0,68

TableA.3: Genes upregulated in 4-week-old *raf36* plants untreated

gene	function	Rat *raf36-1*
AT3G55980	transcription factor_ zinc finger (CCCH-type) family protein	2,01
AT3G48360	transcription regulator_ speckle-type POZ protein-related	1,95
AT1G80840	WRKY40; transcription factor	1,79
AT1G07135	unknown protein_ glycine-rich protein	1,64
AT5G45340	CYP707A3; monooxygenase	1,63
AT1G68840	RAV2; transcription factor	1,56
AT4G37260	MYB73; transcription factor HMA1	1,35
AT1G72520	lipoxygenase	1,18

AT1G49500	unknown protein	1,18
AT4G24380	unknown protein ubiquitin-protein ligase	1,17
AT1G76600	unknown protein	1,15
AT2G23810	unknown protein_ senescence-associated family protein	1,12
AT2G35930	ubiquitin-protein ligase_ U-box domain-containing protein	1,08
AT5G61590	transcription factor	1,08
AT5G06310	unknown protein NHL3	1,07
AT2G44500	unknown protein	1,07
AT1G18300	hydrolase_ MutT/nudix family protein	1,00
AT1G69890	unknown protein	1,00
AT2G40140	transcription factor_ zinc finger (CCCH-type) family protein	0,99
AT2G25735	unknown protein	0,98
AT1G23390	unknown protein_ kelch repeat-containing F-box family protein	0,97
AT1G56660	unknown protein	0,96
AT3G06070	unknown protein	0,96
AT3G50260	transcription factor	0,94
AT5G62670	ATPase	0,92
AT2G38470	WRKY33; transcription factor unknown protein	0,92
AT1G66090	disease resistance protein (TIR-NBS class)	0,90
AT4G23800	transcription factor WRKY53	0,87
AT2G30040	MAPKKK14; kinase	0,86
AT1G12610	DDF1; transcription factor	0,86
AT2G40000	unknown protein	0,85
AT3G59350	kinase	0,85
AT3G19030	unknown protein	0,83
AT3G29000	calcium ion binding	0,82
AT2G18700	ATTPS11; glycosyl transferase	0,80
AT2G42870	unknown protein	0,80
AT1G17420	LOX3; lipoxygenase	0,79
AT3G10190	calmodulin	0,79
AT2G26530	AR781	0,78
AT1G61890	antiporter	0,78
AT3G56400	WRKY70; transcription factor	0,77
AT5G15230	GASA4_ gibberellin-regulated protein 4 (GASA4)	0,77
AT1G19180	unknown protein	0,76
AT5G57630	CIPK21 (CBL-interacting protein kinase 21)	0,76

AT3G15450	unknown protein unknown protein	0,76
AT5G25280	unknown protein_ serine-rich protein-related	0,76
AT1G65390	ATPP2-A5; transmembrane receptor_ disease resistance protein (TIR class)	0,75
AT1G32920	unknown protein	0,75
AT1G74450	unknown protein	0,74
AT1G07040	unknown protein	0,74
AT1G66180	pepsin A_ aspartyl protease family protein	0,74
AT3G23250	myb family transcription factor (MYB15)	0,74
AT5G61600	transcription factor_ ethylene-responsive element-binding family	0,73
AT3G10040	transcription factor	0,73
AT5G47370	HAT2; homeobox-leucine zipper protein 2 (HAT2)	0,73
AT3G49530	ANAC062; transcription factor_ no apical meristem (NAM) family	0,73
AT1G80910	unknown protein J8; heat shock protein binding	0,73
AT4G17230	scarecrow-like transcription factor 13 (SCL13)	0,73
AT5G11070	unknown protein	0,72
AT4G27960	UBC9 (UBIQUITIN CONJUGATING ENZYME 9)	0,72
AT1G19770	ATPUP14; purine transporter	0,72
AT5G54490	PBP1 (PINOID-BINDING PROTEIN 1)	0,72
AT1G56150	unknown protein_ auxin-responsive family protein	0,71
AT5G09220	AAP2 (AMINO ACID PERMEASE 2)	0,71
AT4G08540	unknown protein	0,70
AT1G76650	calcium ion binding	0,69
AT1G20823	ubiquitin-protein ligase	0,69
AT2G47060	kinase	0,68
AT5G10695	unknown protein	0,67
AT3G50930	ATPase	0,66
AT1G27100	unknown protein	0,65
AT1G71030	ATMYBL2; transcription factor	0,65
AT1G70290	ATTPS8; transferase, _ trehalose-6-phosphate synthase	0,65
AT5G47220	ATERF-2; _ ethylene-responsive element-binding factor 2	0,64

Table A.4: Genes downregulated in 4-week-old untreated *raf36* plants

AT1G44000	unknown protein	-0,65
AT4G24190	SHD (SHEPHERD); clavata formation protein	-0,66
AT3G46970	ATPHS2; starch phosphorylase	-0,67

AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3)	-0,68
AT2G31040	unknown protein_ ATP synthase protein I -related	-0,68
AT5G08610	ATP-dependent helicase	-0,68
AT3G50970	XERO2_ dehydrin xero2 (XERO2) / low-temperature-induced protein LTI30	-0,69
AT3G61890	ATHB-12; homeobox-leucine zipper protein 12 (HB-12)	-0,70
AT1G75040	PR5 (PATHOGENESIS-RELATED GENE 5)	-0,70
AT2G21320	transcription factor	-0,75
AT5G49480	ATCP1; sodium-inducible calcium-binding protein (ACP1)	-0,76
AT4G26850	VTC2 (VITAMIN C DEFECTIVE 2)	-0,77
AT1G66100	toxin receptor binding	-0,77
AT4G38840	unknown protein_ auxin-responsive protein	-0,78
AT3G22840	ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN)	-0,85
AT1G62180	APR2 (5primADENYLYLPHOSPHOSULFATE REDUCTASE 2)	-0,86
AT2G38240	unknown protein_ oxidoreductase, 2OG-Fe(II) oxygenase family protein	-0,87
AT4G17090	CT-BMY; beta-amylase	-0,89
AT3G57240	BG3 (BETA-1,3-GLUCANASE 3)	-0,89
AT1G14880	unknown protein	-0,92
AT3G44450	unknown protein	-0,94
AT5G54470	transcription factor/ zinc finger (B-box type) family protein	-0,97
AT4G33870	peroxidase	-0,97
AT2G44670	unknown protein_ senescence-associated protein-related	-1,00
AT5G42020	BIP; luminal binding protein 2 (BiP-2)	-1,04

Table A.5: Genes upregulated in 4-week-old *raf36* plants 1 day after *Alternaria brassicicola* infection

AT4G04610	APR1 (PAPS REDUCTASE HOMOLOG 19)	2,74
AT3G02470	SAMDC (S-ADENOSYLMETHIONINE DECARBOXYLASE)	2,71
AT3G24503	ALDH2C4 (REDUCED EPIDERMAL FLUORESCENCE1)	2,50
AT4G35630	PSAT; phosphoserine transaminase	2,32
AT5G42020	BIP-2 (ATP binding_ luminal binding protein 2)	2,14
AT5G05730	ASA1 (ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1)	2,12
AT2G31460	unknown protein	2,03
AT3G18290	EMB2454; ubiquitin-protein ligase	1,94
AT4G36220	FAH1 (FERULATE-5-HYDROXYLASE 1	1,87
AT2G16500	ADC1_ arginine decarboxylase 1 (SPE1)	1,86

AT3G09440	ATP binding_ heat shock cognate 70 kDa protein 3 (HSC70-3)	1,79
AT5G04590	SIR; sulfite reductase (ferredoxin)	1,76
AT1G50480	THFS (10-FORMYLTETRAHYDROFOLATE SYNTHETASE)	1,73
AT4G15530	transferase _ pyruvate phosphate dikinase family protein	1,73
AT2G24180	CYP71B6 (CYTOCHROME P450 71B6)	1,71
AT4G01050	unknown protein_ hydroxyproline-rich glycoprotein family protein	1,61
AT1G15125	S-adenosylmethionine-dependent methyltransferase	1,53
AT1G64720	CP5	1,51
AT4G36990	HSF4 (HEAT SHOCK FACTOR 4)	1,50
AT5G13750	tetracycline:hydrogen antiporter	1,45
AT2G27050	EIL1 (ETHYLENE-INSENSITIVE3-LIKE 1)	1,45
AT1G59870	ATPase, ABC transporter family protein	1,44
AT3G27770	unknown protein	1,44
AT5G19220	ADG2 (ADPG PYROPHOSPHORYLASE 2)	1,44
AT2G45220	pectinesterase	1,43
AT2G16660	unknown protein_ nodulin family protein	1,42
AT3G24503	ALDH2C4 (REDUCED EPIDERMAL FLUORESCENCE1)	1,42
AT1G51980	metalloendopeptidasee	1,40
AT3G02090	MPPBETA; metalloendopeptidase	1,40
AT5G14040	mitochondrial phosphate transporter	1,39
AT3G18060	transducin family protein / WD-40 repeat family protein	1,36
AT4G29900	ACA10; calcium-transporting ATPase	1,35
AT2G29670	unknown protein	1,35
AT5G13750	tetracycline:hydrogen antiporter	1,35
AT5G17860	calcium:sodium antiporter _ cation exchanger, putative (CAX7)	1,34
AT5G26360	chaperonin	1,34
AT5G48300	ADG1 (ADP GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1)	1,32
AT1G08650	PPCK1 (PHOSPHOENOLPYRUVATE CARBOXYLASE KINASE)	1,32
AT2G16280	acyltransferase	1,31
AT1G37130	NIA2 (NITRATE REDUCTASE 2)	1,29
AT3G60750	transketolase	1,29
AT1G19660	unknown protein_ wound-responsive family protein	1,29
AT1G30090	unknown protein_ kelch repeat-containing F-box family protein	1,28
AT1G24100	UDP-glycosyltransferase	1,28
AT3G49990	unknown protein	1,27

AT5G64820	unknown protein	1,26
AT1G74960	FAB1 (FATTY ACID BIOSYNTHESIS 1)	1,25
AT5G61160	AACT1; transferase	1,24
AT4G32020	unknown protein	1,23
AT2G29630	catalytic_ thiamine biosynthesis family protein	1,23
AT3G01470	ATHB-1; transcription factor_ homeobox-leucine zipper protein 5 (HAT5)	1,23
AT5G42090	unknown protein	1,22
AT1G26580	unknown protein	1,22
AT5G40450	unknown protein	1,22
AT1G31970	DEAD/DEAH box helicase, putative	1,22
AT5G46250	RNA recognition motif (RRM)-containing protein	1,22
AT2G40000	unknown protein	1,21
AT2G29980	FAD3 (FATTY ACID DESATURASE 3)	1,21
AT3G19260	LAG1 HOMOLOG 2 (LONGEVITY ASSURANCE GENE1 HOMOLOG 2)	1,20
AT3G48000	ALDH2B4 (ALDEHYDE DEHYDROGENASE 2)	1,19
AT1G74100	sulfotransferase	1,19
AT5G53450	ORG1; kinase	1,19
AT2G42100	structural constituent of cytoskeleton_ actin	1,18
AT4G04640	ATPC1_ ATP synthase gamma chain 1, chloroplast (ATPC1)	1,18
AT5G08530	FMN binding / NADH dehydrogenase (ubiquinone)	1,17
AT4G23100	RML1 (ROOT MERISTEMLESS 1)_ gamma-glutamylcysteine synthetase (GSH1)	1,17
AT5G35935	unknown protein_ copia-like retrotransposon family	1,17
AT2G30440	serine-type peptidase_ chloroplast thylakoidal processing peptidase	1,17
AT4G15760	oxidoreductase_ monooxygenase, putative (MO1)	1,17
AT1G70700	unknown protein	1,17
AT5G15640	binding_ mitochondrial substrate carrier family protein	1,17
AT5G49720	KOR1 (KORRIGAN)	1,16
AT1G19670	ATCLH1 (CORONATINE-INDUCED PROTEIN 1)	1,16
AT5G46760	transcription factor_ basic helix-loop-helix (bHLH) family protein	1,16
AT1G76180	ERD14 (EARLY RESPONSE TO DEHYDRATION 14)_ dehydrin (ERD14)	1,15
AT3G26670	unknown protein	1,15
AT3G46970	ATPHS2; phosphorylase/ transferase	1,15

AT1G18070	translation release factor_ EF-1-alpha-related GTP-binding protein	1,13
AT1G14920	GAI (GA INSENSITIVE)	1,13
AT5G60410	ATSIZ1; DNA-binding family protein	1,12
AT3G19260	LAG1 HOMOLOG 2 (LONGEVITY ASSURANCE GENE1 HOMOLOG 2)	1,12
AT2G39730	RCA (RUBISCO ACTIVASE)	1,12
AT1G26110	unknown protein	1,11
AT3G10060	FK506 binding / FKBP-type peptidyl-prolyl cis-trans isomerase	1,11
AT5G02500	HSC70-1; ATP binding_ heat shock cognate 70 kDa protein 1 (HSC70-1)	1,11
AT1G54100	ALDH7B4; aldehyde dehydrogenase	1,11
AT5G39050	transferase	1,10
AT1G31660	unknown protein_ bystin family	1,10
AT5G63980	SAL1; 3prim(2prim),5prim-bisphosphate nucleotidase	1,10
AT5G19990	ATSUG1; 26S proteasome AAA-ATPase subunit (RPT6a)	1,10
AT1G03740	kinase	1,10
AT1G13260	RAV1; transcription factor	1,10
AT4G21790	TOM1 (TOBAMOVIRUS MULTIPLICATION 1)	1,09
AT5G16150	carbohydrate transporter	1,09
AT4G38970	fructose-bisphosphate aldolase	1,09
AT5G13740	carbohydrate transporter	1,08
AT3G33002	unknown protein_ pseudogene, ribosomal protein S2p family	1,08
AT1G01140	CIPK9 (CBL-INTERACTING PROTEIN KINASE 9)	1,08
AT2G24820	oxidoreductase_ Rieske (2Fe-2S) domain-containing protein	1,07
AT3G30775	ERD5 (PROLINE OXIDASE)	1,07
AT5G08650	translation elongation factor_ GTP-binding protein LepA	1,07
AT3G54110	ATPUMP1 (UNCOUPLING PROTEIN PUMP2)	1,07
AT3G19553	cationic amino acid transporter	1,05
AT4G34290	unknown protein_ SWIB complex BAF60b domain-containing protein	1,05
AT3G13460	unknown protein	1,05
AT1G78680	gamma-glutamyl hydrolase (GGH1)	1,05
AT2G31430	pectinesterase	1,05
AT2G05710	RNA binding / aconitate hydratase	1,05
AT5G43940	formaldehyde dehydrogenase (glutathione)_ alcohol dehydrogenase class III / GSH-FDH (ADHIII)	1,05
AT1G68620	unknown protein	1,05

AT5G03610	carboxylic ester hydrolase	1,05
AT1G21630	calcium ion binding	1,04
AT3G56050	kinase	1,04
AT4G37520	peroxidase 50 (PER50) (P50) (PRXR2)	1,04
AT5G64460	unknown protein	1,04
AT4G38630	AT-MCB1 (MULTIUBIQUITIN CHAIN BINDING PROTEIN 1)	1,04
AT2G36380	ATPase, ABC transporter family protein	1,03
AT1G12800	S1 RNA-binding domain-containing protein	1,03
AT1G14170	nucleic acid binding_ KH domain-containing protein	1,03
AT1G22710	SUC2; carbohydrate transporter	1,03
AT1G24510	T-complex protein 1 epsilon subunit, TCP-1-epsilon	1,03
AT5G35630	GS2 (GLUTAMINE SYNTHETASE 2)	1,02
AT1G70330	ENT1, (EQUILIBRATIVE NUCLEOTIDE TRANSPORTER 1)	1,02
AT4G39840	unknown protein	1,02
AT1G54990	unknown protein	1,02
AT5G03900	unknown protein	1,01
AT1G72150	PATL1 (PATELLIN 1)	1,01
AT4G25640	antiporter/ drug transporter_ MATE efflux family protein	1,00
AT2G32240	unknown protein	1,00
AT1G77490	L-ascorbate peroxidase_thylakoid-bound (tAPX)	1,00
AT3G07810	RNA binding / heterogeneous nuclear ribonucleoprotein	0,99
AT3G10985	unknown protein_ wound-responsive protein-related	0,99
AT1G58360	AAP1; amino acid permease	0,99
AT1G80380	kinase_ phosphoribulokinase	0,99
AT1G80300	AATP1(ATP carrier protein 1)	0,99
AT2G40330	unknown protein_ Bet v I allergen family protein	0,99
AT1G65960	GAD2 (GLUTAMATE DECARBOXYLASE 2)	0,98
AT2G17420	NTRA_ thioredoxin reductase 2	0,98
AT5G14120	unknown protein_ nodulin family protein	0,98
AT2G34500	CYP710A1; monooxygenase	0,98
AT4G12290	copper ion binding_ copper amine oxidase	0,98
AT2G31660	protein transporter_ importin beta-2 subunit family protein	0,98
AT1G08510	FATB; acyl carrier_ acyl-(acyl carrier protein)	0,97
AT5G57630	CIPK21 (CBL-interacting protein kinase 21)	0,97
AT4G00370	ANTR2; organic anion transporter	0,97
AT1G20840	carbohydrate transporter	0,97

AT2G14910	unknown protein	0,97
AT4G32410	CESA1 (CELLULASE SYNTHASE 1)	0,97
AT3G07390	AIR12; auxin-induced protein (AIR12)	0,96
AT3G04730	IAA16; auxin-responsive protein	0,95
AT1G33610	protein binding_ leucine-rich repeat family protein	0,94
AT2G40800	unknown protein	0,94
AT2G31410	unknown protein	0,94
AT5G05170	CESA3 (CELLULASE SYNTHASE 3)	0,93
AT2G02180	TOM3_ tobamovirus multiplication protein 3 (TOM3)	0,93
AT2G37220	RNA binding / nucleic acid binding_ 29 kDa ribonucleoprotein	0,93
AT1G49670	oxidoreductase/ zinc ion binding_ ARP protein (REF)	0,93
AT1G13360	unknown protein	0,93
AT5G58330	malate dehydrogenase/ oxidoreductase (NADP)	0,92
AT5G21160	unknown protein_ La domain-containing protein	0,92
AT3G09020	glycosyltransferase sugar-binding DXD motif-containing protein	0,92
AT1G27760	unknown protein_ interferon-related developmental regulator family protein	0,92
AT1G48850	EMB1144; chorismate synthase	0,91
AT1G22570	transporter_ proton-dependent oligopeptide transport (POT) family protein	0,91
AT5G42870	unknown protein_ lipin family protein	0,91
AT3G59970	MTHFR1; methylenetetrahydrofolate reductase 1	0,91
AT2G31740	methyltransferase	0,91
AT5G17910	unknown protein	0,91
AT3G47620	transcription factor_ TCP family transcription factor	0,91
AT1G68490	unknown protein	0,91
AT4G11790	unknown protein_ Ran-binding protein 1 domain-containing protein	0,90
AT2G35050	ATP binding / serine/threonine kinase	0,90
AT1G01090	PDH-E1 ALPHA (PYRUVATE DEHYDROGENASE E1 ALPHA)	0,90
AT1G62430	ATCDS1; phosphatidate cytidylyltransferase	0,90
AT3G25890	AP2 domain-containing transcription factor	0,90
AT3G54110	ATPUMP1 (UNCOUPLING PROTEIN PUMP2)	0,89
AT5G44680	DNA-3-methyladenine glycosylase I_ methyladenine glycosylase family protein	0,89
AT1G16880	unknown protein_ uridylyltransferase-related	0,88
AT3G57290	EIF3E_ eukaryotic translation initiation factor 3E	0,87

AT4G14210	PDS3 (PHYTOENE DESATURASE)	0,87
AT2G03890	inositol or phosphatidylinositol kinase	0,87
AT1G54270	EIF4A-2; eukaryotic translation initiation factor 4A-2	0,86
AT2G23390	unknown protein	0,86
AT3G02350	transferase, glycosyl transferase family 8 protein	0,86
AT1G08930	ERD6; early-responsive to dehydration stress protein (ERD6)	0,86
AT4G08960	phosphatase activator	0,86
AT3G03440	unknown protein_ armadillo/beta-catenin repeat family protein	0,85
AT3G61440	ATCYSC1; L-3-cyanoalanine synthase	0,85
AT3G07180	unknown protein_ GPI transamidase component PIG-S-related	0,85
AT3G62770	unknown protein_ transport protein-related	0,84
AT4G21390	kinase	0,84
AT3G02360	phosphogluconate dehydrogenase (decarboxylating)	0,84
AT1G20020	oxidoreductase_ ferredoxin--NADP(+) reductase	0,84
AT1G27980	carboxy-lyase_ pyridoxal-dependent decarboxylase family protein	0,84
AT5G18290	transporter_ major intrinsic protein-related / MIP-related	0,84
AT5G28920	unknown protein	0,83
AT3G19490	ATNHD1; sodium:hydrogen antiporter	0,83
AT5G07440	GDH2 (GLUTAMATE DEHYDROGENASE 2	0,83
AT5G18490	unknown protein	0,83
AT5G02770	unknown protein	0,83
AT1G62570	disulfide oxidoreductase	0,83
AT3G02580	STE1 (STEROL 1); C-5 sterol desaturase_ delta 7-sterol-C5-desaturase	0,82
AT1G23080	PIN7 (PIN-FORMED 7)	0,82
AT1G68550	transcription factor	0,82
AT5G46110	APE2 (ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONMENT)	0,82
AT5G59160	TOPP2; protein serine/threonine phosphatase	0,82
AT2G37480	unknown protein glutamate N-acetyltransferase	0,82
AT1G42970	GAPB (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE B SUBUNIT)	0,81
AT4G05310	unknown protein_ ubiquitin family protein	0,81
AT1G09620	leucine-tRNA ligase _ tRNA synthetase class I (I, L, M and V) family protein	0,81
AT1G29355	unknown protein	0,81
AT2G45290	transketolase_ transketolase	0,81

AT3G16000	MFP1 (MAR BINDING FILAMENT-LIKE PROTEIN 1)	0,81
AT5G12890	UDP-glycosyltransferase	0,81
AT1G64650	unknown protein	0,81
AT5G67310	CYP81G1; monooxygenase	0,80
AT5G18480	Transferase_glycogenin glucosyltransferase (glycogenin)-related	0,80
AT3G07700	unknown protein_ ABC1 family protein	0,80
AT1G24160	unknown protein	0,80
AT3G14990	catalytic_ 4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein	0,80
AT3G23700	RNA binding_ S1 RNA-binding domain-containing protein	0,80
AT5G62000	ARF2 (AUXIN RESPONSE FACTOR 2)	0,80
AT4G36400	electron carrier_ FAD linked oxidase family protein	0,80
AT3G57890	unknown protein_ tubulin-specific chaperone C-related	0,80
AT5G28040	transcription regulator	0,79
AT3G07100	protein binding / protein transport protein Sec24	0,79
AT2G47940	DEGP2; serine-type peptidase	0,79
AT1G07280	unknown protein	0,79
AT5G02780	unknown protein_ In2-1 protein	0,79
AT3G55050	catalytic/ protein phosphatase type 2C	0,79
AT5G03420	unknown protein_ dentin sialophosphoprotein-related	0,79
AT3G60120	hydrolase, _ glycosyl hydrolase family 1 protein	0,78
AT3G52200	LTA3; acyltransferase	0,78
AT3G30390	amino acid permease	0,78
AT1G61350	unknown protein_ armadillo/beta-catenin repeat family protein	0,78
AT5G01710	unknown protein	0,78
AT5G55610	unknown protein	0,78
AT4G36760	ATAPP1_ aminopeptidase P	0,77
AT2G19480	nucleosome assembly protein (NAP)	0,77
AT1G48920	nucleic acid binding_ nucleolin	0,77
AT5G25340	unknown protein	0,77
AT4G13770	CYP83A1 (CYTOCHROME P450 83A1); oxygen binding	0,77
AT3G14620	CYP72A8; monooxygenase	0,77
AT5G23660	MTN3_ nodulin MtN3 family protein	0,77
AT3G49110	peroxidase 33 (PER33) (P33) (PRXCA)	0,77
AT2G31780	ubiquitin-protein ligase/ zinc finger (C3HC4-type RING finger) family protein	0,77

AT2G03760	ST; sulfotransferasee	0,77
AT1G78660	gamma-glutamyl hydrolase	0,76
AT2G15560	unknown protein	0,76
AT5G40610	glycerol-3-phosphate dehydrogenase (NAD+)	0,76
AT5G08560	nucleotide binding_ transducin family protein	0,76
AT5G53480	protein transporter_ importin beta-2	0,76
AT2G38170	CAX1; calcium:hydrogen antiporter	0,76
AT5G17630	antiporter/ glucose transporter unknown protein	0,76
AT5G61010	exocyst subunit EXO70 family protein	0,76
AT1G43690	unknown protein_ ubiquitin interaction motif-containing protein	0,76
AT1G72880	acid phosphatase survival protein SurE	0,76
AT4G01560	unknown protein_ brix domain-containing protein	0,75
AT3G15510	ATNAC2; transcription factor_ no apical meristem (NAM) family protein (NAC2)	0,75
AT3G02570	mannose-6-phosphate isomerase	0,75
AT2G25490	EBF1 (EIN3-BINDING F BOX PROTEIN 1)	0,75
AT1G78670	gamma-glutamyl hydrolase	0,75
AT5G16220	unknown protein_ octicosapeptide	0,75
AT4G30190	AHA2; ATPase_ ATPase 2, plasma membrane-type, putative	0,75
AT2G22330	CYP79B3; monooxygenase	0,74
AT5G60850	OBP4; transcription factor	0,74
AT3G13070	unknown protein_ CBS domain-containing protein	0,74
AT1G52780	unknown protein	0,74
AT1G60200	splicing factor PWI domain-containing protein	0,74
AT3G66654	peptidyl-prolyl cis-trans isomerase	0,74
AT3G06550	unknown protein_ O-acetyltransferase-related	0,74
AT1G31800	monooxygenase _ cytochrome P450 family protein	0,74
AT3G47800	aldose 1-epimerase hydrolase/ protein serine/threonine phosphatase	0,73
AT4G36790	carbohydrate transporter	0,73
AT5G55630	KCO1; calcium-activated potassium channel	0,73
AT1G52870	unknown protein_ peroxisomal membrane protein-related	0,73
AT2G43710	SSI2; acyl-[acyl-carrier protein] desaturase	0,73
AT1G01300	aspartic-type endopeptidase/ pepsin A	0,73
AT1G76990	ACR3_ ACT domain containing protein	0,73
AT2G41410	calmodulin	0,73
AT4G28610	PHR1 (PHOSPHATE STARVATION RESPONSE 1)	0,73

AT4G17615	CBL1 (CALCINEURIN B-LIKE PROTEIN 1)	0,72
AT2G25450	unknown protein_ 2-oxoglutarate-dependent dioxygenase	0,72
AT4G34710	ADC2 (ARGININE DECARBOXYLASE 2)	0,72
AT5G23280	transcription factor_ TCP family transcription factor	0,72
AT1G63840	ubiquitin-protein ligase/ zinc finger (C3HC4-type RING finger) family protein	0,72
AT1G53320	phosphoric diester hydrolase/ tubby family protein (TULP7)	0,72
AT5G64440	amidase	0,72
AT1G12050	fumarylacetoacetase	0,72
AT3G01500	CA1 (CARBONIC ANHYDRASE 1)	0,72
AT4G39780	transcription factor	0,72
AT5G65310	ATHB5_ homeobox-leucine zipper protein 5 (HB-5)	0,72
AT5G47210	nuclear RNA-binding protein	0,72
AT4G00720	shaggy-related protein kinase theta / ASK-theta (ASK8)	0,72
AT5G24690	unknown protein	0,71
AT4G23600	CORI3 (CORONATINE INDUCED 1)	0,71
AT1G50450	unknown protein	0,71
AT3G56200	amino acid permease	0,71
AT4G08593	unknown protein	0,71
AT1G12110	NRT1.1; transporter	0,71
AT3G10980	SAG20 (WOUND-INDUCED PROTEIN 12)	0,71
AT1G18500	2-isopropylmalate synthase	0,71
AT3G48560	CSR1 (CHLORSULFURON/IMIDAZOLINONE RESISTANT 1)	0,71

Table A.6: Genes downregulated in 4-week-old *raf36* plants 1 day after *Alternaria brassicicola* infection

AT1G15790	unknown protein	-0,71
AT1G76800	unknown protein_ nodulin	-0,71
AT3G29000	calcium ion binding	-0,71
AT1G03870	FLA9 (fasciclin-like arabinogalactan-protein 9)	-0,72
AT1G64970	G-TMT (GAMMA-TOCOPHEROL METHYLTRANSFERASE)	-0,72
AT4G17090	CT-BMY; beta-amylase	-0,72
AT2G22870	EMB2001; GTP binding unknown protein	-0,73

AT5G10380	ubiquitin-protein ligase	-0,73
AT4G02410	kinase_ lectin protein kinase family protein	-0,74
AT4G36670	carbohydrate transporter	-0,74
AT1G10522	unknown protein	-0,75
AT4G14450	ATBET12	-0,75
AT3G15520	peptidyl-prolyl cis-trans isomerase	-0,75
AT1G76960	unknown protein	-0,76
AT1G17420	LOX3; lipoxygenase	-0,76
AT1G10340	protein binding_ ankyrin repeat family protein	-0,76
AT5G51720	unknown protein	-0,76
AT1G75040	PR5 (PATHOGENESIS-RELATED GENE 5)	-0,76
AT3G55980	transcription factor_ zinc finger (CCCH-type) family protein	-0,76
AT1G05670	transferase	-0,76
AT3E22410	seen in ABF59441 (102 aa), glutaredoxin-like	-0,77
AT3G28540	nucleoside-triphosphatase	-0,77
AT3G57240	BG3 (BETA-1,3-GLUCANASE 3)	-0,78
AT2G42530	unknown protein_ cold-responsive protein (cor15b)	-0,78
AT3G19030	unknown protein	-0,79
AT1G30730	electron carrier_ FAD-binding domain-containing protein	-0,79
AT1G14880	unknown protein	-0,79
AT3G23920	beta-amylase	-0,80
AT4G12720	hydrolase_ MutT/nudix family protein	-0,80
AT3G50960	electron transporter	-0,81
AT1G65490	unknown protein	-0,81
AT3G22840	ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN)	-0,82
AT1G72060	serine-type endopeptidase inhibitor	-0,82
AT1G07135	unknown protein_ glycine-rich protein	-0,82
AT2G37460	unknown protein_ nodulin MtN21 family protein	-0,83
AT2G20670	unknown protein	-0,83
AT2G44840	ATERF13; ethylene-responsive element-binding protein	-0,83
AT5G26690	heavy-metal-associated domain-containing protein	-0,84
AT4G14365	ubiquitin-protein ligase	-0,84
AT2G24600	protein binding_ ankyrin repeat family protein	-0,84
AT4G32870	unknown protein	-0,85
AT1G72520	lipoxygenase	-0,85
AT1G33960	AIG1 (AVRRPT2-INDUCED GENE 1)	-0,86

AT4G32480	unknown protein	-0,86
AT5G58120	ATP binding / nucleoside-triphosphatase	-0,86
AT1G68440	unknown protein	-0,86
AT2G46400	WRKY46; transcription factor	-0,86
AT4G33870	peroxidase	-0,86
AT1G65390	ATPP2-A5; transmembrane receptor_ disease resistance protein (TIR class)	-0,87
AT2G38780	unknown protein	-0,87
AT1G58290	HEMA1; glutamyl-tRNA reductase	-0,88
AT4G11890	kinase	-0,89
AT5G55460	protease inhibitor/seed storage	-0,89
AT1G60950	FED A; electron transporter	-0,90
AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3)	-0,90
AT5G22520	unknown protein	-0,91
AT3G52430	PAD4 (PHYTOALEXIN DEFICIENT 4)	-0,92
AT5G01540	kinase	-0,92
AT5G24530	oxidoreductase, 2OG-Fe(II) oxygenase family protein	-0,92
AT5G10695	unknown protein	-0,93
AT5G03210	unknown protein	-0,93
AT3G23230	ethylene-responsive factor	-0,95
AT1G24145	unknown protein	-0,95
AT5G22690	ATP binding / transmembrane receptor_ disease resistance protein (TIR-NBS-LRR class)	-0,98
AT4G02200	unknown protein	-0,99
AT2G28190	CSD2 (COPPER/ZINC SUPEROXIDE DISMUTASE 2)	-1,00
AT2G32670	ATVAMP725 kinase	-1,02
AT1G77760	NIA1 (NITRATE REDUCTASE 1)	-1,03
AT2G24160	unknown protein_ pseudogene, leucine rich repeat protein family	-1,04
AT2G17040	ANAC036; transcription factor_ no apical meristem (NAM) family protein	-1,06
AT4G23800	transcription factor WRKY53; transcription factor	-1,07
AT1G72060	serine-type endopeptidase inhibitor	-1,10
AT3G51920	CAM9 (CALMODULIN 9)	-1,18
AT5G39670	calcium ion binding EMB2744	-1,22
AT5G63160	transcription regulator_ speckle-type POZ protein-related	-1,29
AT5G58950	kinase	-1,41
AT4G29905	unknown protein	-1,51

Table A.7: Genes upregulated in 4-week-old untreated *MKK2EE* plants

AT3G54290	unknown protein	1,49
AT3G48360	protein binding / transcription regulator_ speckle-type POZ protein-related	0,99
AT4G23800	transcription factor WRKY53; transcription factor	0,80
AT1G68840	RAV2; DNA-binding protein RAV2 (RAV2) / AP2 domain-containing protein RAP2.8	0,78
AT3G62550	unknown protein_ universal stress protein (USP) family protein	0,76
AT5G25370	PLDALPHA3 (PLD ZETA 1); phospholipase D_ phospholipase D, putative (PLDZETA)	0,67
AT4G23800	transcription factor WRKY53; transcription factor	0,67
AT3G15450	unknown protein unknown protein	0,67
AT2G25735	unknown protein	0,66

Table A.8: Genes downregulated in 4-week-old untreated *MKK2EE* plants

AT1G51090	metal ion binding_ heavy-metal-associated domain-containing protein	-0,66
AT1G66100	toxin receptor binding_ thionin, putative	-0,67
AT1G14780	unknown protein RDR1 (RNA-DEPENDENT RNA POLYMERASE 1)	-0,68
AT2G42530	unknown protein_ cold-responsive protein (cor15b)	-0,68
AT4G17470	palmitoyl-(protein) hydrolase_ palmitoyl protein thioesterase family protein	-0,69
AT3G21670	transporter_ nitrate transporter (NTP3)	-0,69
AT3G61890	ATHB-12; transcription factor_ homeobox-leucine zipper protein 12 (HB-12)	-0,70
AT5G61820	unknown protein	-0,70
AT3G21890	transcription factor/ zinc finger (B-box type) family protein	-0,73
AT4G23600	CORI3 (CORONATINE INDUCED 1); transaminase	-0,73
AT4G18440	adenylosuccinate lyase/ catalytic_ adenylosuccinate lyase, putative	-0,74
AT2G29350	SAG13; oxidoreductase_ tropinone reductase, putative	-0,74
AT2G31040	unknown protein_ ATP synthase protein I -related	-0,75
AT3G44450	unknown protein	-0,76
AT1G48100	polygalacturonase unknown protein	-0,76
AT5G63160	protein binding / transcription regulator_ speckle-type POZ protein-related	-0,79
AT4G01080	unknown protein	-0,85
AT3G50970	XERO2_ dehydrin xero2 (XERO2) / low-temperature-induced protein LTI30 (LTI30)	-0,87
AT5G24470	APRR5 (PSEUDO-RESPONSE REGULATOR 5); transcription regulator	-0,90
AT1G70700	unknown protein	-0,92

AT2G15020	unknown protein	-0,92
AT5G54470	transcription factor/ zinc finger (B-box type) family protein	-0,95
AT2G43510	ATTI1_ trypsin inhibitor, putative	-0,95
AT4G33870	peroxidase	-1,02
AT3G45140	LOX2 (LIPOXYGENASE 2)	-1,04
AT1G62180	APR2 (5primADENYLYLPHOSPHOSULFATE REDUCTASE 2)	-1,05
AT2G38240	unknown protein_ oxidoreductase, 2OG-Fe(II) oxygenase family protein	-1,12
AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3); transaminase_ aminotransferase, putative	-1,13
AT3G28220	unknown protein_ meprin and TRAF homology domain-containing protein	-1,13
AT2G33380	RD20 (RESPONSIVE TO DESSICATION 20/calcium-binding	-1,16
AT5G49480	ATCP1; sodium-inducible calcium-binding protein (ACP1)	-1,23
AT4G17090	CT-BMY; beta-amylase (CT-BMY)	-1,28
AT1G52400	BGL1 (BETA-GLUCOSIDASE HOMOLOG 1); hydrolase	-1,31
AT2G39030	N-acetyltransferase_ GCN5-related N-acetyltransferase (GNAT) family protein	-1,33

Table A.9: Genes upregulated in 4-week-old *MKK2EE* plants 1 day after *Alternaria bassicicola* infection

AT3G02470	SAMDC (S-ADENOSYLMETHIONINE DECARBOXYLASE)	1,93
AT3G24503	ALDH2C4 (REDUCED EPIDERMAL FLUORESCENCE1); aldehyde dehydrogenase	1,90
AT4G04610	APR1 (PAPS REDUCTASE HOMOLOG 19) (PRH19)	1,77
AT5G42020	BIP; ATP binding_ luminal binding protein 2	1,66
AT3G32990	unknown protein_ pseudogene, ATP synthase C subunit	1,56
AT3G54290	unknown protein	1,52
AT2G31460	unknown protein	1,51
AT4G35630	PSAT; phosphoserine transaminase	1,48
AT2G16500	ADC1_ arginine decarboxylase 1 (SPE1)	1,38
AT3G18290	EMB2454; protein binding / ubiquitin-protein ligase	1,34
AT4G01050	unknown protein_ hydroxyproline-rich glycoprotein family protein	1,34
AT1G59870	ATPase, ABC transporter family protein	1,31
AT5G40450	unknown protein	1,29
AT1G50480	THFS (10-FORMYLTETRAHYDROFOLATE SYNTHETASE); ATP binding	1,22
AT4G36220	FAH1 (FERULATE-5-HYDROXYLASE 1); ferulate 5-hydroxylase_ cytochrome P450 84A1 (CYP84A1)	1,22
AT5G19220	ADG2 (ADPG PYROPHOSPHORYLASE 2)	1,21

AT4G29900	ACA10; calcium-transporting ATPase	1,21
AT3G27770	unknown protein	1,17
AT1G37130	NIA2 (NITRATE REDUCTASE 2)	1,17
AT2G16660	unknown protein_ nodulin family protein	1,16
AT2G28630	acyltransferase_ beta-ketoacyl-CoA synthase family protein	1,15
AT1G26580	unknown protein	1,15
AT3G60750	transketolase	1,14
AT2G27050	EIL1 (ETHYLENE-INSENSITIVE3-LIKE 1); transcription factor	1,14
AT3G24503	ALDH2C4 (REDUCED EPIDERMAL FLUORESCENCE1); aldehyde dehydrogenase	1,12
AT5G04590	SIR; sulfite reductase (ferredoxin)	1,11
AT5G64820	unknown protein	1,10
AT2G40330	unknown protein_ Bet v I allergen family protein	1,10
AT2G39730	RCA (RUBISCO ACTIVASE)_ ribulose bisphosphate carboxylase	1,10
AT3G09440	heat shock cognate 70 kDa protein 3 (HSC70-3)	1,09
AT5G08650	GTP binding / translation elongation factor	1,08
AT4G04640	ATPC1_ ATP synthase gamma chain 1	1,06
AT2G29670	unknown protein	1,06
AT1G16720	oxidoreductase	1,05
AT2G40000	unknown protein	1,03
AT5G60410	ATSIZ1; DNA binding	1,03
AT5G05170	CESA3 (CELLULASE SYNTHASE 3); cellulose synthase, catalytic subunit (Ath-B)	1,02
AT5G13750	tetracycline:hydrogen antiporter	1,01
AT2G32240	unknown protein	0,98
AT1G76180	ERD14 (EARLY RESPONSE TO DEHYDRATION 14)	0,98
AT4G15530	kinase/ transferase _ pyruvate phosphate dikinase family protein	0,97
AT1G74960	FAB1 (FATTY ACID BIOSYNTHESIS 1); fatty-acid synthase	0,97
AT1G21630	calcium ion binding	0,97
AT3G46970	ATPHS2; starch phosphorylase	0,97
AT3G49990	unknown protein	0,96
AT1G65960	GAD2 (GLUTAMATE DECARBOXYLASE 2); calmodulin binding	0,96
AT3G18060	transducin family protein / WD-40 repeat family protein	0,96
AT5G42090	unknown protein	0,95
AT5G35630	GS2 (GLUTAMINE SYNTHETASE 2)	0,95
AT3G56050	kinase	0,95
AT3G48000	ALDH2B4 (ALDEHYDE DEHYDROGENASE 2)	0,95
AT1G09620	ATP binding / leucine-tRNA ligase	0,94

AT1G18070	GTP binding / translation factor	0,93
AT3G33002	unknown protein_ pseudogene, ribosomal protein S2p family	0,93
AT1G12800	S1 RNA-binding domain-containing protein	0,93
AT5G05730	ASA1 (ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1	0,93
AT1G08650	PPCK1 (PHOSPHOENOLPYRUVATE CARBOXYLASE KINASE	0,91
AT1G24100	UDP-glycosyltransferase	0,91
AT2G16280	acyltransferase_ very-long-chain fatty acid condensing enzyme	0,90
AT5G14120	unknown protein_ nodulin family protein	0,89
AT5G08530	FMN binding / NADH dehydrogenase (ubiquinone)	0,88
AT4G36760	ATAPP1_ aminopeptidase P	0,87
AT5G57630	CIPK21 (CBL-interacting protein kinase 21); kinase	0,87
AT3G19260	LAG1 HOMOLOG 2 (LONGEVITY ASSURANCE GENE1 HOMOLOG 2)	0,87
AT5G17910	unknown protein	0,87
AT1G14170	nucleic acid binding_ KH domain-containing protein	0,87
AT1G20020	oxidoreductase_ ferredoxin--NADP(+) reductase	0,87
AT2G30440	serine-type peptidase	0,86
AT3G09020	transferase/ alpha 1,4-glycosyltransferase family protein	0,86
AT3G01470	ATHB-1; transcription factor_ homeobox-leucine zipper protein 5 (HAT5)	0,85
AT1G19660	unknown protein_ wound-responsive family protein	0,85
AT2G45220	enzyme inhibitor/ pectinesterase	0,84
AT2G24180	CYP71B6 (CYTOCHROME P450 71B6); monooxygenase	0,84
AT1G22570	proton-dependent oligopeptide transport (POT) family protein	0,84
AT3G26670	unknown protein	0,83
AT1G72416	heat shock protein binding	0,83
AT1G15125	S-adenosylmethionine-dependent methyltransferase	0,83
AT1G74100	sulfotransferase	0,83
AT3G19553	cationic amino acid transporter	0,82
AT4G16660	ATP binding_ heat shock protein 70	0,82
AT3G02090	MPPBETA; metalloendopeptidase	0,82
AT2G23390	unknown protein	0,82
AT5G15640	mitochondrial substrate carrier family protein	0,82
AT2G42100	structural constituent of cytoskeleton	0,81
AT3G16000	MFP1 (MAR BINDING FILAMENT-LIKE PROTEIN 1)	0,81
AT2G29980	FAD3 (FATTY ACID DESATURASE 3)	0,81
AT5G26360	chaperonin, putative	0,81
AT1G42970	GAPB (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE B SUBUNIT	0,80

AT1G72150	PATL1 (PATELLIN 1); transporter	0,80
AT4G13770	CYP83A1 (CYTOCHROME P450 83A1); oxygen binding	0,80
AT2G29630	Unknown protein_ thiamine biosynthesis family protein	0,80
AT1G80380	uridine kinase-related	0,80
AT1G24510	ATP binding / T-complex protein 1 epsilon subunit	0,79
AT3G13460	unknown protein	0,79
AT3G10060	FK506 binding / peptidyl-prolyl cis-trans isomerise	0,79
AT5G19990	ATSUG1; ATPase_ 26S proteasome AAA-ATPase subunit (RPT6a)	0,79
AT5G61010	exocyst subunit EXO70 family protein	0,78
AT1G03740	kinase	0,78
AT3G57410	VLN3 (VILLIN 3); actin binding	0,78
AT5G53450	ORG1; kinase	0,78
AT1G69850	NTL1; calcium ion binding	0,78
AT5G03900	unknown protein	0,78
AT1G30090	unknown protein_ kelch repeat-containing F-box family protein	0,78
AT4G36550	ubiquitin-protein ligase_ U-box domain-containing protein	0,78
AT2G18440	GUT15 (GENE WITH UNSTABLE TRANSCRIPT 15)	0,78
AT1G31660	unknown protein_ bystin family	0,78
AT1G31970	ATP-dependent helicase	0,77
AT5G21160	unknown protein_ La domain-containing protein	0,77
AT2G43590	chitinase	0,77
AT3G25690	CHUP1 (CHLOROPLAST UNUSUAL POSITIONING 1)	0,77
AT2G14910	unknown protein	0,76
AT2G43590	chitinase	0,76
AT1G13260	RAV1; transcription factor	0,76
AT4G34290	unknown protein_ SWIB complex BAF60b domain-containing protein	0,76
AT3G14620	CYP72A8; heme binding / iron ion binding / monooxygenase/ oxygen binding	0,75
AT5G49720	KOR1 (KORRIGAN); hydrolase	0,75
AT5G40450	unknown protein	0,75
AT4G32020	unknown protein	0,75
AT2G28000	CPN60A; 60 kDa chaperonin alpha subunit	0,75
AT4G00370	ANTR2; organic anion transporter	0,75
AT2G45290	transketolase	0,75
AT1G51500	CER5 (ECERIFERUM 5); ATPase,	0,75
AT2G24820	oxidoreductase_ Rieske (2Fe-2S) domain-containing protein	0,75
AT4G39780	AP2 domain-containing transcription factor	0,75

AT1G78680	gamma-glutamyl hydrolase (GGH1)	0,75
AT5G48300	ADG1 (ADP GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1)	0,75
AT4G23100	RML1 (ROOT MERISTEMLESS 1)_ glutamate-cysteine ligase / gamma-glutamylcysteine synthetase (GSH1)	0,74
AT5G42870	unknown protein_ lipin family protein	0,74
AT2G40800	unknown protein	0,74
AT2G37220	29 kDa ribonucleoprotein / RNA-binding protein cp29	0,74
AT3G57290	EIF3E_ eukaryotic translation initiation factor 3E / eIF3e (TIF3E1)	0,74
AT2G31410	unknown protein	0,74
AT1G51110	structural molecule_ plastid-lipid associated protein PAP	0,73
AT1G16880	unknown protein_ uridylyltransferase-related	0,73
AT5G61160	AACT1; transferase	0,73
AT5G43940	glutathione-dependent formaldehyde dehydrogenase / GSH-FDH (ADHIII)	0,73
AT3G23700	RNA binding_ S1 RNA-binding domain-containing protein	0,73
AT1G49670	oxidoreductase/ zinc ion binding_ ARP protein (REF)	0,72
AT5G17860	calcium:sodium antiporter/ cation:cation antiporter_ cation exchanger, putative (CAX7)	0,72
AT2G46340	SPA1 (SUPPRESSOR OF PHYA-105 1); phytochrome A supressor spa1 (SPA1)	0,71
AT3G19260	LAG1 HOMOLOG 2 (LONGEVITY ASSURANCE GENE1 HOMOLOG 2)	0,71
AT5G63780	ubiquitin-protein ligase/ zinc finger (C3HC4-type RING finger) family protein	0,71
AT5G13740	carbohydrate transporter	0,71
AT3G16370	carboxylic ester hydrolase	0,71
AT2G11240	unknown protein_ non-LTR retrotransposon family (LINE)	0,71
AT2G36380	ATPase _ ABC transporter family protein	0,70
AT2G35050	protein kinase	0,70
AT5G13750	tetracycline:hydrogen antiporter	0,70
AT3G30720	unknown protein	0,69
AT4G38600	KAK (KAKTUS)_ ubiquitin-transferase family protein	0,69
AT1G07280	unknown protein	0,69
AT4G12290	copper ion binding, putative	0,69
AT1G68550	AP2 domain-containing transcription factor	0,68
AT5G24470	APRR5 (PSEUDO-RESPONSE REGULATOR 5); transcription regulator	0,68
AT5G55630	KCO1; calcium-activated potassium channel	0,68
AT2G33770	ubiquitin conjugating enzyme	0,68
AT3G10340	ammonia ligase	0,68
AT4G21790	TOM1 (TOBAMOVIRUS MULTIPLICATION 1)_	0,68
AT5G63980	SAL1; 3prim(2prim),5prim-bisphosphate nucleotidase/ FIERY1 protein (FRY1)	0,67

AT3G07810	heterogeneous nuclear ribonucleoprotein / hnRNP	0,67
AT3G27560	ATN1; kinase	0,67
AT3G06550	unknown protein_ O-acetyltransferase-related	0,67
AT5G24300	transferase_ starch synthase	0,67
AT1G20840	carbohydrate transporter	0,67
AT2G37480	unknown protein glutamate N-acetyltransferase	0,67
AT2G15560	unknown protein	0,67
AT1G58360	AAP1; amino acid permease	0,67
AT5G52180	unknown protein	0,67
AT1G08930	ERD6; carbohydrate transporter	0,66
AT3G52200	LTA3; acyltransferase	0,66
AT2G42810	PP5_ serine/threonine protein phosphatase	0,66
AT5G17630	glucose transporter unknown protein	0,66
AT5G58330	malate dehydrogenase	0,66
AT2G03890	inositol or phosphatidylinositol kinase	0,66
AT1G54990	unknown protein	0,66
AT5G14590	isocitrate dehydrogenase (NADP+)	0,66
AT4G32410	CESA1 (CELLULASE SYNTHASE 1)	0,66
AT3G07180	unknown protein_ GPI transamidase component PIG-S-related	0,66
AT4G36990	HSF4 (HEAT SHOCK FACTOR 4)	0,65
AT2G20890	THF1	0,65
AT1G52870	unknown protein_ peroxisomal membrane protein-related	0,65
AT1G27980	carboxy-lyase	0,65
AT5G35220	EGY1 (ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW-GREEN 1)	0,65
AT1G52780	unknown protein	0,65
AT4G11790	unknown protein_ Ran-binding protein 1 domain-containing protein	0,65
AT4G39330	oxidoreductase	0,64
AT4G08593	unknown protein	0,64
AT5G53460	GLT1_ glutamate synthase (NADH)	0,64

Table A.10: Genes downregulated in 4-week-old *MKK2EE* plants 1 day after *Alternaria bassicicola* infection

AT2G23120	unknown protein	-0,65
AT3G51920	CAM9 (CALMODULIN 9); calcium ion binding_ calmodulin-9 (CAM9)	-0,65
AT2G46400	WRKY46; transcription factor	-0,66
AT1G73080	kinase _ leucine-rich repeat transmembrane protein kinase	-0,66
AT2G33380	RD20 (RESPONSIVE TO DESSICATION 20); calcium-binding RD20 protein	-0,66
AT3G19030	unknown protein	-0,66
AT1G61340	unknown protein_ F-box family protein	-0,66
AT3G23220	transcription factor_ ethylene-responsive element-binding protein	-0,66
AT2G01180	ATPAP1 (PHOSPHATIDIC ACID PHOSPHATASE 1) / PAP2 family protein	-0,66
AT2G47180	transferase, transferring hexosyl groups	-0,67
AT2G28190	CSD2 (COPPER/ZINC SUPEROXIDE DISMUTASE 2)	-0,67
AT2G22870	EMB2001; GTP binding unknown protein	-0,68
AT5G49480	ATCP1; sodium-inducible calcium-binding protein (ACP1)	-0,68
AT5G64310	AGP1 (ARABINOGALACTAN-PROTEIN 1)	-0,70
AT3G15520	peptidyl-prolyl cis-trans isomerase	-0,70
AT1G62290	aspartic-type endopeptidase/ pepsin A	-0,70
AT1G19960	unknown protein_expressed protein	-0,70
AT1G65390	ATPP2-A5; transmembrane receptor_ disease resistance protein (TIR class), putative	-0,72
AT1G20823	ubiquitin-protein ligase_ zinc finger (C3HC4-type RING finger) family protein	-0,74
AT5G10695	unknown protein unknown protein	-0,74
AT4G32480	unknown protein	-0,77
AT1G07135	unknown protein_ glycine-rich protein	-0,77
AT3G23230	transcription factor_ ethylene-responsive factor	-0,79
AT1G73260	endopeptidase inhibitor	-0,80
AT1G05670	UDP-glucosyl transferase	-0,80
AT1G17420	LOX3; lipoxygenase	-0,82
AT5G63160	transcription regulator_ speckle-type POZ protein-related	-0,82
AT3G22840	ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN)	-0,82
AT5G03210	unknown protein	-0,86
AT2G21640	unknown protein	-0,86
AT2G44840	ATERF13; transcription factor_ ethylene-responsive element-binding protein	-0,89
AT3G10500	ANAC053; transcription factor_ no apical meristem (NAM) family protein	-0,91
AT2G24850	TAT3 (TYROSINE AMINOTRANSFERASE 3)	-0,92

AT2G37970	binding_ SOUL heme-binding family protein	-0,92
AT3G23920	beta-amylase	-0,94
AT1G72520	lipoxygenase	-0,95
AT2G42530	cold-responsive protein (cor15b)	-1,04
AT3G44300	NIT2 (NITRILASE 2)_	-1,25

Curriculum vitae

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Education and professional experience

From 03/05 **PhD thesis at the Institute of Plant Molecular Biology, Prof. H. Hirt, Max F. Perutz Laboratories, University of Vienna**
Topic: The role of MAPKKKC5 in MKK2 mediated stress signalling in *Arabidopsis thaliana*

08/06-02/07 **Scientific training in the plant stress group of Prof. Jaakko Kangasjärvi, University of Helsinki, Finland**
Topic: Evaluation of the ozone sensitivity of different plant signalling mutants

01/05 **Graduation as 'Diplombiologe technisch orientiert (t.o.)' at the University of Stuttgart, Germany**

01/04-01/05 **Diploma thesis at the University of Stuttgart, Germany, Institute of Plant Molecular biology and Plant Virology, in collaboration with C. Koncz, Max Planck Institute for Plant Breeding Research, Cologne, Germany,**
Topic: Examination of the function of the putative SnRK1 substrate ATAF1 in *Arabidopsis thaliana* signalling pathways

04/03-07/03 **Internship at the Queensland Institute of Medical Research, Brisbane, Australia, Department of Population Studies and Human Genetics (Dr. P. Parson)**
Topic: Isolation of plant secondary metabolites and examination of their anti-cancer effects

04/02-06/02 **Research project at the Institute for Industrial Genetics, University of Stuttgart**
Topic: Investigation of the netropsin-resistence in *Streptomyces flavopersicus*

12/00 **Completion of undergraduate studies at the University of Stuttgart, finished with ‚Vordiplom'**

from 10/98 **Undergraduate and graduate studies of 'Technical Biology' at the University of Stuttgart**

06/98 **Graduation at the Albert-Schweitzer-Gymnasium Dillingen/Saar, Germany, finished with 'Abitur'**

Conferences with poster presentations, publications

8-11 february 2009	International Conference on Plant Abiotic Stress Tolerance, Vienna (poster)
17-22 august 2008	XVI Congress of the Federation of European Societies of Plant Biology (FESPB), Tampere, Finland (poster)
12-15 september 2007	4th Trinationale Arabidopsis Meeting, Vienna (poster)
27-29 august 2007	PhD Summer School on Environmental Signaling, Utrecht, Netherlands (poster)
26-29 september 2006	3d Trinationale Arabidopsis Meeting, Tübingen, Germany (poster)

Stumpp, T., Himbert, S., Altenbuchner, J.: Cloning of the netropsin resistance genes from Streptomyces flavopersicus NRRL 2820., *J Basic Microbiol*. 2005 ;45 (5):355-62