The role of mitogen-activated protein kinase signalling to regulate leaf growth in response to environmental signals

A thesis submitted for the degree of

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Declaration

I hereby declare that this thesis has been composed by me, this work has not been submitted for any other degree or professional qualification, the work is my own and contribution from others has been clearly indicated.

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Date: 25th Dec. 2015

Abstract

Better understanding how plants growth is in tune with limiting environment is fundamental in efforts to improve stress tolerance. *Arabidopsis thaliana* provides a model system to study the signalling pathways that transmit signals for stress adaptation. Drought is one of the most prevalent abiotic stress to consider. I have set up a screen for stress induced mitogen-activated protein kinase signalling pathways using inducible overexpression of gain of function MAPK kinases (MKKs). Among the 10 *Arabidopsis* MKKs I have found two; MKK7 and MKK9 that significantly altered the growth of both roots and shoots including leaves. I have characterised the expression of these MKKs on the protein level, and the induction of two downstream MAPKs, MPK3 and MPK6. I set up experimental conditions that mimick mild drought stress using mannitol that limit leaf growth by 50 percent. Using the estradiol inducible overexpression lines for MKK7 and MKK9, as well as insertional mutant lines of *mkk7*, *mkk9*, *mpk3* and *mpk6* I tested the involvement of these MAPK signalling pathways in transducing drought stress and thereby regulating cell proliferation and leaf growth. I used the mitotic cell cycle marker, CycB1;1-DB-GUS reporter, flow cytometry and cell size imaging through DIC and confocal microscopy in these experiments. In paralell to these drought experiments we found that dark stress also induces the MAPK signalling pathway. To characterise the role of MKK7, MKK9, MPK6 and MPK3 in dark-induced growth arres of leaves, I set up deetiolation experiments and imaged emerging leaves upon transfer to light. I found that MPK6 is involved in the initial leaf growth after dark arrest. This work was later followed up and led to the conclusion that the MAPK pathway may regulate auxin transport. I also set out to investigate whether drought and the MKK7, MKK9 MAPK signalling might regulate cell proliferation through the RETINOBLASTOMA RELATED (RBR) protein and E2F transcription factors. To this end, I showed that drought stress influence RBR phosphorylation and promotes RBR interaction with E2FB. I made crosses with the inducible MKK7 and MKK9 lines and the E2FA-GFP, E2FB-GFP to look for the regulation of RBR-E2FB interaction when these MPK pathways are induced. In summary, my research identified the MKK7 and MKK9 pathways regulate leaf growth and cell proliferation. Modulating these signalling pathways could help to obtain crops that better adapt their growth to limiting environmental conditions.

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Dedication

I dedicate this thesis to my family for nursing me with affection and love and their dedicated partnership for success in my life.

Abbreviations

ABA	Abscisic acid
°C	Degree Celsius
cDNA	Copy or complementary deoxyribonucleic acid
Col-0	Columbia-0 ecotype of *Arabidopsis thaliana*
dag	days after germination
DAI	days after induction
DAT	days after transfer
DIC	Differential interference contrast (microscopy)
DNA	Deoxyribonucleic acid
GFP	Green Fuorescence Protein
GOF	Gain of Function
GUS	β-glucuronidase
hrs	hours
H_2O_2	Hydrogen peroxide
kDa	Kilo Dalton
MPK (MAPK)	Mitogen Activated Protein Kinase
MAPKK (MKK)	MAPK Kinase
MAPKKK (MAP3K)	MAPKK Kinase
mg	microgram
min	minute
ml	millilitre
mM	millimolar
nm	nanometer
N_2	nitrogen
PI	propidium iodide
PTPM	post-translational protein modification
QC	quiescent centre
RB	Retinoblastoma protein
RBR	Retinoblastoma related protein
ROS	Reactive Oxygen Species
S	Serine

SD	Standard deviation
SE	Standard error
T	Threonine
T-DNA	Transfer-DNA
Tris	2-Amino-2-hydrxymethylpropan-1.3-diol
5' utr	5' untranslated region
Y	Tyrosine
WT	wild-type
µl	microlitre
µm	micrometer
µM	micromolar
½ GM	half strength germination medium

Chapter 1: Introduction

1.1 Environmental stresses and corresponding signalling pathways

Plants are sessile in nature that necessitates sensitive response and rapid adaptation to changes in environment. Environmental conditions can be used as cues that direct plant growth, such as nutrient signals, light or gravity (Vandenbrink *et al.*, 2014). Other external factors surrounding plants are the various forms of stresses that limit their growth or endanger their survival. As a response to environmental conditions, plants evolved sophisticated perception of the myriads of signals that elicit intracellular signal transduction mechanisms that function to integrate the information to select proper adaptive responses. A prevalent mechanism of signal transduction is through protein phosphorylation cascades (Hirayama and Shinozaki, 2010). Signalling mechanisms are connected to stress responses that involve the modification of physiological and biochemical mechanisms to enable adaptation and facilitate plant survival (Xiong *et al.*, 2002). Cellular damage and impaired physiological functions are induced by environmental stresses (e.g. drought, heat, low temperature) singularly or most likely in combinations (Larcher, 2003). The paramount significance of abiotic stresses for crop performance necessitates the detailed characterisation of molecular adaptation mechanisms that enable the plant to reset the disturbed homeostatic equilibrium upon stress conditions.

In this context, osmotic stress can be induced by a number of major climatic factors such as salinity, drought, low or high temperature (which all can result in cellular turgor loss). When osmotic stress persists this can lead to the membranes to become disorganized, and proteins may denature resulting in the loss of activity (Mahajan and Tuteja, 2005). In this condition, frequently high levels of reactive oxygen species (ROS) are produced and more oxidative damage occur subsequently, hence inhibition of photosynthesis and metabolic dysfunction contribute to growth

perturbances, reduced fertility, and (Woo *et al.*, 2013). However, these processes can be highly variable and can largely vary from species to species the optimum environments. To a given stress condition one species can be fully resistant while another largely susceptible dependent on the multitude, duration and timing of the exposure (Larcher, 2003; Munns and Tester, 2008).

In our changing climatic environment, it is extremely important to improve crops resistance by developing resistant varieties for better adaptation. The studies using the model plant *Arabidopsis thaliana* (thale cress) provided important mechanistic insights for the genetic and molecular understanding of how plants adapt to stress conditions. This knowledge provides a platform that can be used to improve stress tolerance in crops. *Arabidopsis* became the major model organism in plant biology due to its, short generation time, small genome size and simple transformation methods (Buell and Last, 2010; Koornneef and Meinke, 2010). Additionally, the availability of its genome sequence, large genomic resources and mutant collection, make *Arabidopsis* the most studied plant species (Spannagl *et al.*, 2011). However, the basic developmental, biotic and abiotic stress tolerance mechanisms have common biological basis. Therefore, the genes and corresponding functions identified in *Arabidopsis* can be directly employed to identify homologous genes in crop species and use these candidate genes to facilitate breeding for stress tolerance as it was shown by overexpressing the maize nuclear transcription factor Y subunit B-2 that was identified through genetic screening of Arabidopsis for drought tolerance (Feuillet *et al.*, 2011). However, it must be said that not in all the cases the identified orthologs may have similar functions in different plant species. The regulatory mechanisms might have diverged, modified or adapted to distinct responses (Movahedi *et al.*, 2011; Rensink and Buell, 2004). The availability of *Arabidopsis* genome sequence some 15 years ago provided a large leap for discovering the molecular details of regulatory mechanisms (Arabidopsis Genome, 2000). The ever expanding number of genome sequences available for many crops as well as closely related and evolutionary distant plant species opened the way to begin exploring how regulatory mechanisms evolved.

Another important factor that facilitated molecular genetic studies in *Arabidopsis* is the highly efficient transformation protocols by *Agrobacterium tumefaciens* through floral dipping (Bent, 2006). This largely underpinned the functional characterization of sequences of genes through transfer-DNA (T-DNA)

insertion mutant libraries (Krysan *et al.*, 1999). In addition, other genetic and molecular tools were rapidly developed including full-length complementary DNA (cDNA) libraries for gene isolation and functional studies, gene expression profiling platforms (Rensink and Buell, 2004; Jung *et al.*, 2008).

1.1.1 Drought stress

Drought is one of the primary factors of crop losses worldwide that leads to an average reduction of yields for most major crop plants by more than fifty percent (Bray *et al.*, 2000; Wang *et al.*, 2003). Drought not only affects plant survival but also impacts on all major metabolic and cellular processes and therefore decreases growth and productivity of plants (Krasensky and Jonak, 2012). The influence of drought on plant survival and growth is largely dependent on the strength and duration. The molecular networks underlying growth adaptation to drought stress is much less understood than the drought survival responses. Stress adaptation is highly complex and dependent on parameters such as stress severity, organ or cell identity, and developmental stages (Claeys and Inze, 2013).

1.1.1.1 Experimental conditions to study drought responses

To experimentally administer drought stress in a reproducible way is challenging. A broadly used method is to mimic drought stress by modifying the osmolarity of the medium plants grow *in vitro*. This can be done by including non-metabolisable sugar, such as mannitol. The increased osmolarity by mannitol leads to water deficit of plant tissues and therefore to the loss of water potential. The effects of water deficit were studied on different levels ranging from ecophysiology to cell metabolism (Shinozaki and Yamaguchi-Shinozaki, 1997; Chaves *et al.*, 2003).

In drought conditions that still enable survival, growth is inhibited (Skirycz and Inze, 2010). Growth adaptation mechanisms were studied by administering mild osmotic stress using low concentrations (25-50 mM) of mannitol (Skirycz *et al.*, 2011a). The effect of such mild drought stress can be most feasibly followed on leaf growth. In *Arabidopsis* rosette leaves emerge sequentially over time. At emergence, the majority of leaf cells actively proliferate. Subsequently, cells exit proliferation and enter into cell expansion (Andriankaja *et al.*, 2012). In *Arabidopsis* leaf, this transition typically happens first at the leaf tip and gradually progresses towards the

leaf base. Besides there are dispersed meristematic regions surrounding developing stomata that contribute to leaf growth. How drought affects these two populations of cells to regulate cell proliferation and exit from proliferation are fundamentally important to understand growth adaptation (Donnelly *et al.*, 1999; Kazama *et al.*, 2010; Andriankaja *et al.*, 2012). This transition from cell proliferation to cell expansion is accompanied by a switch from the mitotic cell cycle to endoreduplication. Drought was shown to impact on this transition as well (Setter and Flannigan, 2001). Because the switch to endoreduplication occurs in the G2 phase of the cell cycle, drought might impact at the G2-M phase transition (Beemster *et al.*, 2005). This is indicated by the expression of mitotic specific reporter gene; Cycb1; 1: GUS, which is down-regulated upon drought stress (Skirycz *et al.*, 2011a).

1.1.2.1 Morphological responses of drought stress

In drought conditions, it is paramount to optimise the use of available water by adapting growth rate and other morphological and physiological properties of organs and by changes in biomass allocation (Turner, 1986). Stress avoidance includes phenological number of changes in developmental processes that include a change in stomata density, cuticular resistance, changes in leaf area, orientation and anatomy of leaves and other organs (Morgan, 1984; Jones and Corlett, 1992). Plants tolerate drought by maintaining sufficient cell turgor to allow metabolism to continue under increasing water deficits (Munns, 1988).

1.1.2.2 Cellular and biochemical responses to drought

Metabolic networks are highly dynamic, therefore metabolites can move between different cellular compartments. However, the signalling processes required for homeostasis of basic cellular and metabolic processes in adverse environments are just starting to emerge (Cramer *et al.*, 2011). Metabolic adjustments of plants with different levels of stress tolerance is an important mechanism for stress survival. For example, one of the most studied metabolites that as an osmolyte alters tolerance to osmotic pressure is proline (Szabados and Savoure, 2010). Stress signalling pathways can modulate biosynthetic pathway activities but the mechanisms are little understood. Several other questions remain to be addressed such as how the developmental stage of a plant influences the metabolic adjustment, or how survival and growth responses are coordinated.

1.1.2.3 Abscisic acid is central to stress adaptation

How drought stress or osmotic stress is perceived by plant cells is not fully understood (Wohlbach *et al.*, 2008). Downstream of signal perception are the regulatory pathways involved in eliciting the response to environmental stresses such as drought (Ingram and Bartels, 1996; Ramanjulu and Bartels, 2002). There are two important branches of drought stress signalling pathways we know about, one being abscisic acid (ABA) dependent and the other is ABA independent (Shinozaki and Yamaguchi-Shinozaki, 2007). ABA is a plant stress hormone, that is a central integrator of many plant responses to environmental stresses, particularly osmotic stress (Cramer *et al.*, 2011; Kim *et al.*, 2010)(Fig 1.1). ABA signalling can be very rapid without involving transcriptional activity; a good example is the control of stomatal aperture by ABA through the regulation of ion and water transport processes (Kim *et al.*, 2010). The current model of ABA signalling includes three core components, receptors Pyrabactin resistance/Pyrabactin-like or regulatory components of ABA receptor (PYR/PYL/RCAR), protein phosphatases and protein kinases (Sucrose non-fermenting1-like kinase 2s; SnRK2s). The PYR/PYL/RCAR proteins were identified as soluble ABA receptors by two independent groups (Ma *et al.*, 2009; Park *et al.*, 2009). The 2C-type protein phosphatases (PP2C) including ABI1 and ABI2, were first identified from the ABA-insensitive *Arabidopsis* mutants *abi1-1* and *abi2-1*, and they act as global negative regulators of ABA signalling (Leung and Giraudat, 1998). SNF1-related protein kinase 2 (SnRK2) is a family of protein kinases isolated as ABA-activated protein kinases (Yoshida *et al.*, 2002). Cellular dehydration under water limited conditions induces an increase in endogenous ABA levels that trigger downstream target genes encoding signalling factors, transcription factors, metabolic enzymes, among others (Yamaguchi-Shinozaki and Shinozaki, 2006). In the vegetative phase of plant development, expression of ABA-responsive genes is mainly regulated by bZIP transcription factors (TFs) known as AREB/ABFs, which act in an ABA-responsive element (ABRE) (Fujita *et al.*, 2005; Kang *et al.*, 2002; Yoshida *et al.*, 2010). In contrast, a dehydration responsive cis-acting element, DRE/CRT sequence and its DNA binding ERF/AP2-type TFs, DREB1/CBF and DREB2A, are related to the ABA-independent dehydration and temperature responsive pathways (Shinozaki and Yamaguchi-Shinozaki, 2007). Ethylene is also involved in many stress responses (Stepanova and Alonso, 2009; Yoo *et al.*, 2009). Functional analysis of these

hormone pathways and intricate cross-talks between the different signalling pathways during stress adaptation underlies most stress adaptation responses. Understanding these pathways is fundamental for developing strategies for crop improvement for abiotic stresses and eventually will lead us to develop crop varieties superior in stress tolerance by genetic manipulation (Yamaguchi-Shinozaki and Shinozaki, 2006).

1.1.2.4 Effects of drought on leaf growth

As discussed above, one of the earliest events of drought response is the production of the phytohormone ABA that triggers stomatal closure to reduce evapotranspiration (Chaves *et al.*, 2003). Initially, leaf stomatal conductance may decrease faster than carbon assimilation, leading to increased water use efficiency. However, even under moderate stress, stomatal closure leads to decreased intercellular CO_2 levels causing a reduction in photosynthesis (Chaves and Oliveira, 2004). Furthermore, drought directly inhibits cell division and expansion thereby reducing leaf area for photosynthesis (Munns, 2002). A recent transcription profiling of young growing leaves revealed two main pathways involved in the reduction of leaf growth in drought conditions (Skirycz *et al.*, 2011a), a GA-mediated pathway through the DELLA transcription factors (Claeys *et al.*, 2012), and a pathway mediated by the MKK7, MPK3 MAPK signalling pathway (see below). The reduction in growth may be an adaptive response allowing diversion of energy and assimilates to drive the various drought tolerance mechanisms. It also limits the leaf area available for water loss. However, since leaf growth and photosynthesis are the primary drivers of biomass accumulation, the reduction in leaf area and photosynthesis has major negative consequences for crop yield. A secondary effect of drought is oxidative stress. In a field situation, plants are usually subjected to other stresses accompanying drought such as heat. Under these conditions, the rate of reducing power production can overcome the rate of its use by the Calvin cycle leading to the production of ROS, which are highly destructive to lipids, nucleic acids and proteins (Xiong and Zhu, 2002; Xiong *et al.*, 2002). ROS, particularly hydrogen peroxide (H_2O_2), can also function as a signalling molecule, able to activate MAPK pathways, and thereby affect growth responses (Colcombet and Hirt, 2008).

1.1.2.5 System biology approaches to improve drought tolerance

` The system biology is an area or approach that involves the systematic and comprehensive molecular analysis of plant responses to abiotic stress such as gene regulation, protein functions, metabolism that utilise transcriptomics, metabolomics, proteomics (Fig 1.1). The aim is to connect these molecular changes with cellular, physiological and plant phenotypes. Thus, in parallel with molecular profiling platforms it was important to develop methods for high throughput phenotyping at fully controlled environmental conditions (phenomics and cellular imaging systems) (Skirycz and Inze, 2010). The promise of systems biology on one hand is to expand our current knowledge on the framework of stress signalling pathways and their connections to diverse regulatory mechanisms, while on the other hand it leads to the discovery of novel stress adaptation mechanisms. System biology approaches can also help building mechanistic models that predict how molecular changes lead to physiological stress adaptation responses that modulate plant growth.

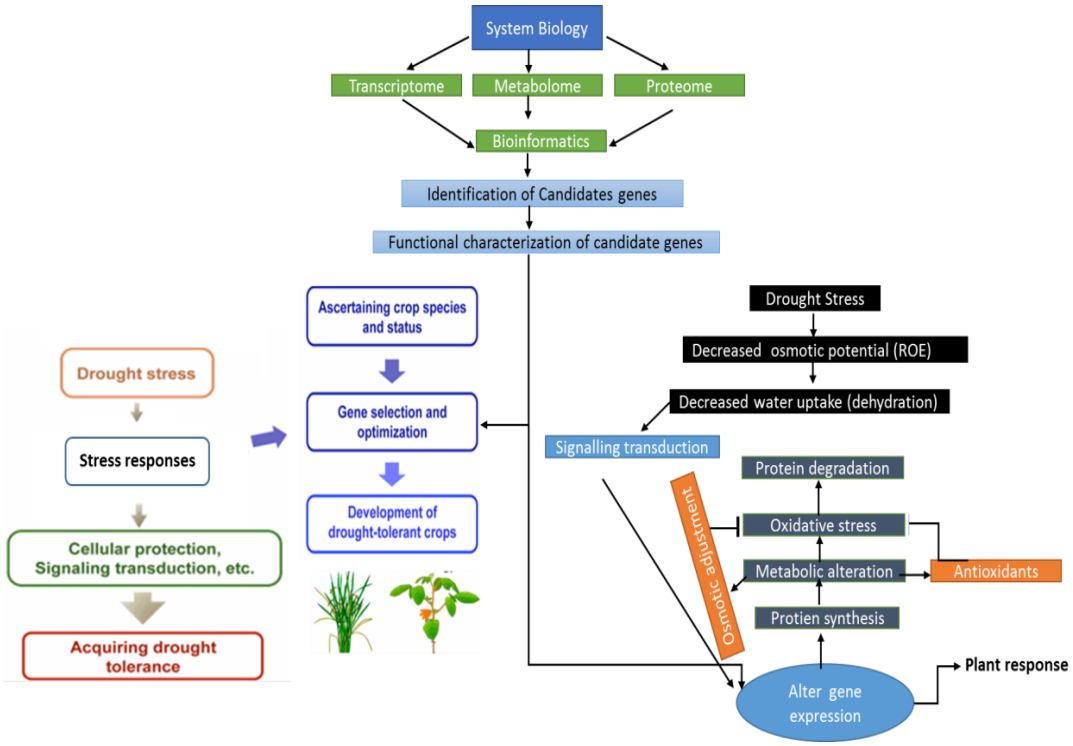

Figure 1.1 A scheme of how the integration of stress physiology, molecular and systems biology can contribute to our understanding of stress tolerance. Adapted image (Fujita *et al.*, 2010). System biology approaches can be used to identify candidate genes that linked to drought tolerance. The next step is functionally characterize these genes to understand their biological role.

1.2 MAPKs provide a central signalling mechanism for stress adaptation

1.2.1 Post-translational modifications and the role of phosphorylation

After biosynthesis, many proteins undergo chemical modifications, which is crucial for specific functions, these modifications are known as post-translational protein modifications (PTPMs). PTPMs are key in many biological processes such as gene expression regulation (Filipowicz and Hohn, 2012), protein-protein interactions (Duan and Walther, 2015), signalling and regulatory processes (Deribe *et al.*, 2010), protein degradation (Nelson and Millar, 2015) etc. The PTPMs are usually covalent modifications through enzymatic reactions and their results are often conformational changes that regulate the catalytic activity of the protein. Well-studied PTPMs of eukaryotic cells are: phosphorylation, glycosylation (N-linked or O-linked), ubiquitination, sumoylation, methylation, S-nitrosylation, S-palmitoylation, N-acetylation, N-myristoylation.

Phosphorylation is a one of the most studied post-translational modifications, refers to the enzymatic reaction that results in an addition of phosphate group(s) to a protein (Hubbard and Cohen, 1993). In plants phosphorylation is known to play important role in intracellular signal transduction and is involved in regulating cell cycle progression (Gu *et al.*, 1992), differentiation, development (Schiefelbein and Wang, 2014), hormone response (Haruta *et al.*, 2014) etc. In eukaryotic cells phosphorylation can occur at the serine (S), threonine (T) or tyrosine (Y) residues. The enzyme that adds phosphate group to the substrate protein is called protein kinase. Phosphorylation in several cases activates but sometimes inactivates the target protein. The phosphate group donor is ususally the adenosine triphosphate (ATP). Protein phosphorylation is a reversible, dephosphorylation is a process in which the phosphate group is removed from the target. Dephosphorylation of proteins is carried out by protein phosphatases. Dephosphorylation basically reverses the effect of phosphorylation. Kinases and phosphatases provide a dynamic regulation of the phosphorylated proteins within cells. The importance of this regulatory mechanism is reflected by the large number of genes in eukaryotes that code for protein kinases and protein phosphatises. There are more than one thousand protein kinases that were annotated in the *Arabidopsis* genome (Bogre, 2007). The specificity of protein kinases is based on the consensus sequences that located in the vicinity of the target amino acids. There are kinases that can phosphorylate several

substrates if they contain similar kinase-specific consensus domains (Rust and Thompson, 2011). Besides, protein kinases can phosphorylate more than one target amino acids on a single protein if it contains more specific consensus domains. Generally protein kinases can be activated by regulatory substrates through their regulatory subunits.

1.2.2 Mitogen activated protein kinases

One of the most expanded family of protein kinases in plants are the components of the mitogen-activated protein kinase (MPK) cascades, which are encoded by 120 annotated genes in *Arabidopsis* (Jonak *et al.*, 2002; Doczi *et al.*, 2012). MPK signalling cascades are composed of three main signalling elements- MKK-activating kinase (MKKK), MPK-activating kinase (MKK), and the MPK. The MPK pathway is activated through consecutive phosphorylation events of the activation loop. These MPK pathways are connected to extracellular signals and intracellular second messengers and other signalling mechanisms, such as G-proteins, but the mechanisms for these are poorly understood in plants (Fig 1.2 A). The activated MPK phophorylates a variety of cytoplasmic and nuclear substrates, with possible effects on their localization, activity state, stability, and transcript levels, including not only transcriptional and translational regulation but through post-transcriptional regulation such as protein-protein interactions (Popescu *et al.*, 2009; Whitmarsh, 2007; Taj *et al.*, 2010). The MPK substrates are then involved in numerous essential cellular processes. However, knowledge of MPK substrates and how MPK phosphorylation might regulate their activity and function are also rather limited in plants. MPK signalling cascades form a complex interconnected network (Pedley and Martin, 2005). Traditional genetic and biochemical methods have identified MKKK/MKK/MPK signalling modules with overlapping roles in controlling cell division, development, hormone signalling and synthesis, and response to abiotic stress and pathogens. There are also overlapping functions for the same MPK signalling module, for example the *Arabidopsis* MEKK1-MKK4/5-MPK3/6 module was found to particulate in flagellin-mediated innate immunity signalling (Asai *et al.*, 2002) and are involved in a number of other biological processes during growth and development (Nakagami *et al.*, 2005; Takahashi *et al.*, 2007). Multiple upstream signalling components might connect to the same

downstream MPK. For example the *Arabidopsis* MKKK20 was found to be involved in the osmotic stress signal transduction pathway via MPK6 activity under abiotic stress treatment as was found based on results involving the *mkkk20* loss-of-function mutant (Kim *et al.*, 2012)

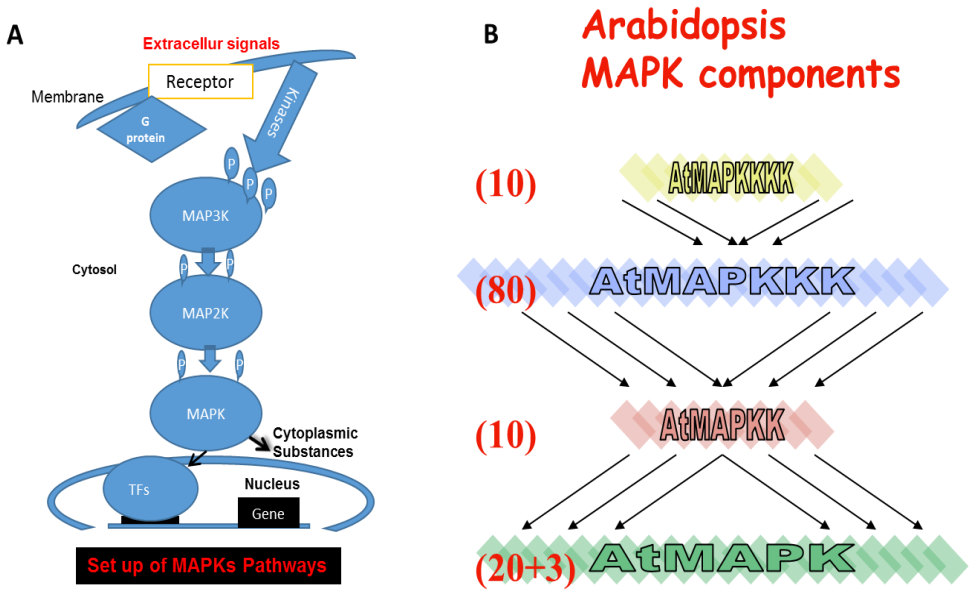

Figure 1.2 The MAP kinase regulatory pathways. (**A)** Architecture of MAPK cascades and (**B**) its complexity in *Arabidopsis*. Signals perceived of environmental stresses at the level of cell receptors then signalling mechanisms involving G-proteins, second messengers or other protein kinases activate the MAPKs through sequential phosphorylation cascades. MAPK then phosphorylate downstream targets e.g. transcription factors that move to the nucleus and bind to a DNA elements to initiate transcription. In left the number of MAPK signalling components are indicated at the various levels of the MAPK signalling cascade.

1.2.3 MAPK signalling pathways in *Arabidopsis*

The mitogen-activated protein (MAP) kinase phosphorylation cascades are conserved signalling modules in all eukaryotes and known to have pivotal roles to regulate cell division, cell growth and stress responses in animals. In *Arabidopsis* genes encoding 20 MPKs, 10 MAPKKs (Table 1.1) and 60-80 putative MAPKKKs

were identified (Fig 1.2 B), and both MPKs and MKKs have been divided into four phylogenetic groups designated A-D (MAP_Kinase_Group, 2002).

MKKKs are serine/threonine kinases that phosphorylate two amino acid in the S/T-X_{3-5}-S/T motif of the MKK activation loop (Colcombet and Hirt, 2008). In turn, MKKs are dual specific kinases that phosphorylate the T-X-Y motif at the activation loop of MPKs. Lastly, MPK are serine/threonine kinase able to phosphorylate a wide range of substrates, including other kinases and/or transcription factors (Popescu *et al.*, 2009).

***Arabidopsis* MAPKK Proteins**	**Gene code**	**Accession No Gene bank protein**	**Group**	**References**
MKK1	At4g26070	AAB97145	A1	(Morris *et al.*, 1997)
MKK2	At4g29810	BAA28828	A1	(Ichimura *et al.*, 1998)
MKK3	At5g40440	BAA28829	B3	(Ichimura *et al.*, 1998)
MKK4	At1g51660	BAA28830	C4	(Ichimura *et al.*, 1998) (Ren *et al.*, 2002)
MKK5	At3g21220	BAA28831	C4	(Ichimura *et al.*, 1998) (Ren *et al.*, 2002) (Hamal *et al.*, 1999)
MKK6	At5g56580	NP_200469	A6	*Arabidopsis* genome project
MKK7	At1g18350	AAF25995	D7	*Arabidopsis* genome project
MKK8	At3g06230	AAF30316	D7	*Arabidopsis* genome project
MKK9	At1g73500	AAG30984	D7	*Arabidopsis* genome project
MKK10	At1g32320	AAF81327	D7	*Arabidopsis* genome project

Table 1.1 Summary and systematic details of MKKs in *Arabidopsis*

MPK cascades in plants are involved in diverse functions that include plant adaptation to abiotic stresses such as cold, salt, touch/wind, wounding, heat, UV radiation, and osmotic shock. Cold and salt stress induces the MKKK1-MKK2-MKK4 signalling module and drought and wounding the MKK1-MPK4 pathway (Teige *et al.*, 2004; Xing *et al.*, 2007). MPK cascades are also involved in stress unrelated processes, including growth and development and auxin signalling. Reverse genetic analysis identified the regulation of stomata patterning in the

epidermis by YODA-MKK4/5-MPK3/6 (Mockaitis and Howell, 2000; Lukowitz *et al.*, 2004; Bergmann, 2004). Utilising gene silencing techniques as well as transient expression in protoplasts or stable transgenic lines for both gain-and loss-of-function analysis were also employed to advance our understanding of the complexity of MPK network and the role of MAPK cascades in plants (Yoo and Sheen, 2008).

1.2.3.1 MAPK signalling pathways in abiotic stress tolerance

MAPK cascades in Arabidopsis are also involved in signalling pathways activated by abiotic stresses such as salt, cold, water stress, oxidative stress and high temperature (Colcombet and Hirt, 2008; Sinha *et al.*, 2011). One of the first studies in this respect showed that under salt and cold stresses, MPK4 and MPK6 are phosphorylated by MKK2, which was shown to be activated by salt, cold, and the stress-induced MEKK1 (Teige *et al.*, 2004), therefore describing a cascade mediated by MEKK1-MKK2-MPK4/MPK6 (Fig 1.3). The MAPKs MPK3 and MPK6 have been connected to ABA-mediated signalling processes: MPK3 was shown to participate in ABA and ROS perception during stomatal closure in *Arabidopsis* guard cells (Gudesblat *et al.*, 2007) and a MAPK cascade consisting of MKK1 and MPK6 mediated the ABA-induced expression of Catalase 1 (CAT1) and H_2O_2 production, in an ABA-dependent signalling cascade. In the same study, the overexpression of MKK1 enhanced drought tolerance in *Arabidopsis* plants and the authors explained the production of H_2O_2 as a signal to facilitate stomatal closure or to further activate MAPKs (Xing *et al.*, 2008). Oxidative stress activates MPK3, MPK4 and MPK6 although by different MAPKs pathways. In response to H_2O_2, MKK1 phosphorylates MPK4 (Teige *et al.*, 2004) and MPK4 is placed downstream of MEKK1, which is activated in the presence of H_2O_2 and functions as a regulator of ROS homeostasis (Nakagami *et al.*, 2006), therefore describing a H_2O_2-induced MEKK1-MKK1-MPK4 cascade (Fig 1.3). The H_2O_2-mediated activation of the MAP3K ANP1 triggers a phosphorylation cascade that activates MPK3 and MPK6, and ANP1 activates the expression of H_2O_2-regulated genes, demonstrating that ANP1 functions in H_2O_2 mediated signal transduction (Kovtun *et al.*, 2000). MPK3 and MPK6 are activated by ozone (O_3) and translocated to the nucleus after O_3 treatment, which suggests that MPK3 and MPK6 are involved in the phosphorylation of transcription factors that regulate O_3-responsive genes (Ahlfors *et al.*, 2004). Recently, it was shown that followed by heat activation, the *Arabidopsis* MPK6

phosphorylated the heat shock protein transcription factor 2 (HSFA2), therefore regulating its nuclear accumulation (Evrard *et al.*, 2013). HSFA4A interacts with MPK3 and MPK6 in both yeast and plant cells, and that HSFA4A is a substrate of MPK3 and MPK6 *in vitro* (Perez-Salamo *et al.*, 2014).

It is intriguing that MPK3 and MPK6 were identified as central regulators of multitudes of stress responses such as plant innate immunity, hypoxia, salt and osmotic stress responses, which control cross-talk between different stresses, hormonal signals and second messengers, such a ROS (Droillard *et al.*, 2002; Teige *et al.*, 2004; Chang *et al.*, 2012; Rasmussen *et al.*, 2012). MAP kinases were reported to phosphorylate other transcription factors such as WRKY33, involved in regulation of ethylene biosynthesis (Li *et al.*, 2012) or MYB44 which regulates ABA sensitivity (Nguyen *et al.*, 2012a). MPK3 and MPK6 can form *in vivo* complexes with ZAT10/STZ regulating plant defence responses (Mittler *et al.*, 2006; Nguyen *et al.*, 2012b), while under salt and osmotic stress, MPK6 interacts with and phosphorylates ZAT6 (Liu *et al.*, 2013). Thus, MPK3 and MPK6 appear to regulate a wide-range of biotic and abiotic defence responses mediated by ROS signals, and coordinate the activity of various transcription factors such as WRKY, MYB, ZAT and HSF, that in turn control the transcription of a large set of target genes.

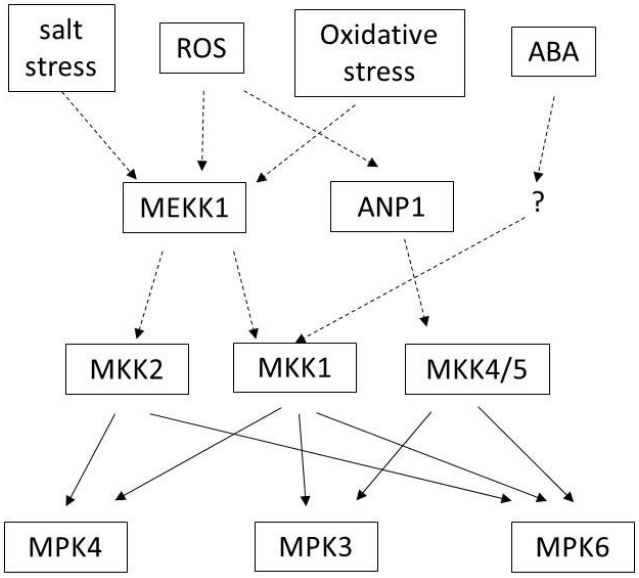

Figure 1.3 Schematic representation of known abiotic stress induced MAP kinase signalling components in Arabidopsis. Solid arrows indicates experimentally proven pathways; dashed arrows show postulated connections.

1.2.3.2 MAPK cascades in cell cycle regulation

In early studies to elucidate the function of MAPK cascades in cell cycle regulation, synchronized alfalfa and tobacco BY-2 suspension culture cells were used to analyse the expression, activity and localization of MAPKs. The *Medicago* MAPK, MKK3, the tobacco MAPKKK named after the *Nicotiana* protein kinase 1 (NPK1) were found to be cell cycle regulated (Cardinale *et al.*, 2002; Nishihama *et al.*, 2001).

One of the best studied mitotic MAPK signalling pathways is the so called PQR signalling cascade that is controlled by the NACK kinesin and targets MAP65 proteins through MPK4 to regulate cytokinesis (Calderini *et al.*, 1998; Nishihama *et al.*, 2001). In accordance, cells in the *mpk4* mutant show aberrant cytokinesis in *Arabidopsis* (Kosetsu *et al.*, 2010a; Beck *et al.*, 2011). Another pathway involves MPK3 and MPK6; single mutants of *mpk3* or *mpk6* have no major developmental phenotypes or cell division abnormalities, while the *mpk3mpk6* double mutant is embryo lethal (Wang *et al.*, 2007). Thus, MPK3 and MPK6 were suggested to have redundant and dose-dependent functions (Xu and Zhang, 2014). In accordance, MPK3 and MPK6 were shown to have overlapping substrate recognition (Popescu *et al.*, 2009; Ellis, 2012), and to play multiple roles including photomorphogenesis (Sethi *et al.*, 2014), specification of cell fate during stomatal development (Wang *et al.*, 2007), regulation of cell proliferation and differentiation in anthers and ovules (Wang *et al.*, 2008), and regulation of cell proliferation during inflorescence development (Meng *et al.*, 2012). While MPK3 and MPK6 have redundant roles in some processes, they are not interchangeable in others (Wang *et al.*, 2008). In one of the pathways, MPK6 is part of the YODA and MKK4/MKK5 MAPK signalling downstream of the ERECTA receptor kinases to regulate meristematic development and cell proliferation (Meng *et al.*, 2012). The YDA pathway, through MPK6, also targets MAP65 to regulate cortical microtubules and cytokinesis (Smekalova *et al.*, 2014). MPK6 can also repress cell proliferation, as suggested by small seed size and decreased root growth rate in the *mpk6* mutant (Lopez-Bucio *et al.*, 2014).

Stress signals are frequently transmitted through the generation of ROS and MPK6 provides a pivotal signalling route by targeting Nitrate reductase 2 (NIA2) to generate niric oxide (NO), which impacts on growth adaptation e.g. root development in *Arabidopsis* (Wang *et al.*, 2010).

MAPK signalling is intricately connected with the cytoskeleton, either directly through the phosphorylation of cytoskeletal proteins, or via interactions with cytoskeletal scaffolding proteins that may bring MAPKs together with their activators, substrates, or components of other pathways (Meister *et al.*, 2013). There are numerous examples in animal and yeast cells for interactions of MAP kinases with microtubular proteins in response to stress or developmental cues. For example MAP kinases regulate microtubular dynamics during osmotic stress in yeast cells (Hagan, 2008).

Despite extensive studies of plant MAPKs and their role in development, only a few MAPK substrates have been identified among microtubular proteins (Komis *et al.*, 2011; Sasabe and Machida, 2012). The microtubule-associated proteins; MAP65-1, MAP65-2 and MAP65-3 are the only ones that were experimentally shown to be regulated by MAPK signalling in mitotic plant cells. Phosphorylation of MAP65-1 by MAP kinase in tobacco (*Nicotiana tabacum*) regulates phragmoplast expansion through microtubule destabilization (Sasabe *et al.*, 2006). *Arabidopsis* MAP65-1 is phosphorylated both by MPK4 and by MPK6 *in vitro* (Smertenko *et al.*, 2006). MAP65-2 and MAP65-3 were also shown to be phosphorylated by MPK4 (Kosetsu *et al.*, 2010a; Sasabe *et al.*, 2011). MAP65-1 and MAP65-2 have redundant functions in *Arabidopsis* with MAP65-3 in cytokinesis (Sasabe *et al.*, 2011).

1.2.3.3 MKK7/9-MPK6 signalling module

In plants, MAP kinase pathways have been shown to play a major role in stress signalling (Pitzschke *et al.*, 2009). The best studied plant MAP kinase is MPK6 of *Arabidopsis*, which regulates various stress responses such as pathogen defence (Asai *et al.*, 2002; Meszaros *et al.*, 2006), cold and salt stress (Teige *et al.*, 2004), as well as developmental processes, such as inflorescence and embryo development (Bush and Krysan, 2007; Wang *et al.*, 2008) and stomatal patterning (Wang *et al.*, 2007). MPK6 has been implicated in the regulation of ethylene biosynthesis and signalling (Hahn and Harter, 2009). In addition to this apparent functional complexity, MPK6 has been assigned as being downstream of at least five of the ten *Arabidopsis* MAP kinase kinases, belonging to all four phylogenetic MKK groups (Asai *et al.*, 2002; Meszaros *et al.*, 2006; Takahashi *et al.*, 2007; Teige *et al.*,

2004; Yoo *et al.*, 2008; Zhou *et al.*, 2009), suggesting an elaborate regulatory network.

MPK6 has been shown to be downstream of MKK9 (Xu *et al.*, 2008; Yoo *et al.*, 2008), which belongs to group D of plant MAP kinase kinases. MKK9 participates in salt signalling, genetic evidence supports both positive and negative regulation of salt tolerance by MKK9 (Alzwiy and Morris, 2007). Furthermore, similarly to its downstream partner MPK6, MKK9 has been also functionally associated with both ethylene biosynthesis and signalling (Xu *et al.*, 2008; Yoo *et al.*, 2008). A dual role in inhibiting polar auxin transport (PAT) and promoting pathogen defence has been recently unravelled for MKK7, the only other MKK of group D for which functional evidence is presently available (Dai *et al.*, 2006; Zhang *et al.*, 2007). Furthermore, both MKK7 and MKK9 have negative as well as positive functions in stomatal cell fate regulation (Lampard *et al.*, 2009). In light of these diverse findings a novel, complex regulatory pathway is emerging at the cross-roads of plant developmental and stress responses, which minimally comprises of MKK7/9 and MPK6.

1.3 Organ growth and cell cycle overview

Plant sizes are remarkably flexible and altered both by environmental conditions (see e.g. bonsai plants) and the genetic makeup. Understanding the molecular regulatory networks underlying plant growth and organ size is becoming a high priority research in plant science. Thus growth is one of the most studied phenomena in multicellular organisms. Cell proliferation plays a central role for defining organ sizes in higher plants. Plant growth can be indeterminate in plants and this is achieved by maintaining proliferation competence in the meristems that contain a pool of undifferentiated cells that proliferate (Inze and De Veylder, 2006). As cells leave the meristematic zone they stop dividing and they differentiate into specific tissues (Jakoby and Schnittger, 2004). Cell division and differentiation are well co-ordinated events, but the molecular mechanisms that maintain the balance between these processes are still not well understood (Magyar, 2008).

1.3.1 Cyclin-dependent kinases (CDKs)

The regulation of cell cycle is conserved in eukaryotes and centred on the cyclin dependent kinases (CDKs). In plants, both the CDKs and the associated cyclins diverge from what we know in yeast and animals (Mironov *et al.*, 1999). A-type CDKs contain a conserved PSTAIRE cyclin-binding motif, and function throughout the cell cycle, similar to their yeast and mammalian counterparts. Plant-specific CDKs include the mitotic CDKB (Weingartner *et al.*, 2003). How the function of this central cell cycle regulatory mechanism is connected to environmental signals and developmental cues is little understood.

1.3.2 E2F/RB regulatory mechanisms

1.3.2.1 The Retinoblastoma protein

The major substrate for CDK control is the Retinoblastoma protein (RB) in animals, which is also conserved in plants and known as RETINOBLASTOMA RELATED (RBR) (Inze and De Veylder, 2006). In animal, cells Rb is a tumour suppressor which is mostly due to its repressive function on genes involved in cell proliferation (Figure 1.4A) (Shao and Robbins, 1995). RBR has been studied mostly in *Arabidopsis*, and it is becoming clear that (as in animal cells) it is a broadly utilized transcriptional regulator that provides a convergence point for signaling pathways to reprogram gene expression not only for the regulation of cell proliferation, but also for cell differentiation and metabolism (Chinnam and Goodrich, 2011). RBR functions as an adaptor protein to dynamically regulate the assembly or disassembly of protein complexes on distinct batteries of genes . These RBR target genes provide the multiple outputs through which RBR operates and effect a number of major yield determinants, including the timing when photosynthetic capacity of cells are switched off, the allocation of photosynthates, the number of cells produced to build an organ by regulating cell proliferation and cell proliferation competence (stem cell maintenance, meristem maintenance), regulating the transition from proliferation to differentiation and promotes cellular differentiation (Gutzat *et al.*, 2012). How RBR might carry out these multiple functions is little understood in plants.

1.3.2.2 RB interacting proteins

We know that many known RB interacting proteins are shared between plants and animals, including the E2F/DP transcription factors, the R1R2R3Myb proteins that form the so-called DREAM complex (Haga *et al.*, 2007), and components of chromatin modifiers, such as the polycomb repressive complex (PRC2) (Gutzat *et al.*, 2012). These complexes are formed to repress gene expression on specific genes. These components of RB complexes are conserved between plants and animals, but RBR complexes can also have plant specific components. It was shown that RBR can be recruited to promoters and regulate transcription not only through E2Fs, but also through other transcription factors, such as SCARECROW (Cruz-Ramirez *et al.*, 2012).

RB complexes are dynamically formed and interchange. This might be directed both by the availability of complex components and by signaling pathways that alter the structure and properties of RBR and/or RBR-associated proteins through posttranslational modification, prevalently by phosphorylation (Munro *et al.*, 2012). In agreement, the *Arabidopsis* RBR is the major target for CDKA function (Nowack *et al.*, 2012). CDKA activity is regulated by the activating CYCDs, CYCA3s and the CDK inhibitor Kip-related proteins (KRP) (Van Leene *et al.*, 2011). It was found that *Arabidopsis* RBR is targeted by CYCD3;1/KRP2/CDKA kinase *in vivo* (Magyar et al., 2012). RBR is also phosphorylated by a number of other protein kinases including MAPKs (Munro *et al.*, 2012) and S6K1 (Henriques *et al.*, 2010).

1.3.2.3 The E2F/RB network

The E2F/RB network is remarkably conserved between the plant and animal kingdoms. E2F and RB homologues have been identified from the unicellular green alga *Chlamydomonas reinhardtii*, to the higher plants including *Arabidopsis* (Inze and De Veylder, 2006). Interestingly, nearly 25% of the *Arabidopsis* genes in total contain consensus E2F binding element(s) within their promoter region, which is significantly higher than what was found in the mammalian genome (~2%), indicating that E2Fs could play a fundamental role to regulate many aspects of plant development (Vandepoele *et al.*, 2005). Functional characterization of the plant E2F and RB homologues initially focussed on their involvement in cell proliferation (Gutierrez, 1998). In analogy to animal systems, plant E2Fs have been classified as

transcriptional activators (E2FA and E2FB), or transcriptional repressor (E2FC). However, most of these data were derived from overexpression studies (Magyar, 2008). Genome wide expression profiling has shown that ectopic co-expression of E2FA with the dimerization partner A (DPA) not only leads to transcriptional activation but also to the transcriptional repression of a large number of genes, perhaps indicating that the same E2F/DP complex can be activator on some genes while repressor on others (Fig 1.4 A,B) (Vandepoele *et al.*, 2005).

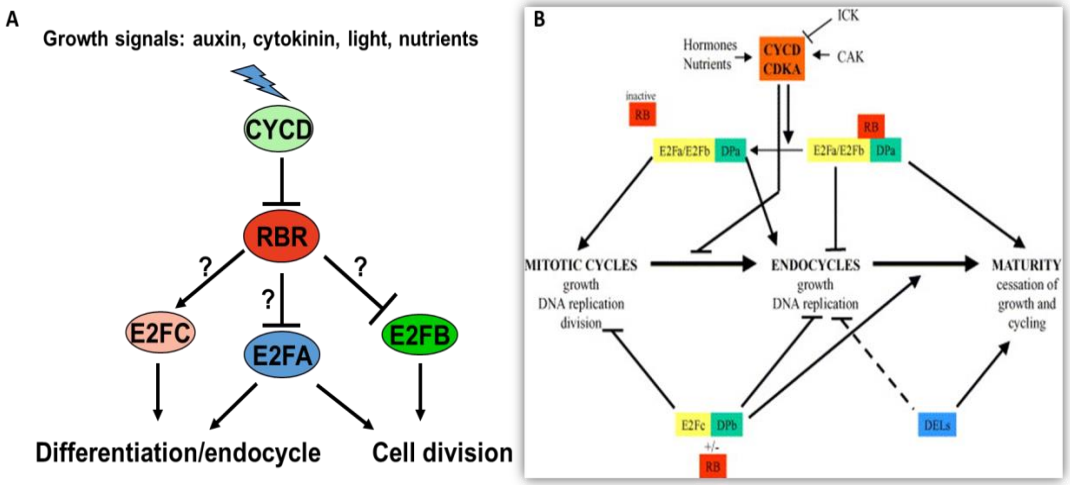

Figure 1.4 Regulation of the cell cycle. (**A**) RBR as a central regulator of cell proliferation. (**B**) The E2F/RB network regulates cell growth and development.

The interactions between E2F and RBR proteins were biochemically characterised during the development of the first leaf pairs in *Arabidopsis* by purifying RBR-E2F complexes in a series of co-immunoprecipitation experiments using specific antibodies against E2Fs, DPs and RBR. These have led to a surprising finding; the two activator E2Fs; E2FA and E2FB are in complex with RBR in young leaves full of proliferating cells, while there was hardly any detectable interaction between these E2Fs and RBR when leaf cells exit proliferation and enter into the cell expansion and differentiation phases (Figure 1.4A). Thus opposite to the canonical model whereby RBR E2F interaction is required for the exit from proliferation, RBR could function together with E2Fs in proliferating cells (Magyar *et al.*, 2012). Furthermore, it was shown that RBR interaction with E2FA is not disrupted by

CDKA phosphorylation upon CYCD3;1 overexpression, but stable, while E2FB interaction with RBR is sensitive to CYCD3;1 level and disrupted when cells actively proliferate e.g. in the presence of sucrose (Magyar *et al.*, 2012).

The ultimate aim to unravel the E2F/RB regulatory networks is to identify the key downstream effectors for the E2F/RB pathway involved in the cell proliferation and differentiation, build a transcriptional regulatory network and connect these to upstream signalling mechanisms that influence plant growth in response to environmental stress conditions.

1.3.3 Endocycle/endoreduplication

Endoreduplication represents extra rounds of DNA synthesis without intervening mitosis leading to increased ploidy levels (Edgar and Orr-Weaver, 2001). This modified cell cycle is coming in differentiated but metabolically active cells in animals, such as in salivary glands or during oogenesis in *Drosophila* and placenta formation in mammals (Lee *et al.*, 2009).

In plants, and specifically in *Arabidopsis* endocycle is common in leaves, hypocotyls root hairs and trichomes. Typically these cells are differentiated but continue to rapidly expand. Because polyploidization is prohibitive of further cell divisions, exit to endocycle leads to terminal differentiation of the cells. Therefore the timing of endocycle during organ development is very important determinant of cell number and final organ sizes (Bogre *et al.*, 2008). The regulation of endocycle onset is also conserved in distant eukaryotic such as human, *Drosophila*, and *Medicago* species. This involves the anaphase-promoting complex/cyclosome (APC/C) activator genes; *CDH1*, *FZR*, and *CCS52* respectively (Edgar and Orr-Weaver, 2001). In *Arabidopsis*, the expression of the *CDH1*/*FZR* orthologous *CCS52A* is controlled by the atypical E2F transcription factor, E2Fe/DEL1 (Boudolf *et al.*, 2004). Endoreduplication and mitotic cell cycle progression are connected to each other. DNA ploidy increased or decreased with premature or delayed exit from the cell division program, respectively (Schnittger *et al.*, 2002; Dewitte and Murray, 2003; Boudolf *et al.*, 2004; Verkest *et al.*, 2005; Churchman *et al.*, 2006).

Main aims

1. To gain insights into the role of MAPK signalling pathways in regulating plant growth through the characterisation of *Arabidopsis* lines with inducible expression of MKK gain of function mutants.

2. Use loss of function MAPK and MKK lines to connect leaf growth to MAPK signalling.

3. Connect the E2F/RB regulatory network to MAPK signalling.

Chapter 2: Materials and methods

All methods are described as accurate as possible and further experimental details are mentioned when required in results. Appendices contain a list of materials and equipments that used in this study (Table A2.1). Protocols for preparation of solutions are described in appendices where indicated. Unless otherwise stated, standard laboratory chemicals were supplied by VWR (VWR International). Therefore, most chemicals were used of AnalaR grade.

2.1 Seed collection and storage

Mature plants were loosely wrapped in drying bags and watering was stopped. Seeds were released mechanically from siliques, whilst in the bags, via rubbing and scratching between hands. The bag was snipped at a corner to release seeds on to a sieve (500 μm pore size) that was placed on top of a clean A4 plain white paper. Sieving was performed 2-3 times and the seeds transferred into a pre-labelled 1.5 ml microfuge tube. Seeds were stored at room temperature. Long term storage of seeds was at -20°C.

2.2 *In vitro* culturing of *Arabidopsis*

2.2.1 Seed sterilisation

Seed sterilisation was carried out under a laminar flow. The approximate number of required seeds was placed into a 1.5 ml microfuge tube; if more than ¼ of the microfuge tube was filled with seeds a subsequent microfuge tube was used. The seeds were soaked in sterile water for ~5 mins prior to sterilisation. Removal of water/solution from the microfuge tube was carried out using a heat sterilised needle attached to a vacuum pump. Seed sterilization was carried out as follows; 1 minute

in 100% EtOH, 10 minutes in a 2.5x dilution of commercial thick bleach, and 4-5 times washing with sterile H_2O. After removal of bleach, seeds were washed with sterile water several times to remove residual bleach. Seeds were finally left in sterile water and stored in +4 °C in the dark.

2.2.2 Culturing media

For the *in vitro* cultivation of *Arabidopsis* plants, half-strength germination medium (½ GM), a modified version of Murashige and Skoog medium (Murashige and Skoog, 1962) was used containing 2.2 g/L MS basal salts (Sigma-Aldrich), 1% sucrose (Sigma-Aldrich), 0.8% plant agar (Duchefa), 10 ml 2-(N-morpholino)ethanesulfonic acid (MES) buffer (see Table A2.2 for MES protocol). The pH of the mixture was set to pH 5.8 using KOH. The media was sterilized by autoclaving at 120 °C for 15 mins, cooled and poured into square or circular cell culture Petri dishes.

2.2.3 Stratification of seeds and plating

Seeds were plated using a p1000 Gilson pipette and excess water, dried under the laminar flow hood prior to sealing with a micro-porous tape. If different lines were used, plating multiple lines on a single plate was favoured and square Petri dishes were preferred. Plates with the plated seeds were stratified for at least 1 day in a +4 °C fridge. Seeds were not stratified for a period of more than 3 days.

For chemical induction of transgenic lines carrying the estradiol-inducible cDNA constructs (See Section 2.3.2), various concentrations (0.001 to 10μM) of 17-β-estradiol (Sigma-Aldrich) was used. Ethanol as the solvent was used for control (mock) treatments.

Plates were overlaid with nylon mesh (Sefar) of 20 μm pore size to prevent roots from growing into the medium. Depending on the experiment, more than 100 seeds were equally distributed on a square 150 mm plate. Various genotypes were grown together with their corresponding control line on the same plate. Germination rate was scored under stereo microscope.

2.2.4 Growth conditions

Plants were grown in a controlled environment growth room under white light, of a fluence rate of 140μmol m-2 sec-1 (+/- 20 μmol m-2 sec-1). The room was kept at 21°C and humidity in the room was kept below 80% and was generally maintained at 60-70%. Respective genotypes/mutants were selected in the presence of Hygromycin (20mg/l) or Norflurazone (50mg/l) depending on the transgenic construct.

2.3 *Arabidopsis* lines used in this work

2.3.1 Wild type line

Columbia-0 (Col-0) ecotype was the wild type (WT) line and the background of every transgenic line used in my work.

2.3.2 β-estradiol induced overexpression of *Arabidopsis* MKKs

The gain-of-function mutant forms (the phosphorylation sites at the activation loop were mutated to asparagines (E) to mimick the active state) of MKKs were generated by site-directed mutagenesis (MKK-GOF) for all 10 *Arabidopsis* MKKs. Each MKK then tagged with c-myc epitope tag at the N-termini and inserted into the expression vector, pER8GW, by Dr Elizabeth Hatzimasoura (PhD thesis). The c-myc epitope is derived from the product of the human c-myc gene and widely used to tag proteins for western blotting. The pER8GW vector was described (Zuo *et al.,* 2000). All these transformed lines 10 *Arabidopsis* MKKs and an unmodified pER8::GUS (empty vector) transformed to Col-0 *Arabidopsis* were provided as homozygous T3 selected lines for me (Table 2.1). pER8::MKK7 and pER8::MKK9 were used to further extensive studies.

2.3.3 Promoter-GUS reporter constructs

Two independent lines of MKK7::GUS as well as MKK9::GUS were kindly provided by Dr. Robert Doczi (Martonvasar, Hungarian Academy of Sciences, Hungary). The CYCB1;1::GUS line was provided by Dr Peter Doerner, Edinburgh University (Colon-Carmona *et al.,* 1999).

2.3.4 T-DNA insertion lines

The homozygous insertion lines of single mutants *mkk7-KO, mkk9-KO* and double mutants *mkk7/9-N5, mkk7/9-N3,* furthermore the *mpk3-KO* and *mpk6-KO* mutants were kindly provided by Dr Aleksandra Skirycz from Dirk Inze group, Ghent University, Belgium. All these mutant lines were derived from the SALK T-DNA collection. The *mpk3-KO* (SALK_151594) and *mpk6-KO* (SALK_073907) were previously published (Wang *et al.*, 2007). The *mkk7-KO, mkk9-KO, mkk7/9-N5, mkk7/9-N3 lines were identified by* Dr Aleksandra Skirycz (unpublished). In the *mkk9-KO* mutant (SALK_071878) the T-DNA insertion is in the 5'utr coding region of the *MKK9* (At1g73500) gene. In the *mkk7-KO* line (*mkk7-N5*; SALK_009430) the insertion in the promoter 231 bp upstream of the START codon. The *mkk7/9-N5* double mutant was produced by crossing the *mkk7-KO* and *mkk9-KO*. Besides, Dr Skirycz was also provided another double mutant line *mkk7/9-N3,* in which the *mkk9-KO* was crossed with another allele of *mkk7* (*mkk7-N3, SALK_129504*). The *mkk7-N3* line is also a promoter mutant, the T-DNA is located 459 bp from the START codon.

2.3.5 Lines with elevated E2Fs or RBR protein expression

The independent E2FB-GFP (pE2FB::gE2FB::gGFP) lines were previously generated by Dr Zoltan Magyar in our laboratory via three-way Gateway cloning (Invitrogen, unpublished data). A GFP antibody was used to detect the presence of the protein in these lines.

The E2FA-GFP (pE2FA::gE2F	**Construct/genotype**	**Selection Marker**	**Origin**	**Notes**
MKK1 (1)	pER8::mycGOFMKK1	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK1 (2)	pER8::mycGOFMKK1	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK2 (1)	pER8::mycGOFMKK2	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: +
MKK2 (3)	pER8::mycGOFMKK2	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK3 (4)	pER8::mycGOFMKK3	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK3 (10)	pER8::mycGOFMKK3	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK4 (3)	pER8::mycGOFMKK4	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: +++
MKK4 (11)	pER8::mycGOFMKK4	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: +++

MKK5 (B1)	pER8::mycGOFMKK5	Hygromycin	E. Hatzimasoura PhD Thesis	Expession: +++
MKK6 (4)	pER8::mycGOFMKK6	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK6 (8)	pER8::mycGOFMKK6	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK7 (1)	pER8::mycGOFMKK7	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: ++
MKK7 (2)	pER8::mycGOFMKK7	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: +
MKK8 (4)	pER8::mycGOFMKK8	Hygromycin	E. Hatzimasoura PhD Thesis	Expression not detected
MKK8 (5)	pER8::mycGOFMKK8	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK9 (1)	pER8::mycGOFMKK9	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: ++
MKK9 (2)	pER8::mycGOFMKK9	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: ++
MKK10 (2)	pER8::mycGOFMKK10	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
MKK10 (3)	pER8::mycGOFMKK10	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
Empty Vector	pER8::GUS	Hygromycin	E. Hatzimasoura PhD Thesis	Expression: not detected
mkk7	T-DNA insertion mutant	Kanamycin	Provided by A. Skirycz	-
mkk9	T-DNA insertion mutant	Kanamycin	Provided by A. Skirycz	-
mkk7/9-N3	Double mutant line	Kanamycin	Provided by Skirycz	-
mkk7/9-N5	Double mutant line	Kanamycin	Provided by A. Skirycz	-
mpk3	T-DNA insertion mutant	Kanamycin	Provided by A. Skirycz	-
mpk6	T-DNA insertion mutant	Kanamycin	Provided by A. Skirycz	-
MKK7::GUS	*proMKK7::GUS*	Kanamycin	Provided by R. Doczi	-
MKK9::GUS	*proMKK9::GUS*	Kanamycin	Provided by R. Doczi	-
CYCB1;1::GUS	*proCYCB1;1::GUS*	Kanamycin	(Colon-Carmona *et al.*, 1999)	-
E2FA	prE2FA82/1-GFP	Norflurazone	(Magyar *et al.*, 2012)	Elevated E2FA expression
E2FB	prE2FB72/5-GFP	Norflurazone	Provided by Z. Magyar	Elevated E2FB expression
E2FC	prE2FC7/2-GFP	Norflurazone	Provided by Z. Magyar	Elevated E2FC expression
RB	prRBR-GFP	Norflurazone	Provided by B. Horvath	Elevated RBR expression

Table 2.1 List of *Arabidopsis* lines used in this study

2.4 Analysis of plant growth rates

Eight days after germination, seedlings were transferred onto media supplemented with 25 mM mannitol (Sigma-Aldrich) or control plates (1/2 GM) by gently lifting the nylon mesh with forceps in sterile conditions. In some of the experiments, the seedlings were transferred one-by-one. For growth rate analysis of seedlings, images were taken with Nikon D70 camera. Alternatively, third pair of leaves were harvested at different time points after transfer. After clearing with 100% methanol, leaves were mounted in 100% lactic acid on microscopy slides. For each experiment, 10 to 15 leaves were photographed with a stereo microscope, photographs of leaves and drawings were used to measure leaf and cell area, respectively, with ImageJ v1.41o (NIH; http://rsb.info.nih.gov/ij/), from which the cell numbers were calculated (Fig 2.1).

Figure 2.1 Leaf surface area measurements with ImageJ. Pictures of *Arabidopsis* seedlings were taken individually with the same magnification by stereo microscope and used to measure surface area of cotyledon or leaf discs with ImageJ software (http://rsb.info.nih.gov/ij/). To define the spatial scale of an image, straight line selection tool was used to make pixel selection that corresponds to a known distance.

Digital images of seedlings were taken on the Nikon SMZ1500 stereo microscope equipped with a Nikon DXM1200 camera with the addition of an external lighting source by using the NIS elements software, NIS Freeware 2.10. For new leaves cleared in methanol, the Hoyer's solution was used for mounting the cleared leaves as described (Masubelele *et al.*, 2005).

2.4.2 Primordia size measurement

Slides were mounted on small drop of Hoyer's solution (this will make

seedlings translucent) and arranged in a row by using needle and tweezers, observing under stereomicroscope open the cotyledon without damaging them, to expose the primordia, examined under Invert Microscope-Nikon Eclipse (Nikon Camera DXM 1200) TE300 DIC/Phase Contrast with 20x magnification, or analysed under the DIC Microscope-Nikon Optiphot 2 40x, or 20x (or 10x depending on age of seedling). Photographs were taken using NIS-element software (Freeware 2.10), then measured the area of leaf primordial by using ImageJ with graticule of scale set using a straight line drawing tool to draw a line of known distance: on the 'Set scale' function the known distance and units of measure were inserted. The pixel value was noted for future use and the 'Global' tab selected if subsequent images were to be measured (Fig 2.1), then analysed the data on excel and plotted them insert the error bar based on standard error (SE) and one-way ANOVA performed with Tukey's multiple comparison the mean of each column with mean of every column. An average of 30 leaf primordia (replicates) were analysed in each condition for every line/mutant. The whole primordia's mean was determined by drawing from the base of the leaf to distal end upper part of the leaf.

2.5 Light-induced de-repression of shoot apical meristem

Seeds were plated on culturing media without sucrose and kept in 4°C for 3 days in dark, then exposed to red light pulse for approx. an hour. After, the culturing plates were wrapped in aluminium foil and kept in dark in an LMS cooled incubator (V) for 3 days (21°C). Three days later, one part of the etiolated seedlings were collected (3 days in dark sample), the rest of the seedlings either kept in the dark for two more days (5 days in dark sample) or transferred in continuous white light (200 µmol m-2 s-1). The light induced seedlings were collected 1 and 2 days later, respectively (3 days in dark+1 day in light and 3 days in dark+2 days in light samples) Dark grown seedlings were collected under green dim light. Seedlings were used to perform β-glucuronidase assay (See Section 2.8) and subsequent differential interference contrast (DIC) microscopy (Nikon Optiphot-2).

2.6 Flow cytometry

Flow cytometry analysis was used to determine DNA content of cells. Flow solutions were always stored in +4 °C conditions.

2.6.1 Harvesting samples

New leaves or cotyledons were harvested from seedlings using micro-fine tweezers. The petiole tissue was removed. For plants younger than 7/8 days old 4 - 6 new leaves were harvested. Small leaves were harvested by flattening the seedlings on the agar, holding out the cotyledons with one tweezer and pinching at the base of both new leaf pairs with the second tweezer. Upon harvesting leaves were immediately placed into a circular petri dish (9 cm diameter) containing nuclei extraction buffer.

2.6.2 Preparation of samples

The Partec kit comprises of two solutions: 1. Nuclei extraction buffer; 2. nucleic acid staining solution (1 mg/mL 4',6-diamidino-2- phenylindole (DAPI) in 1 mL of 45 mM $MgCl_2$, 30 mM sodium citrate, 20mM 3-(N-morpholino) propanesulfonicacid, pH7, and 1%TritonX-100 (Galbraith et al., 1983). One mL of nucleic acid staining solution was added to the extracted nuclei). Flow solutions were decanted into smaller volumes and placed on ice. Contamination between the two solutions was avoided and contamination of sample prep and solution was particularly carefully avoided. For new leaves younger than 6-10 days ~4 drops of nuclei extraction buffer was used. Samples were not left on the nuclei extraction buffer for longer than 5mins.

The samples were prepared using only double edged razor blades, (Boots, Wilkinson sword or Tesco brands). Leaves were centred on the petri dish with the nuclei extraction buffer, any excess buffer was run down the sides of the petri dish. Leaf tissues were homogenized using a gentle but firm tapping motion, avoiding tearing of tissue, and rotation of the angles of chopping was necessary. The mid region of the blade was used to chop larger samples and the ends of the blades were used to chop very small samples. Samples were chopped until they appeared as fine pieces. Added 1.0 - 1.5 ml of DAPI solution with a Gilson pipette. A few drops of the DAPI solution were run down the used edge of the blade increasing the volume of the sample. Blade edges were wiped using a tissue, re-used up to four times but changed for the different lines.

The sample was pre-mixed on the petri dish, by pipetting, and collected into a plastic cuvette with a fine filter placed on top. Samples remained incubated on ice prior to running and were left wrapped in foil in the +4 °C fridge to be run the

following day if needed, (samples were left for a maximum of 2 days in +4 °C).

2.6.3 Running of samples

Samples were placed in a plastic seed box on ice to keep cuvettes dry. Upon turning on the flow cytometer the cleaning was carried out as follows: decontamination solution x1; cleaning solution x2; sterile water x1; cleaning solution x1; final water left running. Water washes were always carried out between samples.

The lower threshold for fluorescence intensity was ~30, higher threshold at ~999 and the gain set at ~485. The first peak (2n) was set at the arbitrary value of 50 and the second peak (4n) at 100 using a flower sample as a control (endoreduplication does not occur in those samples). With these parameters up to five peaks (2n, 4n, 8n, 16n, 32n) could be detected. Samples were run at ~50 cells/second. The gain was readjusted if necessary to meet the 50 and 100 intensity mark. This was always reconfirmed by a control flower sample. The nuclei were analysed with a CyFlow flow cytometer with the FloMax Software (Partec).

2.6.4 Data Analysis

Data was presented as peaks using the Flomax program. Data analysis was also achieved with the use of the Flomax software; values obtained were based on the area calculations of each peak via the software indicating the cell distribution as a percentage of DNA content.

Primarily, analysis was carried out via the automated ‘peak analysis’ tool: (Analysis/Peak Analysis). A minimum of three replicates were used and the data averaged and plotted as a ‘100% stacked column’. Where only 2n and 4n peaks were present, ‘cell cycle analysis’ was additionally carried out, if desired. This indicated percentage of cells in G1, S and G2/M phases, where G1 is equivalent to the 2n cell population and G2/M is equivalent to the 4n cell population and S phase as the valley of cells in between G1 and G2/M. the data of each phase was written down at the time of running sample or later by taking screenshot than transferred to notepad and subsequently on excel for further analysis such as plotting graphs and performed statistical tests.

2.7 Epidermal cell size analysis

The measurement of epidermal cell size of new leave was achieved based on four replicates of transgenic lines with corresponding wild type.

2.7.1 Preparation of samples

Whole seedlings were soaked in absolute methanol for 12 - 36 hours, or longer until seedlings became clear in colour. Methanol was replaced by lactic acid permanently. Leaves were pinched at the petiole and placed on a microscopic slide, adaxial side up, mounted with lactic acid. A cover slip was gently placed on top.

2.7.2 Microscope and functions

Images of the adaxial epidermis were taken using a DIC Nikon Optiphot-2 microscope using Nomarski optics, a DXM1200 camera and NIS-elements AR program. Using the x20 magnification half the leaf epidermis was imaged using the 'Grab Large Image' tool. The first image captured would start at the tip of the leaf and the subsequent image would be right to the previous captured image, with aid of a twenty five percept transparency function, the image was grabbed ('Grab') if transparency was manually met. The focus was also adjusted if needed. This process was repeated until the outer most edge of the leaf was anticipated, the following image was below ('Meander') and moved in the left direction ('Grab'), as described above, until the first image was directly above and again the following image was below the image captured. Upon completion all images were collaged into a single image of half the leaf epidermis ('Finish').

2.7.3 Measurement protocol

Epidermal cell walls were manually drawn using the 'Paint' software (Microsoft), on images from section 2.7.1. If cell walls were not clear in a particular region then the region was avoided in such a way that the absence of cell walls would be apparent, this would avoid that area being considered a large cell and being measured incorrectly. Cell size measurements of epidermal cells were carried out using the free downloadable software 'ImageJ'-'http://imagej.nih.gov/ij/'. Using an image of a gratitude the scale (see above section).

2.8 β-glucuronidase (GUS) assay

Histochemical detection of the β-glucuronidase (GUS) activity (Jefferson *et al.*, 1987) was performed by following a modified protocol of Jim Murray's lab (see Appendices, Table A2.4) with MKK7::GUS, MKK9::GUS and Cycb1;1::GUS reporter lines. Seedlings or dissected leaves were harvested into 2 ml ice-cold 90% acetone in 6-well cell culture plates and incubated for 30 mins in +4 °C. Subsequent steps were carried out in the same microtiter plates. The samples were washed with 2 ml 70% ethanol 3 times to remove chlorophyll, then 2 times with sodium phosphate buffer (pH=7.0). After the last wash 2 ml x-gluc reaction solution was added (see Appendices, Table A2.3) and vacuum infiltration was performed by using Speed vac for 10 min, as recommended by (Donnelly *et al.*, 1999). Subsequently, the samples were overnight incubated at 37 °C in the dark. The reaction was stopped by changing the x-gluc reaction solution to the 'stop' solution (3:1 methanol: acetic acid). Samples remained in this solution for 2 hours or longer until the chlorophyll was cleared. Finally, two washes with 70% ethanol were carried out.

2.8.1 Mounting of samples

When samples were used for DIC microscopy the 70% ethanol was replaced with Hoyer's solution (see Appendices, Table A2.5) and then mounted onto microscope slides under a stereo microscope with the use of fine tweezers. Additional Hoyer's solution was added, if needed, once the cover slip was gently placed clear nail polish was used to seal it. DIC images were taken by Nikon Optiphot-2 microscope.

2.9 Confocal laser scanning microscopy to study root cells

Cellular organization of the root tips of MKK7 and MKK9 overexpression lines were examined under confocal microscopy. The roots of young seedlings were dipped into 10 µg/ml propidium iodide solution for approx. one minute and then washed in distilled water to remove the stain. After this, roots were mounted under a coverslip and Olympus FV1000 confocal laser scanning miscroscope was used to take images of the root tips.

2.10 Protein expression analysis: Western blot

Frozen plant samples were ground in a mortar and total protein was extracted with extraction buffer (see Appendices, Table A2.6). The extraction were centrifuged for 10 minutes (13,000 rpm, 4°C). The supernatant was transferred to new tubes. Protein concentrations were quantified with Bradford method, preparing reaction mixtures of 2 µl supernatant, 800µl distilled water and 200 µl Bradford's dye. The Bradford reaction mixtures were mixed well and transferred to cuvettes for spectrophotometer measurements. For blank 2 µl of extraction buffer was added instead of the supernatant. The protein extract was mixed with 5x SDS loading dye (0.312mM Tris pH 6.8, glycerol 50%, SDS 10%, 250mM DTT) and boiled at 95ºC for 5 minutes. For separation 25-50µg protein extract was loaded in 12% SDS polyacrylamide gels. The separated proteins from the gel were transferred to a PVDF membrane (Immobilon, Millipore) by electroblotting. Subsequently, the membranes were blocked with 5% skimmed milk in TBS-T buffer (0.05 M Tris-HCl, 0.15 M NaCl pH 7.5, 0.01% Tween 20) for 1 hour at room temperature. The following antibodies were used for probing: horseradish peroxidase conjugated anti-c-myc rabbit polyclonal antibody (Sigma-Aldrich) dilution: 1:1000, active MAPK (Erk1/2, Phospho-Specific-p44/42 MAPK) rabbit antibody (Cellsignalling) dilution: 1:1000, horseradish peroxidase conjugated anti-rabbit (Upstate) dilution: 1:10,000, antiAtMPK3 and antiAtMPK6 (Sigma-Aldrich) dilution: 1;1000). The chemiluminescent detection of western signals was carried out with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) using CL-XPosure Film (Thermo Scientific). For loading control the membranes were stained with 0.1% (w/v) Coomassie Brilliant Blue (Sigma-Aldrich) or 0.1% (w/v) Ponceau S (Sigma-Aldrich) in 5% acetic acid solutions.

2.11 Genetic crosses of transgenic lines

In order to further characterize genetic interactions between different lines, genetic crosses were performed. The MKK7 overexpressing line was crossed with the E2FA-GFP and E2FB-GFP-72 lines. For the crossings sepals and petals of immature buds from the maternal plants were carefully removed with a pair of sharp-pointed tweezers, therefore the stigma could be easily accessed. Anthers were used

from the paternal plants to rub onto the stigma of the maternal plants under stereo microscope. After successful crosses siliques were developed, later the mature siliques were collected in an eppendorf tube. F1 and after F2 generation seeds were sown without any selection to reach F3 generation. Typically, 40-48 of F3 generation plants were generated from the crossings (Fig 2.2). The seeds of the F3 generation plants were used for the sequential co-selection of antibiotic resistant growth for the parental antibiotic resistance markers (Norflurazon and Hygromycin B, Sigma-Aldrich).

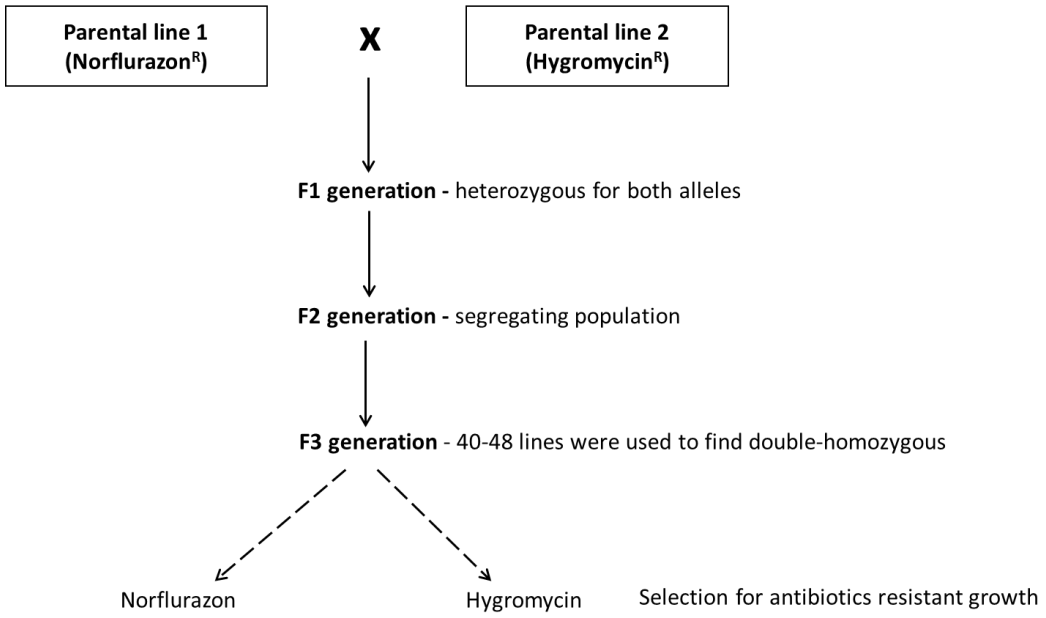

Figure 2.2 Flowchart of genetic crossings. In the genetic crosses stigmas of the Parental line 2 plants (mother plants) were pollinated with the pollen of the Parental line 1 plants (father plants). The presence of both homozygous alleles was selected in the F3 generation by monitoring antibiotics resistance growth.

2.12 Statistical analysis

Standard deviation (SD) and standard error (SE) were calculated and inserted on graphs for all relevant data. One-way analysis of variance (ANOVA) was performed to find out significant differences on the mean of at least triplicate samples. Tukey's test was used when I compared every mean with every other mean. I used Dunnett's multiple comparison tests to compare every mean to a control mean.

Chapter 3: Using inducible overexpression of gain of function MKKs to connect MAPK signalling pathways to leaf and meristem growth and cell proliferation in *Arabidopsis*

3.1 Introduction

In response to an upstream signal, a MAPK kinase kinase (MAPKKK) is activated by phosphorylation and this in turn phosphorylates and activates a MAPK kinase (MKK), which phosphorylates and activates a MAPK. Active MAPKs interact with and phosphorylate a wide range of downstream targets to bring about a specific cellular response (Rodriguez *et al.*, 2010). This architecture of signalling pathway relying on phosphorylation cascade is conserved from yeast to plants and animals. However, mitogen-activated protein kinase (MAPK) cascades have expanded considerably in land plants from a much simpler linear pathway that existed in the common ancestor of yeast animals and plants (Doczi *et al.*, 2012). The completion of *Arabidopsis* genome-sequencing project has revealed the existence of genes encoding 20 MAP kinases (MAPKs), 10 MAPK kinases (MKKs) and over 60 MAPK kinase kinases (MKKKs) suggesting that discrete combinations of kinases could be used to bring about specific responses (Ichimura, 2002).

Specificity in signalling processes might arise from association with interacting partners, subcellular localization of signalling proteins, scaffolding proteins that insulate pathways and by modulating signals amplitude and duration from upstream active kinases (Saito, 2010; Wimmer and Baccarini, 2010; Ovecka *et al.*, 2014; Wortzel and Seger, 2011).

At present the challenge is to determine how the large number of *Arabidopsis* MAPK signalling components (around 120) are organised into pathways and specifically transmit signals to select appropriate cellular responses (Table 3.1).

Gene	Function	Reference
MKK1	defence response	(Meszaros *et al.*, 2006)
MKK2	cold stress signalling, salt stress signalling	(Teige *et al.*, 2004) (Meszaros *et al.*, 2006)
MKK6	cell division	(Calderini *et al.*, 1998) (Soyano *et al.*, 2003) (Meszaros *et al.*, 2006)
MKK3	jasmonate signalling, pathogen response	(Asai *et al.*, 2002) (Takahashi *et al.*, 2007) (Doczi *et al.*, 2007)
MKK4	stomata development, pathogen response	(Asai *et al.*, 2002) (Doczi *et al.*, 2007) (Wang *et al.*, 2007)
MKK5	defence response	(Asai *et al.*, 2002) (Ren *et al.*, 2002) (Wang *et al.*, 2007)
MKK7	systemic acquired resistance, polar auxin transport	(Dai *et al.*, 2006) (Zhang *et al.*, 2007)
MKK8	not identified	(MAP_Kinase_Group, 2002) (Alzwiy and Morris, 2007)
MKK9	negative regulatotion of seed germination, activation of MPK3/MPK6 ethylene and camalexin biosynthesis, stress response, senescence	(Dai *et al.*, 2006) (Zhou *et al.*, 2009)
MKK10	not identified	(MAP_Kinase_Group, 2002) (Alzwiy and Morris, 2007)
MPK4	defence response, salicylic acid biosynthesis, ROS homoeostasis cold stress signalling salt stress signalling	(Teige *et al.*, 2004) (Nakagami et al., 2005) (Qiu JL, 2008)
MPK5	cold stress signalling salt stress signalling	(Teige *et al.*, 2004)
MPK3 and *MPK6*	ethylene biosynthesis, pathogen signalling, stomata development jasmonate signalling	(Asai *et al.*, 2002) (Liu and Zhang, 2004) (Ohashi-Ito and Bergmann, 2006) (Doczi *et al.*, 2007) (Kanaoka *et al.*, 2008) (Lampard *et al.*, 2008)

Table 3.1 Overview of known MKK and MPK functions in plants

To start addressing the functions of plant MAPK pathways, previously in our laboratory gain-of-function (GOF) mutant forms of all the Arabidopsis *MKK* genes were produced by site directed mutagenesis of the activation loop mimicking phosphorylation by exchange of phosphoacceptor S/T amino acids to glutamic acid (see Section 2.3.2). Arabidopsis lines were generated that expressed the *MKK-GOF* genes under the control of the estradiol-inducible promoter of the pER8GW vector (Zuo and Chua, 2000)(Figure 3.1). The chemical inducible system is a powerful tool for conditional and experimental induction of specific signalling pathways allow sensitive and rapid (within 15-60 mins) induction of gene expression and by using different β-estradiol concentrations, it is possible to some extent to titrate the effect. These lines have been provided me for my studies.

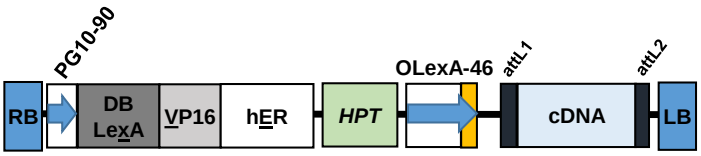

Figure 3.1 T-DNA of the pER8GW expression vector. The pER8GW is the Gateway cloning compatible version of the pER8 vector (Zuo *et al.*, 2000; Shelden and Roessner, 2013). The PG10-90 promoter region is continuously expressing the XVE protein. The XVE coding sequence consists of the DNA-binding domain of LexA bacterial repressor protein (DB LexA), the transcription activation domain of VP16 and the C-terminal region of the human estrogen receptor (hER). The HPT is a resistance gene, encodes the Hygromycin phopshotransferase. The OLexA-46 represents 8 copies of the LexA operator sequence fused to a 35S minimal promoter, which is located at the 5' end of the inserted cDNA and contains the TATA-box, the binding site for the RNA polymerase II complex. The attL1 and attL2 are the attachment sites for Gateway cloning. In the presence of β-estradiol, the XVE protein is able to bind to the OLexA-46 and initiate the transcription of the inserted cDNA. (RB: right border, LB: left border).

Previously, it was shown that constitutive overexpression of *MKK7* gives rise to meristemless seedlings, whereas constitutive overexpression of *MKK9* led to dwarf plants with asymmetric meristems. Moreover microarrays and the transcription profiles data showed the major role of MKK7 and MKK9 action in stress responses, shut down of protein translation and arrest of cell proliferation (Elizabeth Hatzimasoura PhD thesis). This suggests MKK7/9 cascades play critical functions in both cell proliferation and protein translation.

3.1.1 Hypothesis

MKK7 and MKK9 and downstream MAPKs arrest meristem and leaf growth.

3.2 Objectives

1. Examine the sequence similarities among the 10 *Arabidopsis* MKKs.
2. Characterise the estradiol-inducible MKK lines for the inducible expression of MKKs.
3. Find the activation of downstream MAPKs when GOF-MKK7 and MKK9 are inducibly overexpressed.
4. Characterise the leaf growth, meristem arrest cellular phenotypes, cell cycle parameters when MKK7 and MKK9 are inducibly overexpressed.

3.3 Results

3.3.1 Comparison of the 10 MKKs with bioinformatic tools

First I compared the 10 *Arabidopsis* MKK proteins by preparing a multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2). Then I also analysed their amino acid sequences in the PROSITE protein domain database http://prosite.expasy.org/ for protein motif detection. In all the ten MKKs the MKK domain was detected, however the MKK4 also contains a nuclear transport domain (NTF2), which is localised at the C terminal end of the MKK4 protein (Fig 3.2).

```
MKK4      MRPIQSPPGVSVPVKSRPRRRPDLT-LPLPQRDVSLAVPLPLPPTSGGSG--------GS 51
MKK5      MKPIQSPSGVASPMKNRLRKRPDLS-LPLPHRDVALAVPLPLPPPS-------------S 46
MKK7      --------------MALVRKRRQIN-LRLP-----------VPPLSV------------- 21
MKK9      --------------MALVRERRQLN-LRLP-----------LPPISD------------- 21
MKK8      --------------MVMVRDNQFLN-LKLSP----IQAPTTIPPCRF------------- 28
MKK10     --------------MTLVRERRHQEPLTLS-----------IPPLIYHG----------- 24
MKK1      ----MNRGSLCPNP-ICLPPL-EQSISKFL---------TQSGTFK-----DGDLRVNKD 40
MKK2      ----MKKGGFSNNLKLAIPVAGEQSITKFLRKGFGSLCRTQSGTFK-----DGDLRVNKD 51
MKK6      ---MVKIKSNLKQLKLSVPAQ-ESPISSFL---------TASGTFH-----DGDFLLNQK 42
MKK3      ---MAALEELKKKLSPLFDAEKGFSSSSSLDPN-DSYLLSDGGTVNLLSRSYGVYNFNEL 56
                                                        .
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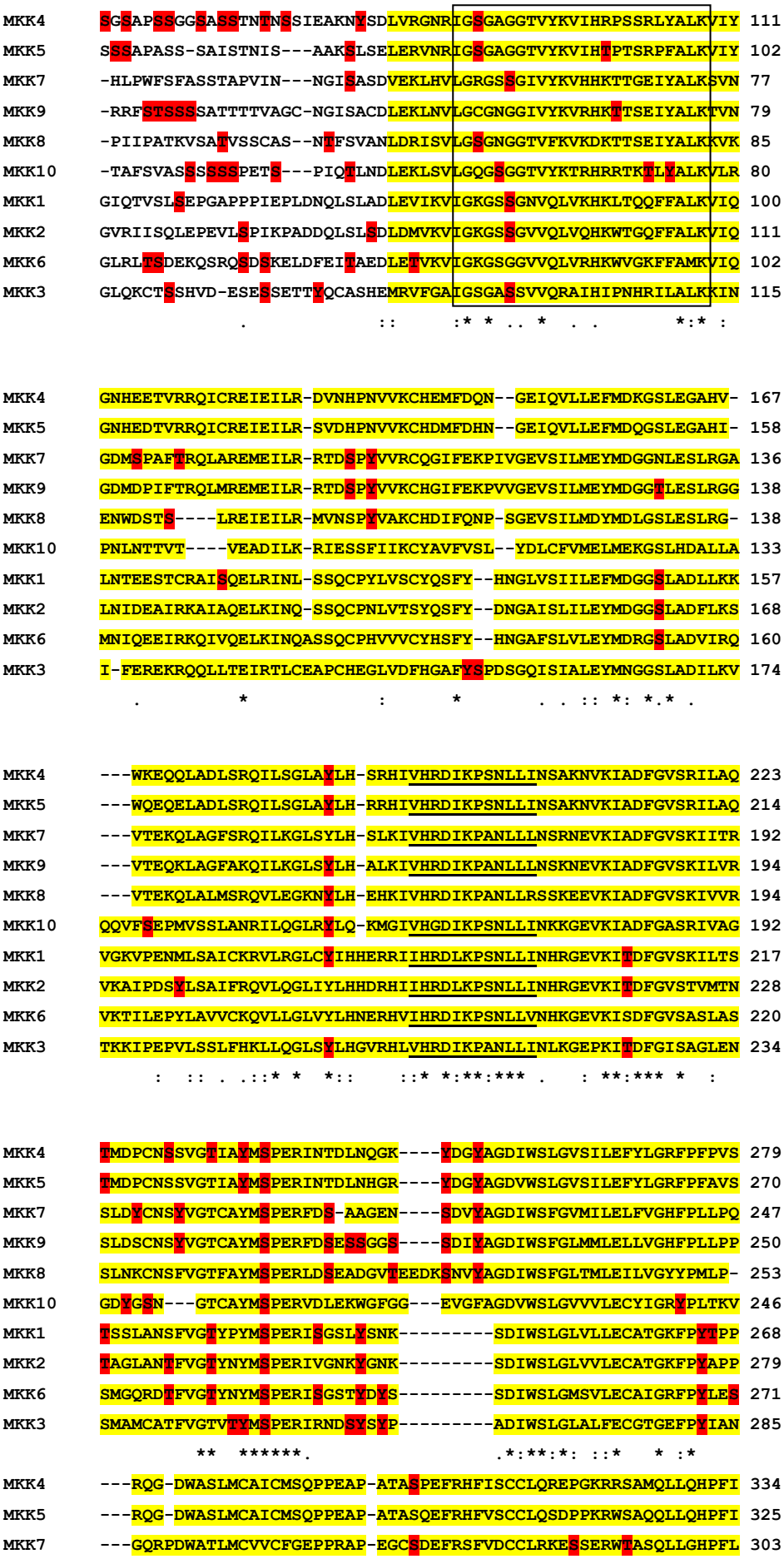
MKK4 SGSAPSSGGSASSTNTNSSIEAKNYSDLVRGNRIGSGAGGTVYKVIHRPSSRLYALKVIY 111
MKK5 SSSAPASS-SAISTNIS---AAKSLSELERVNRIGSGAGGTVYKVIHTPTSRPFALKVIY 102
MKK7 -HLPWFSFASSTAPVIN---NGISASDVEKLHVLGRGSSGIVYKVHHKTTGEIYALKSVN 77
MKK9 -RRFSTSSSSATTTTVAGC-NGISACDLEKLNVLGCGNGGIVYKVRHKTTSEIYALKTVN 79
MKK8 -PIIPATKVSATVSSCAS--NTFSVANLDRISVLGSGNGGTVFKVKDKTTSEIYALKKVK 85
MKK10 -TAFSVASSSSSSPETS---PIQTLNDLEKLSVLGQGSGGTVYKTRHRRTKTLYALKVLR 80
MKK1 GIQTVSLSEPGAPPPIEPLDNQLSLADLEVIKVIGKGSSGNVQLVKHKLTQQFFALKVIQ 100
MKK2 GVRIISQLEPEVLSPIKPADDQLSLSDLDMVKVIGKGSSGVVQLVQHKWTGQFFALKVIQ 111
MKK6 GLRLTSDEKQSRQSDSKELDFEITAEDLETVKVIGKGSGGVVQLVRHKWVGKFFAMKVIQ 102
MKK3 GLQKCTSSHVD-ESESSETTYQCASHEMRVFGAIGSGASSVVQRAIHIPNHRILALKKIN 115
MKK4 GNHEETVRRQICREIEILR-DVNHPNVVKCHEMFDQN--GEIQVLLEFMDKGSLEGAHV- 167
MKK5 GNHEDTVRRQICREIEILR-SVDHPNVVKCHDMFDHN--GEIQVLLEFMDQGSLEGAHI- 158
MKK7 GDMSPAFTRQLAREMEILR-RTDSPYVVRCQGIFEKPIVGEVSILMEYMDGGNLESLRGA 136
MKK9 GDMDPIFTRQLMREMEILR-RTDSPYVVKCHGIFEKPVVGEVSILMEYMDGGTLESLRGG 138
MKK8 ENWDSTS----LREIEILR-MVNSPYVAKCHDIFQNP-SGEVSILMDYMDLGSLESLRG- 138
MKK10 PNLNTTVT----VEADILK-RIESSFIIKCYAVFVSL--YDLCFVMELMEKGSLHDALLA 133
MKK1 LNTEESTCRAISQELRINL-SSQCPYLVSCYQSFY--HNGLVSIILEFMDGGSLADLLKK 157
MKK2 LNIDEAIRKAIAQELKINQ-SSQCPNLVTSYQSFY--DNGAISLILEYMDGGSLADFLKS 168
MKK6 MNIQEEIRKQIVQELKINQASSQCPHVVVCYHSFY--HNGAFSLVLEYMDRGSLADVIRQ 160
MKK3 I-FEREKRQQLLTEIRTLCEAPCHEGLVDFHGAFYSPDSGQISIALEYMNGGSLADILKV 174
MKK4 ---WKEQQLADLSRQILSGLAYLH-SRHIVHRDIKPSNLLINSAKNVKIADFGVSRILAQ 223
MKK5 ---WQEQELADLSRQILSGLAYLH-RRHIVHRDIKPSNLLINSAKNVKIADFGVSRILAQ 214
MKK7 ---VTEKQLAGFSRQILKGLSYLH-SLKIVHRDIKPANLLLNSRNEVKIADFGVSKIITR 192
MKK9 ---VTEQKLAGFAKQILKGLSYLH-ALKIVHRDIKPANLLLNSKNEVKIADFGVSKILVR 194
MKK8 ---VTEKQLALMSRQVLEGKNYLH-EHKIVHRDIKPANLLRSSKEEVKIADFGVSKIVVR 194
MKK10 QQVFSEPMVSSLANRILQGLRYLQ-KMGIVHGDIKPSNLLINKKGEVKIADFGASRIVAG 192
MKK1 VGKVPENMLSAICKRVLRGLCYIHHERRIIHRDLKPSNLLINHRGEVKITDFGVSKILTS 217
MKK2 VKAIPDSYLSAIFRQVLQGLIYLHHDRHIIHRDLKPSNLLINHRGEVKITDFGVSTVMTN 228
MKK6 VKTILEPYLAVVCKQVLLGLVYLHNERHVIHRDIKPSNLLVNHKGEVKISDFGVSASLAS 220
MKK3 TKKIPEPVLSSLFHKLLQGLSYLHGVRHLVHRDIKPANLLINLKGEPKITDFGISAGLEN 234
MKK4 TMDPCNSSVGTIAYMSPERINTDLNQGK----YDGYAGDIWSLGVSILEFYLGRFPFPVS 279
MKK5 TMDPCNSSVGTIAYMSPERINTDLNHGR----YDGYAGDVWSLGVSILEFYLGRFPFAVS 270
MKK7 SLDYCNSYVGTCAYMSPERFDS-AAGEN----SDVYAGDIWSFGVMILELFVGHFPLLPQ 247
MKK9 SLDSCNSYVGTCAYMSPERFDSESSGGS----SDIYAGDIWSFGLMMLELLVGHFPLLPP 250
MKK8 SLNKCNSFVGTFAYMSPERLDSEADGVTEEDKSNVYAGDIWSFGLTMLEILVGYYPMLP- 253
MKK10 GDYGSN---GTCAYMSPERVDLEKWGFGG---EVGFAGDVWSLGVVVLECYIGRYPLTKV 246
MKK1 TSSLANSFVGTYPYMSPERISGSLYSNK---------SDIWSLGLVLLECATGKFPYTPP 268
MKK2 TAGLANTFVGTYNYMSPERIVGNKYGNK---------SDIWSLGLVVLECATGKFPYAPP 279
MKK6 SMGQRDTFVGTYNYMSPERISGSTYDYS---------SDIWSLGMSVLECAIGRFPYLES 271
MKK3 SMAMCATFVGTVTYMSPERIRNDSYSYP---------ADIWSLGLALFECGTGEFPYIAN 285
MKK4 ---RQG-DWASLMCAICMSQPPEAP-ATASPEFRHFISCCLQREPGKRRSAMQLLQHPFI 334
MKK5 ---RQG-DWASLMCAICMSQPPEAP-ATASQEFRHFVSCCLQSDPPKRWSAQQLLQHPFI 325
MKK7 ---GQRPDWATLMCVVCFGEPPRAP-EGCSDEFRSFVDCCLRKESSERWTASQLLGHPFL 303

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MKK9     ---GQRPDWATLMCAVCFGEPPRAP-EGCSEEFRSFVECCLRKDSSKRWTAPQLLAHPFL 306
MKK8     -------DQAAIVCAVCFGEPPKAP-EECSDDLKSFMDCCLRKKASER------------ 293
MKK10    ---GDKPDWATLFCAICCNEKVDIP-VSCSLEFRDFVGRCLEKDWRKRDTVEELLRHSFV 302
MKK1     EHKKGWSSVYELVDAIVENPPPCAPSNLFSPEFCSFISQCVQKDPRDRKSAKELLEHKFV 328
MKK2     NQEETWTSVFELMEAIVDQPPPALPSGNFSPELSSFISTCLQKDPNSRSSAKELMEHPFL 339
MKK6     EDQQNPPSFYELLAAIVENPPPTAPSDQFSPEFCSFVSACIQKDPPARASSLDLLSHPFI 331
MKK3     EGPVN------LMLQILDDPSPTPPKQEFSPEFCSFIDACLQKDPDARPTADQLLSHPFI 339
                      :.  :        *    * ::  *:  *:. .   *

MKK4     LRASPSQNRSPQNLHQLLPPPRPLSSSSSPTT---------------------------- 366
MKK5     LKATGGPN-----LRQMLPPPRPLPSAS------------------------------- 348
MKK7     RESL------------------------------------------------------- 307
MKK9     REDL------------------------------------------------------- 310
MKK8     ---------------------------------------------------------
MKK10    KNR-------------------------------------------------------- 305
MKK1     KMFEDSDTNLSAYFTDAGSLIPPLAN---------------------------------- 354
MKK2     NKYDYSGINLASYFTDAGSPLATLGNLSGTFSV--------------------------- 372
MKK6     KKFEDKDIDLGILVGTLEPPVNYLR----------------------------------- 356
MKK3     TKHEKERVDLATFVQSIFDPTQRLKDLADMLTIHYYSLFDGFDDLWHHAKSLYTETSVFS 399

MKK4     ---------------------------------------------------------
MKK5     ---------------------------------------------------------
MKK7     ---------------------------------------------------------
MKK9     ---------------------------------------------------------
MKK8     ---------------------------------------------------------
MKK10    ---------------------------------------------------------
MKK1     ---------------------------------------------------------
MKK2     ---------------------------------------------------------
MKK6     ---------------------------------------------------------
MKK3     FSGKHNTGSTEIFSALSDIRNTLTGDLPSEKLVHVVEKLHCKPCGSGGVIIRAVGSFIVG 459

MKK4     ---------------------------------------------------------
MKK5     ---------------------------------------------------------
MKK7     ---------------------------------------------------------
MKK9     ---------------------------------------------------------
MKK8     ---------------------------------------------------------
MKK10    ---------------------------------------------------------
MKK1     ---------------------------------------------------------
MKK2     ---------------------------------------------------------
MKK6     ---------------------------------------------------------
MKK3     NQFLICGDGVQAEGLPSFKDLGFDVASRRVGRFQEQFVVESGDLIGKYFLAKQELYITNL 519

MKK4     -
MKK5     -
MKK7     -
MKK9     -
MKK8     -
MKK10    -
```

```
MKK1      -
MKK2      -
MKK6      -
MKK3      D 520
```

Figure 3.2 Multiple sequence alignment and analysis of *Arabidopsis* MKK proteins. ClustalW software was used to align the amino acid sequences of the ten *Arabidopsis* MAPKK proteins (http://www.ebi.ac.uk/Tools/msa/clustalw2). Predicted protein kinase domain of each MKKs are highlighted with yellow, in MKK3 a nuclear transport domain (NTF2) is also predicted and highlighted with green (http://prosite.expasy.org). Turquoise colour indicates MPK docking sites ((Doczi *et al.*, 2012)), T, S or Y amino acids highlighted with red colour are predicted phosphorylation sites from *The Arabidopsis Protein Phosphorylation Database* (http://phosphat.uni-hohenheim.de/). ATP binding pocket (boxed) and protein kinase active sites (underlined) were predicted using the EMBL-EBI InterPro Protein sequence analysis and classification tool (http://www.ebi.ac.uk/interpro/).

In their catalytic domain, protein kinases contain two conserved regions that are essential for their function: the ATP binding site and the protein kinase active site. By using the EMBL-EBI InterPro Protein sequence analysis and classification tool (http://www.ebi.ac.uk/interpro/) (Mitchell *et al.*, 2015), I identified the predicted conserved regions in the sequence of the 10 Arabidopsis MKK proteins. In the N-terminal end of the kinase catalytic domain (which is highlighted with yellow in Fig 3.2), a 23 amino acid region (Fig 3.2, boxed) comprises the ATP binding site of MKKs. The predicted region includes a glycine-rich stretch close to a lysine residue, which is directly involved in ATP binding. At the central part of the catalytic domain, using the InterPro tool it was also predicted a 12 amino acid-length region on the MKKs (out of MKK8) that corresponds to the active site of the MKKs (Fig 3.2, underlined). The sequence contains a conserved catalytic aspartic acid residue that is necessary for the kinase activity (Knighton *et al.*, 1991).

Other important signatures on the MKK proteins are the D-sites, these motifs are necessary for the interactions with complementary binding motifs that present on their MAP kinase substrates (Doczi *et al.*, 2012). I marked all the D-sites that are located in the N-terminal end of MKKs (Fig 3.2, highlighted in turquoise). The identified D-motifs mainly comprise from one to three positively charged amino acids (e.g. arginine, lysine) followed by alternating hydrophobic amino acid residues, such leucine (Doczi *et al.*, 2012).

Finally, I identified predicted phosphorylation sites (serine, threonine, tyrosine) in the MKKs sequences by using the PhosPhAt 4.0 Phosphorylation site predictor tool (The Arabidopsis Protein Phosphorylation Database, http://phosphat.uni-hohenheim.de/) (Durek *et al.*, 2010).

3.3.2 Phylogenetic tree of 10 the MKKs in *Arabidopsis*

To analyse the relationship between the *Arabidopsis* MKK proteins I prepared a phylogenetic tree (Fig 3.3). The MKK7 and MKK9 whole proteins showed high degree of similarities, because of their 79% identical amino acids.

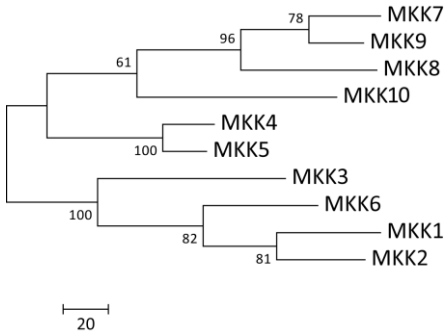

Figure 3.3 Phylogenetic relationships between *Arabidopsis* MKK proteins. MEGA 6 software was used to draw an unrooted phylogenetic tree based on Maximum Parsimony clustering with 500 bootstrap replicates to show the degree of relationship between the Arabidopsis full-length MKK proteins.

Hamel *et al.,* 2006 compared the kinase domains of three plant species MKKS (Arabidopsis, rice and poplar) and distributed them into four clades (Figure A3.1). Each clades contain both monocots and dicots MKKs, however in a seven-member subgroup within the clade D (the authors called this subgroup as the MKK7-9 clade) no any rice ortholog could be placed (Figure A3.1). This subgroup contains the Arabidopsis MKK7, MKK8 and MKK9 and the poplar MKK7, MKK9, MKK11-1 and MKK11-2. This subgroup appeared to be evolutionarily distinct from the monocot MKKs (Hamel *et al.*, 2006).

3.3.3 Characterisation of β-estradiol induction of pER8::MKK lines

The series of 10 MKKs overexpression transgenic lines was generated in the background of Columbia wild type. Two independent transgenic lines were selected

for each *MKK* genes (out of MKK5) and homozygous lines were produced. I began characterizing these pER8::MKK lines to confirm the expression of the MKK proteins, the β-estradiol inducibility of the expression using c-myc antibody that detects the c-myc epitope fused at the N-termini of the proteins. Transgenic MKK lines were grown in ½ GM media on plates. Seven days after germination (dag), the MKK lines were transferred into liquid ½ GM medium containing 5 µM β-estradiol and without β-estradiol (0.05% ethanol as a mock) for 3 hrs induction time. Also included the negative control empty vector (See Section 2.3.2), hereafter seedlings were harvested into liquid N_2. Western blot was performed with commercially available c-myc antibody. The 0 hour sample was also harvested, which was just before the treatment (directly harvested from plates). The Western blot results are shown in Fig 3.4.

The c-myc antibody recognised two aspecific protein bands of 70 and 35 kDa that was also present in the negative control of empty vector-transformed line. In the MKK4(3), MKK4(11) and MKK5(B1) lines the c-myc antibody detected a specific band of around 65 kDa that showed a largely constitutive MKK expression, was present in the starting 0h sample as well as in the treatment with and without β-estradiol (see predicted molecular sizes of MKK proteins in Table 3.2). I found estradiol-dependent expression in the MKK2(1), MKK7(1), MKK7(2), MKK9(1) and MKK9(2) lines. The induction of MKK7(2) was weaker then MKK7(1) and also very weak expression could be observed in the absence of β-estradiol and 0 hour as well. MKK9(2) showed stronger expression than MKK9(1) but was also showed some weak expression in the absence of β-estradiol. I could not detect any expression of the MKK protein in the MKK1(1), MKK1(2), MKK2(5), MKK3(4), MKK3(10), MKK6(4), MKK6(8), MKK8(4), MKK8(5), MKK10(2), MKK10(3).

To investigate the possible reason for the constitutive expression of MKK4, I set up another experiment with the MKK4(3) and MKK4(11) lines using an induction time of 3h and β-estradiol concentration of 5 µM and detected the MKK4 protein through the c-myc-epitope (Fig 3.4 down). The MKK4(3) was induced nicely by β-estradiol, while MKK4(11) was expressed both in absence and presence of β-estradiol (Fig 3.4 lower panel). I decided to focus my further experiments on the MKK4, MKK7 and MKK9 lines.

MKKs	Predicted Size (kDa)	Predicted size with the c-myc epitope tag (kDa)
MKK1	39.2	54.2
MKK2	39.8	54.8
MKK3	57.5	72.5
MKK4	40.1	55.1
MKK5	37.1	52.1
MKK6	39.8	54.8
MKK7	34.3	49.3
MKK8	32.5	47.5
MKK9	34.3	49.3
MKK10	34	49

Table 3.2 Predicted molecular weight of *Arabidopsis* MKK proteins from *https://www.arabidopsis.org/*.

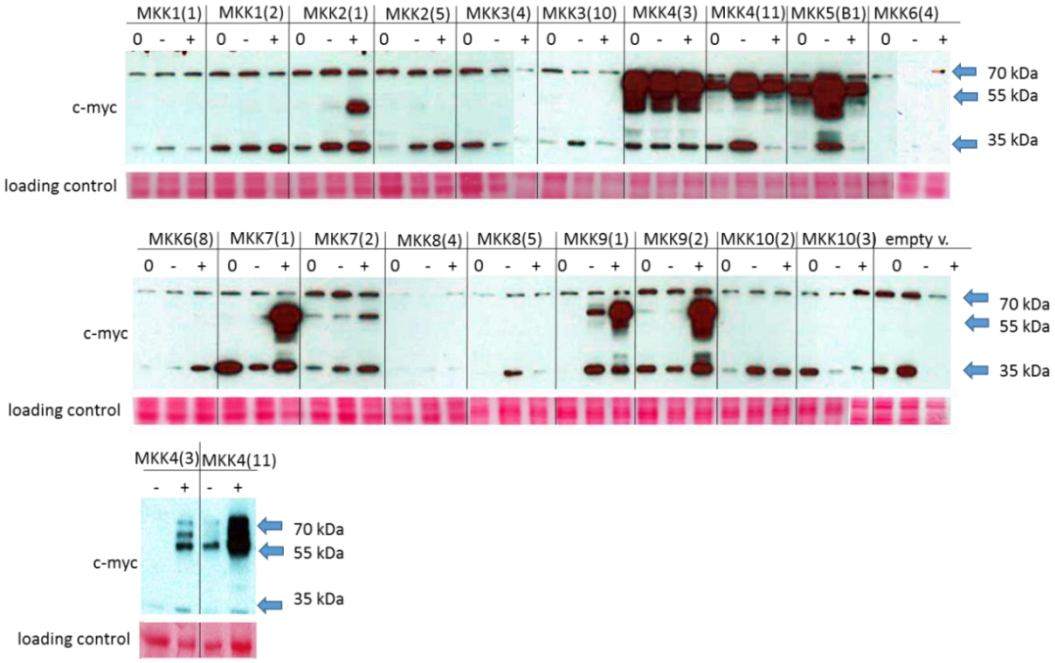

Figure 3.4 Western blot analyses of estradiol-inducible MKK overexpression lines. The myc epitope tagged MKKs expressed under the control of the estradiol-inducible promoter in these homozygous pER8::MKK lines. One-week old plants were treated with 5 µM β-estradiol or 0.05% ethanol as mock, samples were harvested at 0 and 3-hour time points. Then total protein was extracted and 50 µg protein extracts were used to perform western blot with c-myc specific antibody. Note that MKK4(3) and MKK4(11) signals were saturated, therefore the western blot was repeated using 25 µg protein of the mock and β-estradiol treated samples of these two lines (see down lane).

3.3.4 Characterization of seedling sizes upon β-estradiol treatment

I chose the homozygous MKK lines that showed estradiol-inducible MKK protein expression; MKK4(11), MKK7(1), MKK7(2), MKK9(1), MKK9(2) for further phenotypic investigation. In the initial experiment, I studied the seedling growth of these lines on plates in the presence and absence of 5 µM β-estradiol (Fig 3.5). The homozygous transgenic MKK lines were grown with negative control WT-Columbia for 7 dag on ½ GM and then transferred onto media containing 5 µM β-estradiol and without β-estradiol for 4 days. The germination rate was recorded for all the lines 36h after the start of the experiment and was similar for all transgenic lines and the WT. Representative images are shown (Fig 3.5). β-estradiol had no visible effect on the growth of wild type (WT) seedlings, but the growth of MKK4(11), MKK7(1), MKK7(2), MKK9(1), MKK9(2) were all strongly inhibited when MKK expression is induced upon β-estradiol treatment. I decided to focus on the two closely related MKKs, MKK7 and MKK9 in the following experiments. I chose the homozygous lines MKK7 (1) and MKK9 (1) that both show high expression which is β-estradiol dependent as well is inducible growth-arrest phenotype.

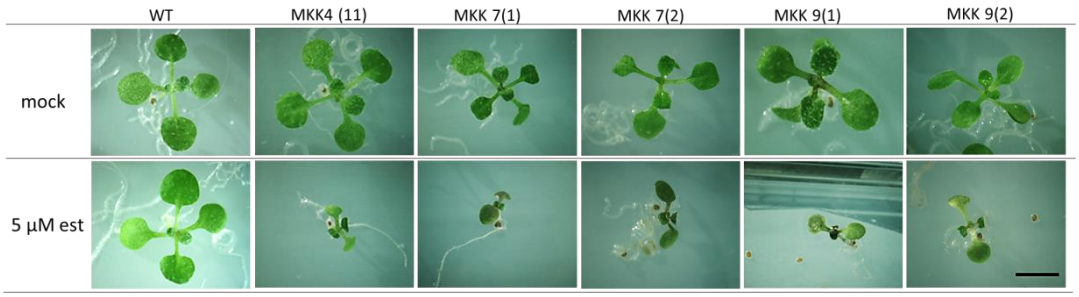

Figure 3.5 Growth inhibitions in five inducible MKK overexpressing lines. Seven days old wild type (WT) and homozygous pER8::MKK seedlings were transferred to culture media supplemented by 5 µM β-estradiol (est) or 0.05% ethanol (mock). Pictures were taken 4 days after estradiol-induction. Scale bar: 10 mm.

3.3.5 Time-dependent β-estradiol induction of MKK7 and MKK9 lines

Two independent homozygous lines pER8::MKK7(1) and pER8::MKK9(1) with negative control pER8::GUS were selected for further studies to investigate time dependent induction of myc-MKK7 and myc-MKK9 protein expression.

Because previously we had positive results with low concentrations of β-estradiol, in this experiment I decided to use 0.1 μM β-estradiol. The MKK7 and MKK9 lines were grown on ½ GM media after 8 dag seedlings were transferred onto ½ GM media containing 0.1 μM β-estradiol and 0.1% ethanol for mock for time course induction 3, 6 and 24 hrs after transferred into β-estradiol to determine the MYC-MKK7 and MKK9-MYC protein levels. The Western blot with the c-myc antibody showed a strong induction of both MYC-MKK7 and MYC-MKK9 proteins, both were high already at the earliest 3h time point, and decreased somewhat at the 6 and 24h time points (Fig 3.6). MKK9 was induced to a somewhat greater level compared to MKK7 while as expected there was no MKK protein detected with c-myc antibody in the empty vector control. Surprisingly, I detected MYC-MKK7 and MKK9-MYC at the 0h samples. The reason for this (0h) expression is not clear (Fig 3.6). The detected molecular weight of the MYC-MKK7, MYC-MKK9 proteins were around 65 kDa as seen before (Fig 3.4).

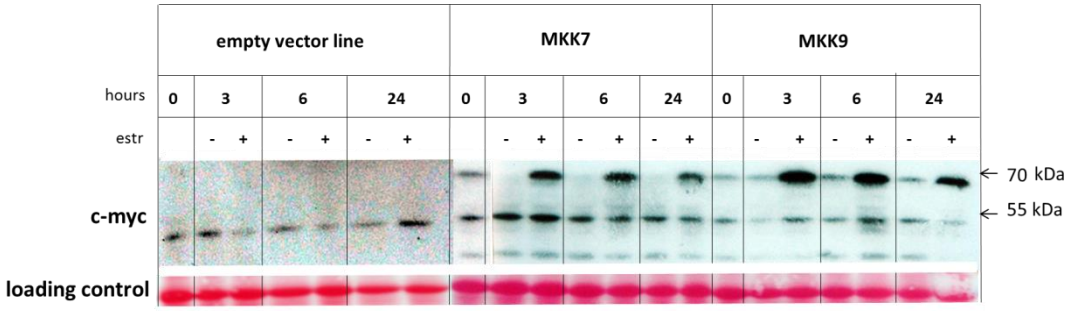

Figure 3.6 Time-dependent inductions of MKK7 and MKK9. One week old seedlings of lines empty vector-, MKK7 and MKK9 were treated with 0.1 μM β-estradiol (estr) and samples were harvested at 0, 3, 6, 24-hour time points. Western blot with 30 μg total protein extract was performed using antibody against the c-myc epitope. Estradiol-induced expression of MKK7 and MKK9 is highest after 3-hour β-estradiol induction and gradually decreases within 24 hours. In the empty vector line that used as a negative control, estradiol-dependent protein expression was not detected.

3.3.6 MKK7 and MKK9 phosphorylated and targeted downstream MPKs

Having seen the estradiol-dependent induction of MKK7(1) and MKK9(1) lines I next wanted to investigate whether the overexpression of these gain of

function MKKs would lead to induced MAPK activities. It was already shown that MPK3 and MPK6 are the two MAPKs downstream of MKK7 (Yoo *et al.*, 2008). To detect active MAPKs I used a commercially available antibody that was developed against the phosphorylated active form of the animal ERK1 (P-ERK), which is also known to recognise active MAPKs in plants (Meszaros *et al.*, 2006). MKK7(1) and MKK9(1) and empty vector were grown on ½ GM for 7 days and transferred onto media containing 0, 1, 5 and 10 µM β-estradiol for 6 hrs for induction. I detected the expression of MKKs through their myc epitope using c-myc antibody (Fig 3.7 up). Both MYC-MKK7 and MKK9-MYC was the strongest detected at 1 µM β-estradiol treatment at the molecular weight of around 60 kDa. This band is completely missing in the empty vector control. I also used a positive control, the EBP1-myc (Fig 3.7 up, lane pc), which was strongly detected by the c-myc antibody.

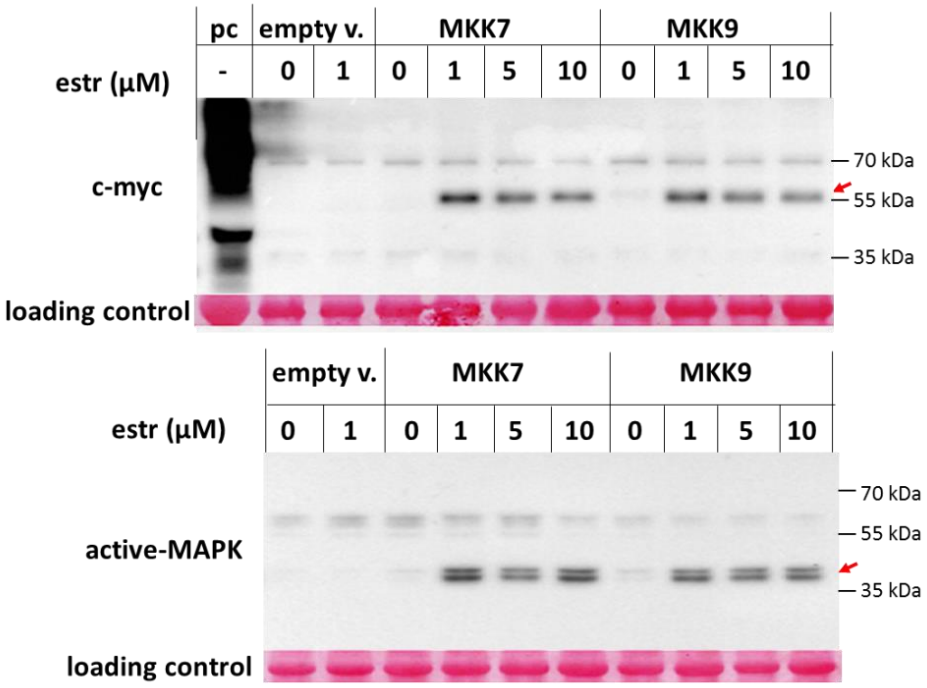

Figure 3.7 Overexpression of MKK7 and MKK9 activates downstream MAPKs. Western blot was performed on total protein extracts from one week old seedlings 6 hours after different concentration of β-estradiol treatment (estr). **Up**: Detection of the myc-tagged MKK7 and MKK9 proteins after estradiol induction using antibody against the c-myc epitope (pc= positive control). The red arrows indicates both the MKK7 and MKK9 proteins above 55 kDa molecular weight. **Down**: detection of the activated MAPK proteins by active-MAPK antibody (P-ERK). Arrow indicates the specific P-ERK signals.

In the same samples of β-estradiol treated empty vector, MKK7(1) and MKK9(1) also set out to detect the active MAPKs using the P-ERK antibody. I found two bands of around 40 kDa that could represent active MAPKs (Fig 3.7 down). There were strong signals with P-ERK only in the MKK7(1) and MKK9(1) samples treated with β-estradiol (Fig 3.7 down). This well corresponded with the induced expression detected with c-myc antibody in the upper panel of Figure 3.7. I conclude that the overexpression of gain of function MKK7 and MKK9 indeed able to activate downstream MAPKs in *Arabidopsis* seedlings.

3.3.7 Identify whether MPK3 or MPK6 are the downstream MAPKs to MKK7 and MKK9 using T-DNA insertional *mkk* and *mpk* mutants

MPK3 and MPK6 proteins were identified in previous experiment as targets of MKK7 and MKK9 (Yoo *et al.*, 2008). In this experiment I aimed to detect MPK3, MPK6 proteins using MPK3- and MPK6-specific antibodies as well as the active forms of MAPKs using P-ERK in the following mutant backgrounds; *mkk7-KO*, *mkk9-KO*, *mkk7/mkk9* double mutant (dKO1, dKO2), *mpk3-KO*, *mpk6-KO* (see Section 2.3.4). If the MKK7 and MKK9 are upstream activators of MPK3 and MPK6, I expected the proteins to be present but not active (not detected by the P-ERK antibody). On the other hand, MPK3 and MPK6 were exprected to be missing in the *mpk3-KO* or *mpk6-KO* mutants, respectively. This would confirm the complete knock out of the MPK3 and MPK6 proteins in these mutants.

All these mutant lines were plated alongside with WT onto ½ GM plates for 8 days, treated with 25mM mannitol for 15 mins to induce the MAPK signalling pathways and also generated samples without mannitol treatment as a control. MPK3-specific antibody detected a 40 kDa band in all samples without (-) and with (+) mannitol except in the *mpk3* knock out mutant, as expected (Fig 3.8). Similarily, the MPK6-specific antibody detected a specific protein of around 44 kDa in all samples except the *mpk6-KO* knock out. The Western blot with the P-ERK antibody did not work, and therefore the activity state of MAPKs could not be determined. I conclude that the *mpk3* and *mpk6* mutants are true null mutants and lack all detectable corresponding proteins.

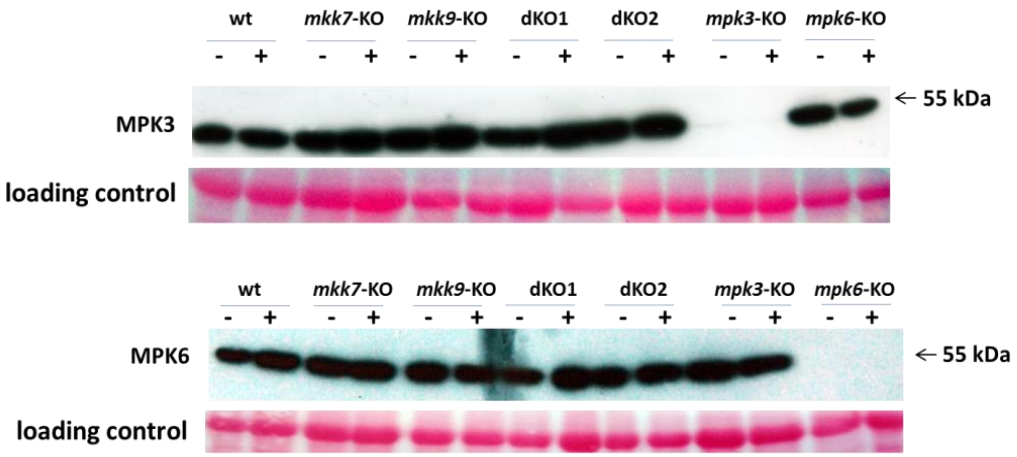

Figure 3.8 Western blot analyses of *mkk* and *mpk* T-DNA insertion mutant lines. One-week old wild type and mutant lines were treated with 25 mM mannitol for 15 minutes and then whole seedlings were harvested for protein extraction. Western blots using MPK3 and MPK6 specific antibodies were performed on 30 µg total protein extract (dKO1: *mkk7*/*mkk9-N3*; dKO2: *mkk7*/*mkk9-N5*).

3.3.8 Quantitation of surface area of newly emerging leaves and cotyledons upon β-estradiol induced expression of MKK7 and MKK9

Since we showed the clear induction of MKK7 and MKK9 proteins with β-estradiol and the activation of downstream MAPKs (see Fig 3.7), I decided to move on the detailed characterisation how these MAPK pathways inhibit plant growth. Cotyledons are embryonic leaves that are already present on the seedlings when they germinate, playing a major role as a food reservoir during seedling development. Cotyledons also provide photosynthetic surface area at early stage of seedling development. The growth of cotyledon is mostly through cell expansion, specifically so at the later stages of cotyledon growth. New leaves emerge at the shoot meristem during seedling development at around 4-6 dag. These true leaves initially growth through proliferation, which is gradually halted and there is a second phase of growth through cell expansion that in *Arabidopsis* is accompanied by endoreduplication.

In this experiment, I grew seedlings on ½ GM plates for 7 days and then transferred onto the ½ GM media supplemented with 0, 0.1 and 1 µM β-estradiol for 4 days induction time. Approximately 85% and 95% seeds were germinated in 2 day and 3 day after plating respectively and the germination rate was same in both

MKKs lines and the WT (Data not shown). Therefore any difference in growth cannot be accounted for due to germination differences.

I investigated the effect of inducible MKK7 and MKK9 expression on cotyledon and leaf growth. For this experiment, I grew MKK7(1) and MKK9(1) lines alongside with empty vector transformed line on ½ GM medium for 6-7 days when the true first leaves just became visible and possible to collect for measurement, but were smaller than 0.2-05 mm which represents leaves where cells fully proliferate (Fig 3.9 up). I timed the transfer to β-estradiol to the emerging leaf size. The aim of the experiment was to look for the change in cotyledon size that rely on cell expansion and leaf size that depends on cell proliferation at this early stage. In this experiment I used 2 different β-estradiol concentrations 0.1 and 1 μM and 0 as a control. I took images under stereo microscope at 0, 1, 2, 3, 4 days after transfer, selected seedlings that were fully straight facing upwards and measured cotyledon and leaf sizes by drawing around their areas and using imageJ I calculated the surface area (Fig 3.9).

The visual inspection of images aready cleary show that β-estradiol treatment strongly reduced the growth of MKK7 and MKK9 seedlings, leading to reduced cotyledon and leaf sizes (see fig 3.5). The control empty vector line was not effected at 0.1 μM β-estradiol, but I did see a mild effect at 1 μM β-estradiol, indicating that at this concentration this drug have aspecific effect. Quantitation of the images show that growth inhibition of both cotyledon and new leaf is strong in MKK7(1). New leaf growth was much more strongly effected that cotyledon, reaching around 95% inhibition with 1 μM β-estradiol. I also measured leaf and cotyledon sizes at earlier time point and found growth reduction already at 1 day after transfer to β-estradiol. In the Fig 3.9 I show only the 4 days after transfer results. I conclude that both MKK7(1) and MKK9(1) overexpression reduces cotyledon growth and thus cell expansion and also reduces young leaf growth and thus cell proliferation-dependent growth (Fig 3.9).

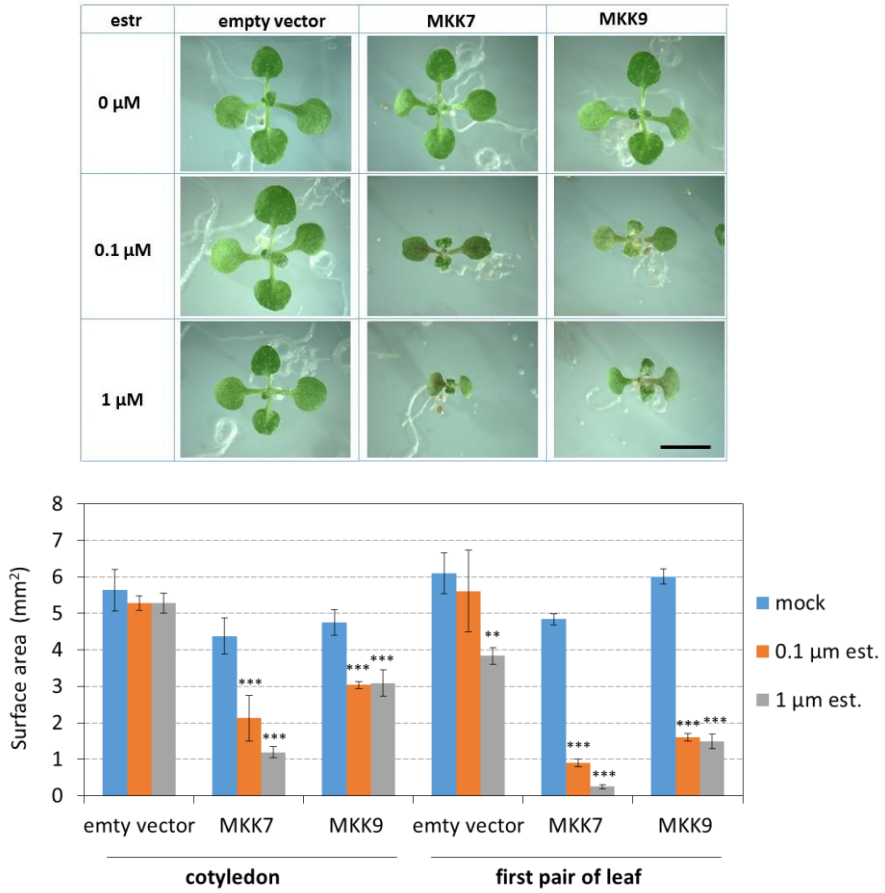

Figure 3.9 Overexpression of MKK7 and MKK9 resulted in reduced cotyledon and leaf sizes. Empty vector line (emty.v.), MKK7 and MKK9 seedlings were germinated and grown on ½ GM media for 7 days and then transferred to media containing mock (0.01% ethanol), 0.1 or 1 µM β-estradiol (estr). Surface areas of cotyledon and the first leaf pair were measured 4 days later as described in Section 2.4. **Up**: pictures of representative seedlings after 4-day β-estradiol treatment. Scale bar shows 10 mm. **Down**: ImageJ quantification of cotyledon and primary leaf surface areas. Data were generated based on three biological replicates. Error bars represent SE. Note that overexpression of MKK7 and MKK9 inhibits leaf growth in dose-dependent manner. Statistical analysis based on One-way ANOVA Dunnett's test was performed to calculate the significant differences compared to the mock treated empty vector line (* $p<0.05$, ** $p<0.005$, *** $p<0.001$).

3.3.9 Measurement of root length upon estradiol-induced MKK7 and MKK9 expression

While both cotyledon and leaf growth is determinate, root growth is indeterminate. Roots grow at the tip by cell proliferation in the meristematic zone and subsequently cell by expansion when meristematic cells exit to the elongation zone. I also looked at the effect of MKK7 and MKK9 inducible overexpression on root lengths and compared it with empty vector (Fig 3.10). Seedlings of the three

lines, empty vector (e.v.) MKK7(1) and MKK9(1) were grown on vertical plates on ½ GM medium for 6 days and then transferred onto medium containing 1 μM β-estradiol (Fig 3.10). The position of root tips were marked just following the transfer and followed for 3 days period. Images of representative roots are shown at day 3 after transfer, and quantitation results of 0, 1, 2 and 3 days after of around 10 roots are shown in Fig 3.10 as root length from the time of transfer.

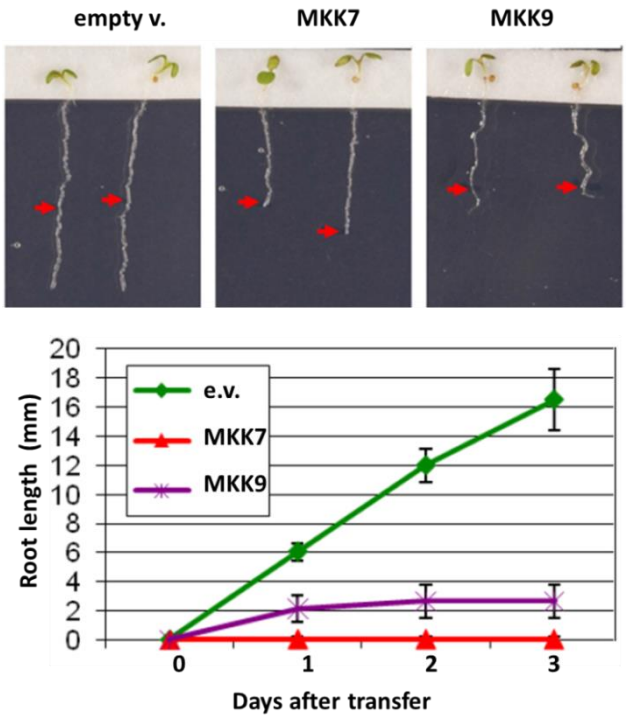

Figure 3.10 Overexpression of MKK7 and MKK9 resulted in reduced root growth. Six days old vertically grown empty vector line (e.v.), MKK7 and MKK9 seedlings were transferred to media containing 1 μM β-estradiol. **Up**: images of two representative plants of each genotypes. Red arrows indicate the root length of plants transferred onto the β-estradiol containing media. **Down**: line chart shows the daily root growth after transferring plants onto the β-estradiol containing media. Error bars represent SE.

While the empty vector line grew of around 16 mm during the 3 days period, the root growth of MKK7(1) was completely inhibited. MKK9(1) did grow somewhat, but the inhibition was also large, around 90%. I conclude that growth inhibition by MKK7 and MKK9 is not specific to leaves and cotyledon, but also happens in root. Therefore the effect might be on a general growth process.

3.3.10 Confocal microscopy analysis of root of meristem structure when MKK7 and MKK9 are inducibly overexpressed

Root meristem has regular cell divisions that are predictable and can be seen by confocal sections of propidium iodide (PI) stained roots. Because I found that root growth is sensitively affected by the estradiol-induced MKK7 and MKK9 expression, I decided to use very low concentration of β-estradiol; 0.01 and 0.1 μM. The confocal microscope images are shown in (Fig 3.11). Six days old seedlings were transferred onto media 0, 0.01 and 0.1 μM β-estradiol and the confocal microscopy analysis was performed at 3 days after transfer (Fig 3.11).

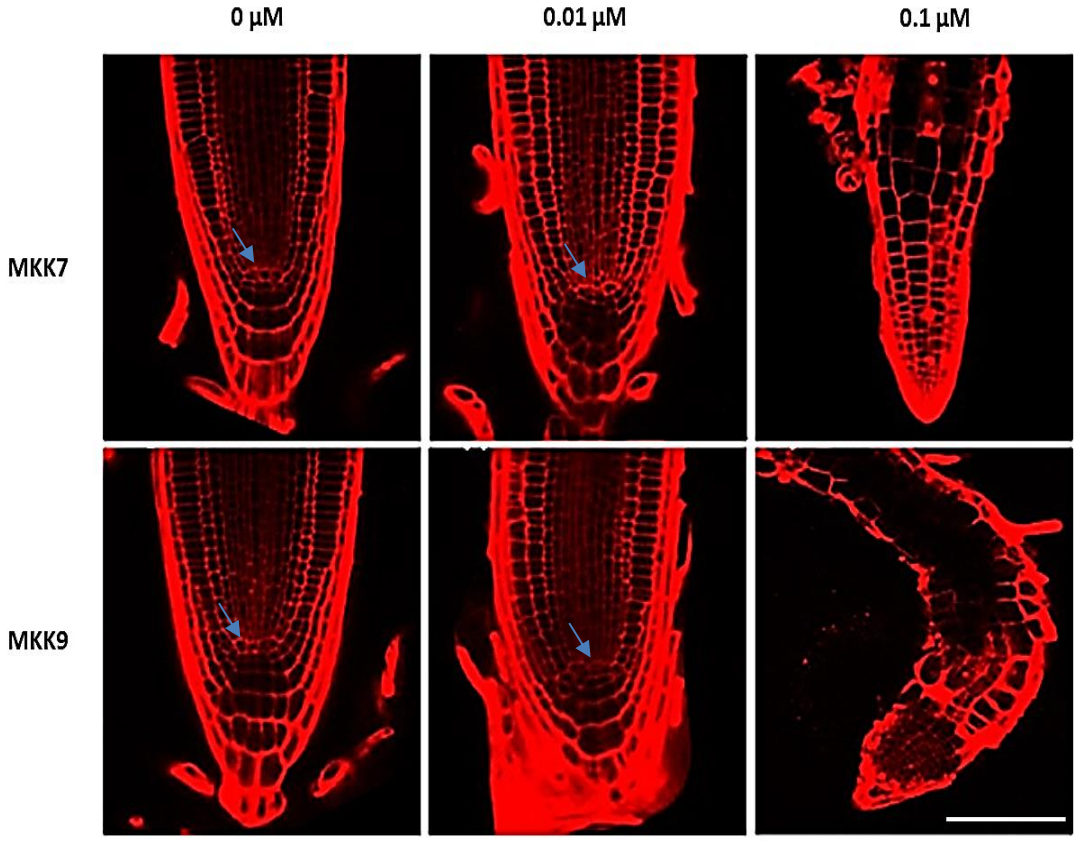

Figure 3.11 Cellular organization of root tips in response to induced MKK7 and MKK9 overexpression. Confocal images of PI stained root tips following treatments with 0, 0.01 and 0.1 μM concentration of β-estradiol. Blue arrows show QC region. Scale bar: 100 μM.

Compared to the mock treatment (0 μM) the 0.01 μM β-estradiol already had an effect on root meristem organisation. In the case of MKK7(1) line the organisation of the quiescent centre (QC) region appears to be more disorganised as

indicated by blue arrow in Fig 3.11. Normally the QC cells are in the middle of the meristem and downward there are the columella stem cells. The orientation of cell division is normally perpendicular at this region, but it became disorganised in MKK7(1) at 0.01 μM β-estradiol. At 0.1 μM β-estradiol the root meristem became disorganised both at MKK7(1) and MKK9(1) lines. Root meristem was largely lost and cells started differentiating to root hairs. Many oblique cell division planes were apparent in both lines, as well as the cells became isodiametric and enlarged (Fig 3.11).

3.3.11 Analysis of epidermal pavement cells during leaf development upon induced expression of MKK7 and MKK9

I observed strongly reduced leaf sizes that is most likely due to the inhibition of cell proliferation as well as reduced cotyledon sizes that could be due to reduced cell expansion. I decided to focus on young leaves (true leaves) with cell proliferation activity and study how the inhibition of leaf growth by induced MKK7 and MKK9 expression effect pavement cell morphology and sizes. Cell proliferation in leaves follow a gradient. At the earliest young stage, most cells in a leaf primordium proliferate, but as leaves become older, cell proliferation is gradually arrested (Andriankaja *et al.*, 2012). In addition on the epidermal layer stomata meristemoids also contribute to the increase in cell numbers by producing pavement cells and stomata linage cells through asymmetric cell division. It was shown that the MKK4/MKK5 and the MKK7/MKK9 signalling regulate stomata development at multiple points, inhibit the production of stomata and leads to low stomata density and more pavement cells (Lampard *et al.*, 2014). The size and number of epidermal pavement cells contributed by cell proliferation and expansion that determine final organ sizes.

To investigate how epidermal cell size is controlled by the overexpression of MKK7 and MKK9 I set up an experiment with MKK7 and MKK9 seedlings and empty vector after 7 dag on ½ GM media, having tiny leaf primordia, were transferred onto media containing 0, 0.1, 1 μM β-estradiol for 24 hrs induction time, methanol cleared new leaf images were taken under DIC microscope and manually hand drawing cell contours in Paint. Cell areas were analysed by imageJ with calibrated scale (Fig 3.12).

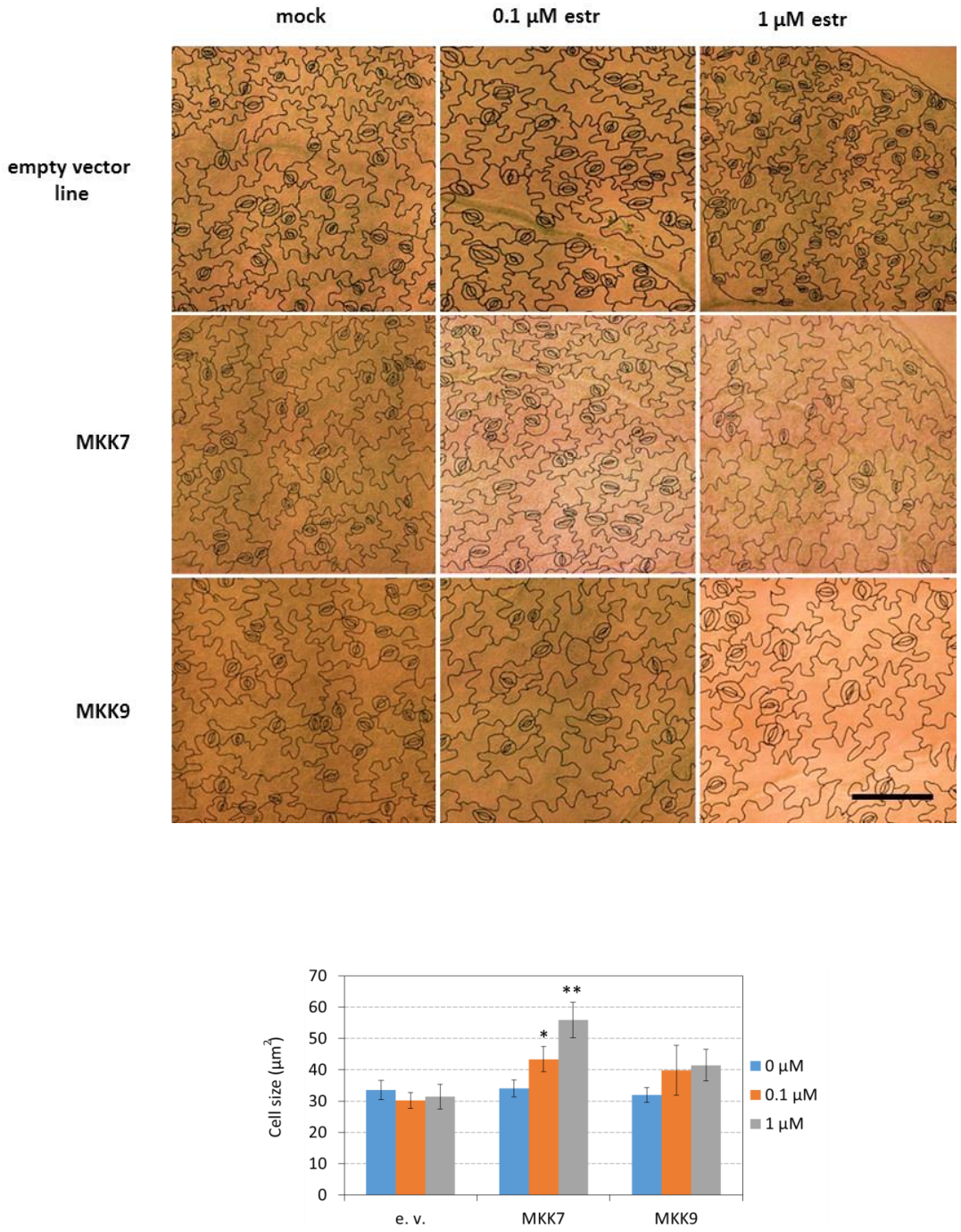

Figure 3.12 Overexpression of MKK7 resulted in larger epidermis cells. Empty vector line (e.v.), MKK7 and MKK9 were germinated on ½ GM medium and 8 days later transferred to media containing mock (0.01% ethanol), 0.1 or 1 µM β-estradiol. Pictures of epidermal cells were taken 24 hours later and cell sizes were measured by ImageJ. Scale bar: 50 µm.

The seedlings of MKK7 from media containing 0.1 and 1 µM β-estradiol showed larger epidermal cell sizes, which was progressively larger in 1 µM concentration as compare to 0 and 0.1 µM of β-estradiol. MKK9 plantlets had also larger epidermal cells in the 0.1 and 1 µM (but with much less difference). The increase in cell size was less in MKK9 than in MKK7 (Fig 3.12). The increase in cell size was in spite of the large inhibition of final leaf sizes (Fig 3.9), which indicated that there is a much reduced total cell number in these cells. The β-estradiol induced and un-induced empty vector showed more of less the same epidermal cell sizes (Fig 3.12).

3.3.12 Ploidy analysis of young leaves upon the estradiol-induced expression of MKK7 and MKK9

Abiotic stress is known to halt leaf growth, but the mechanism how it effects cell cycle is not well understood. As cells leave cell proliferation they start endoreduplication. With ploidy analysis it is possible to determine the time when cells exit cell proliferation into endoreduplication. I aimed to measure ploidy parameters in young leaves with proliferating cells when MKK7 and MKK9 are inducibly overexpressed. MKK7 and MKK9 and empty vector lines of 7 dag old seedlings were transferred to 0.1 µM β-estradiol and mock treatment and samples were taken 2 and 4 days after induction (DAI). At 2 days after induction there were no cells with 8n content except in empty vector line at 0.1 µM β-estradiol. This indicates that at this time point most cells still proliferate in the young leaf and thus cycle between G1 and G2 DNA content. At 4 DAI however, the untreated samples and WT control treated with β-estradiol all show an 8n DNA content of around 5-10%, but this was completely inhibited in the MKK7 and MKK9 lines when treated with β-estradiol to induce MKK7 and MKK9 expression (Fig 3.13). This clearly showed that overexpression of MKK7 and MKK9 blocked endoreduplication. Because of the reduced number of cells in leaves with induced MKK7 and MKK9 expression, it is likely that also cell proliferation is blocked.

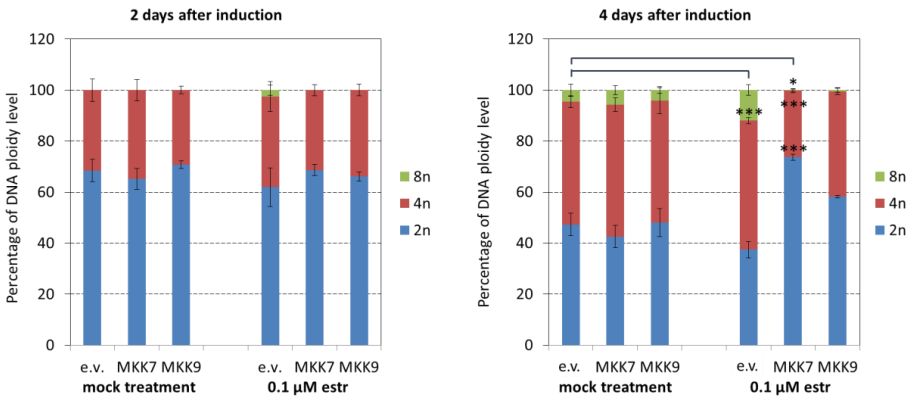

Figure 3.13 Cell cycle and ploidy analysis of MKK7 and MKK9 overexpression lines. Flow cytometer analysis of empty vector line (e.v.), MKK7 and MKK9 seedlings treated with mock (0.01% ethanol), 0.1 or 1 µM β-estradiol (estr) for 2 days (left) or 4 days (right). Error bars represent SD. Statistical analysis was performed comparing 2n, 4n and 8n values independently in every line with the corresponding values of the mock treated empty vector line (One-way ANOVA, Dunnett's test (* $p<0.05$, ***$p<0.001$).

3.4 Chapter discussion

MAPKs provide a versatile module for to multitudes of signals, including a variety of stresses, developmental signals, such as during stomata development and meristem growth (Ding *et al.*, 2015; Jalmi and Sinha, 2015; Moustafa *et al.*, 2014). The use of MAPK signalling modules can also be a powerful strategy to increase plant stress tolerance (Moustafa, 2014). We employed here a gain of function genetic screen to inducibly overexpress from all phylogenic clades a number of MKKs that were mutated to become gain of function by mimicking the activating phosphorylation by glutamic acid. We successfully used the inducible expression system developed by the Chua lab (Zuo *et al.*, 2000) that places the expression cassette under the control of the XVE chimeric transcription factor binding site in a minimal promoter. In order to be able to detect the overexpressed MKK, I used the myc epitope tag. On Western blotting I detected the overexpression of MKK7 and MKK9 constructs that were estradiol dose and time dependent. The constitutive ectopic overexpression of MKK7 and MKK9 gave a strong developmental phenotype that was reminiscent of the activation tagging of MKK7 published earlier (Dai *et al.*, 2006). This developmental phenotype was connected to auxin transport (Dai *et al.*, 2006).

Chapter 4: MKK7/9 are involved in leaf emergence during de-etiolation

4.1 Introduction

The light is an important environmental factor that fundamentally influence plant growth development (Mulligan *et al.*, 1997). There is also interplay with dark-arrest of growth and gaseous hormone ethylene (Smalle *et al.*, 1997). Plant growth patterns dramatically changed in the absence of light is called skotomorphogenesis, and in the presence, which is called photomorphogenesis. The process that occurs when dark grown seedlings emerge to light is called de-etiolation, which represent the one of the major developmental transition in plants (Deng and Quail, 1999; Fankhauser and Chory, 1997; Fankhauser, 2001; Chory and Wu, 2001; Neff *et al.*, 2000). Light perception and signal transduction through phytochrome mediates well-defined responses that can be exploited through genetic screens for mutants, which led to the identification of light signalling components. Photoreceptor activation through phytochromes of PHYA to PHYE (Clack *et al.*, 1994), through subsequent signalling mechanisms the downstream responses are activated that include light-modulated gene expression, posttranslational modification and nucleo-cytoplasmic compartmentalisation that influence the expression of numerous downstream effector genes related to multiple cellular and developmental processes (Fig 4.1).

Whole-genome transcriptome analysis comparing dissected dark arrested shoot apex and emerging leaf primordia upon light exposure followed in a time course uncovered the gene expression program associated with the arrest of cell division and rapid initiation of growth and proliferation (Lopez-Juez *et al.*, 2008). This microarray was compared to other transcription profiling studies, such as following leaf development starting from a leaf primordia representing mostly

proliferating cells with a size of around <3 mm long to fully developed leaves (Beemster *et al.*, 2005). The transcriptional changes by light activation in a finely resolved time course found a large number of differentially expressed genes >5700 also between the shoot apex including the meristem and leaf primordia where growth is supported by cell proliferation) and the cotyledon where growth is mainly through cell expansion. In this study it was found that light triggers rapid cell growth and protein translation and a coordinated progression in the cell cycle. The dark arrest of cell cycle and entry into proliferation was correlated with a change in the abundance of the E2FC and E2FB transcription factors, the repressor E2FC being abundant in dark, while E2FB accumulates in the light (Lopez-Juez *et al.*, 2008). In the same study it was shown that the ratio of E2FC and E2FB are regulated by light through light-signalling components including Constitutive photomorphogenic 1 (COP1) and (Deetiolated1) DET1 (Lopez-Juez *et al.*, 2008). Interestingly, in this study it was shown that MPK6 is strongly upregulated in dark-arrested meristem and rapidly disappears after light exposure (Lopez-Juez *et al.*, 2008). In a subsequent study it was shown that the DET1-dependent regulation of E2FC and E2FB abundance regulate the E2F-related transcription factor DP-E2F-like1 (DEL1) which act on the regulation of CDKB1;1 to regulate cell division (Berckmans *et al.*, 2011a). It was shown that the meristem activity and cell cycle progression are fully under photoreceptor control (Berckmans *et al.*, 2011a).

To follow gene expression changes, promoter-reporter constructs provide valuable tools. GUS reporter gene is one of the most used technologies to investigate the local gene activity. Reporter constructs have been developed for cell cycle and were used to investigate local induction of cell proliferation in the developing leaf (Beemster *et al.*, 2005; Colon-Carmona *et al.*, 1999) as well as in leaf primordia upon transfer from dark to light (Lopez-Juez *et al.*, 2008). The cell proliferation activity progresses from leaf apex to base and form a longitudinal gradient. The reporter for actively proliferating cells, and for entry into proliferation at the G1 to S transition is the *CYCD3;1:GUS* (Dewitte and Murray, 2003), while the *CYCB1;1:GUS* reports mitotic activity (Colon-Carmona *et al.*, 1999). In dark-grown seedlings without sucrose no visual expression observed for *CYCB1;1:GUS* or *CYCD3;1:GUS*, suggesting a full arrest of cell proliferation after germination (Carabelli *et al.*, 2008). And activation of APC component genes between 6 and 24 h of following transfer into light indicate the loss of primordial expression of

CYCB1;1:GUS, because they were expressed later then mitotic cyclin gene (Lopez-Juez *et al.*, 2008). Our analysis (dark-light transition) lays the foundation to identify biologically important individual growth phenomena and anticipated factors, some of their molecular switches as well as integrators among them, and may help to build a network of elementary processes underlying the development of leaves.

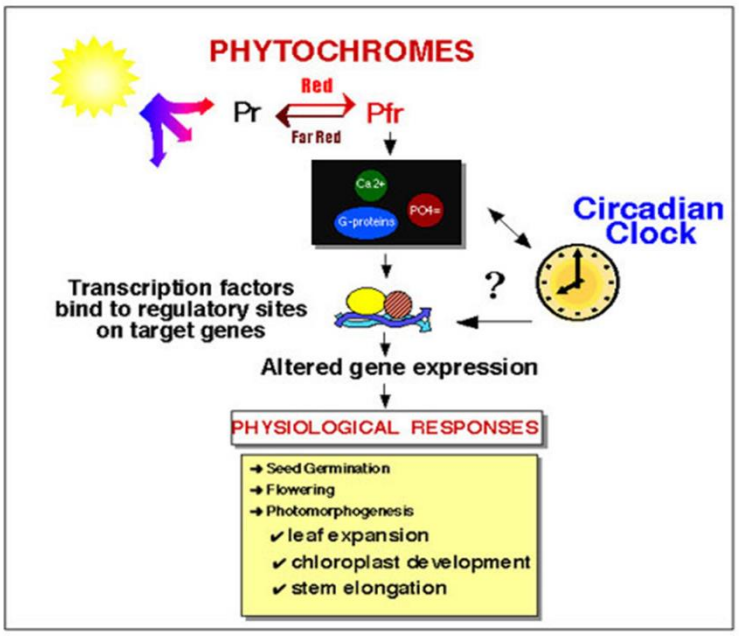

Figure 4.1 The phytochrome signal transduction pathway. Photoreceptors and circadian clocks are universal mechanisms for sensing and responding to the light environment. In addition to regulating daily activities, such as flowering they govern many plant processes, including movements of organs such as leaves and petals, stem elongation, metabolic processes such as respiration and photosynthesis and expression of a large number of different genes https://www.mcdb.ucla.edu/Research/Tobin/research.html.

4.1.1 Hypothesis

Light provide a synchronous growth for leaves from a total arrest in dark and a rapid outgrowth upon light transfer. From the fact that MPK6 accumulates in the dark arrested meristem, and the accumulation of auxin and ethylene in the meristem suggest that MPK6 and its upstream regulator MKK7/9 might be involved in this process. To test this hypothesis I examined the expression of MKK7 and MKK9 using the pMKK7-GUS and pMKK9-GUS lines during the dark to light transfer condition as well as the leaf primordia growth in mutants of *mkk7*, *mkk9*, *mpk3* and *mpk6*.

4.2 Objectives

1. To investigate the spatial and temporal expression of MKK7 and MKK9 during the dark to light transition of seedlings by using GUS reporter genes.
2. To study the involvement of MKK7/9 and MPK3/6 MAPKs signalling pathways in leaf growth upon light using mutants and quantitating leaf size under dark/light transition in time.
3. Study leaf initiation rate and its link to flowering time in the same MAPK signalling mutants.

4.3 Results

4.3.1 Leaf primordia size analysis under dark-light transition

The aim of this work is to follow and quantitate leaf growth when dark arrested seedlings are transferred to light in the following MAPK signalling mutant combinations compared to wild type Col-0, *mkk7-KO*, *mkk9-KO*, *mkk7/mkk9*-N3, *mpk3-KO*, *mpk6-KO*. These insertion knock out (KO) mutants were described in Section 2.3.4. To measure leaf primordium surface area, I cleared the seedlings and mounted on slides after removing the cotyledon and placing the meristem in an orientation that leaves can be viewed from the side. The *mkk* and *mpk* mutants were plated on ½ GM without sucrose. Three days stratification at 4ºC followed by 1 hour red light pulse were done to synchronise germination. Seedlings were incubated (grown) for 3d under continuous dark and then transferred to light for 1 and 2d light period. As a control, I also collected samples when seedlings were kept for the entire period of 5d in dark. In the 3d dark there is a visible bulge of leaf primordia for L1-2, but the difference in the area of the primordia among the genotypes: WT, *mkk7-KO*, *mkk9-KO*, *mkk7/9-N3*, *mpk3-KO* and *mpk6-KO* mutants is marginal, but One-way ANOVA analysis did indicate that *mkk7*, *mpk3* and *mpk6* mutants are already larger in the dark-arrested meristem. This suggests that these MAPK signalling components might have a role in maintaining the dark-imposed growth arrest. When seedlings were transferred from the 3 d dark to 1 d light and 2 d light, the leaf primordia sizes were dramatically increased they became in average around four times larger than the initial 3 d dark (Fig 4.2).

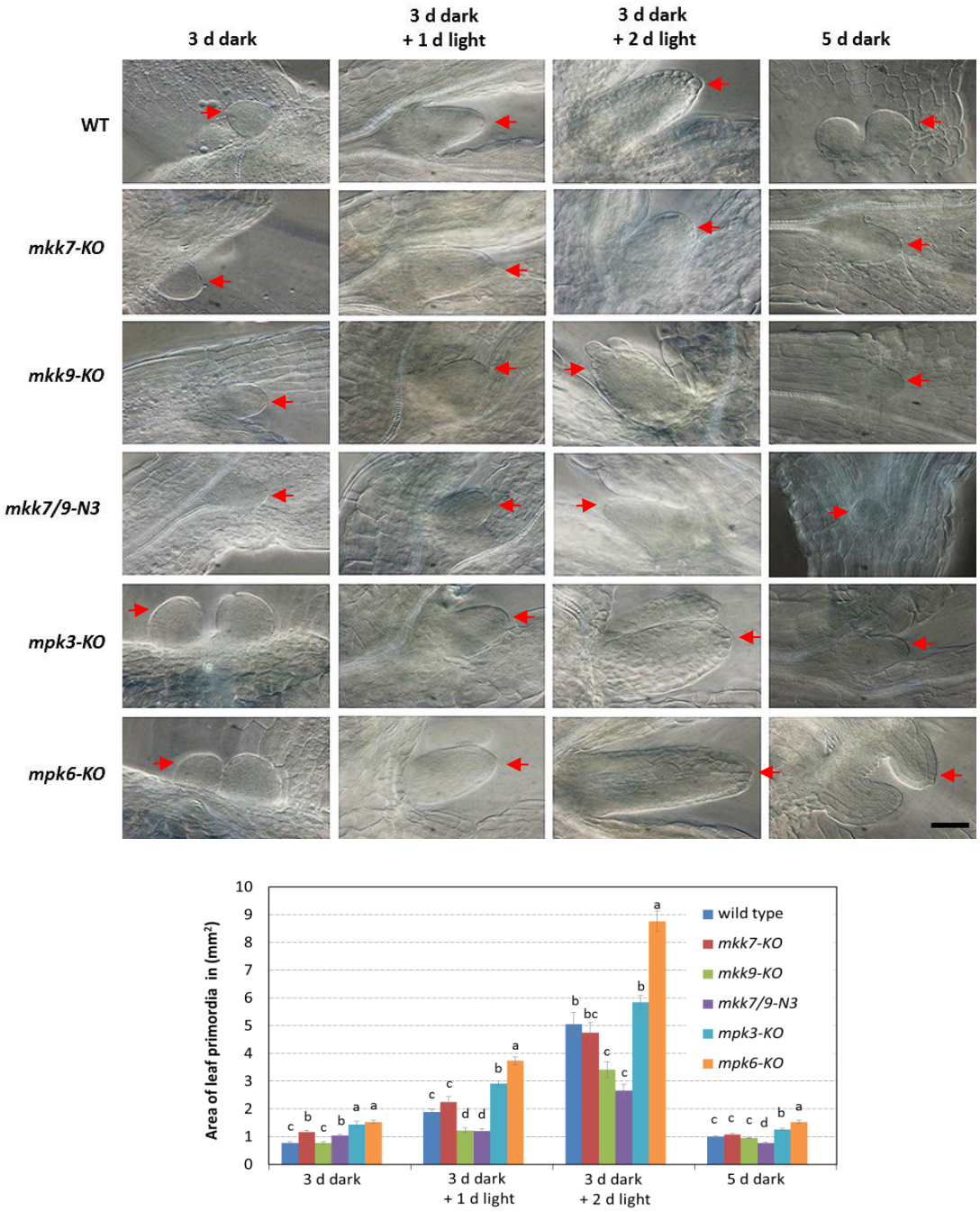

Figure 4.2 Light-induced de-repression of shoot apical meristem revealed the different role of MKKs/MPKs in leaf growth. To examine the meristem activity "light-induced de-repression of the shoot apical meristem" experiment was performed. Three-day old dark grown seedlings were transferred to continuous light and the expansion rate of the developing leaf primordia was measured. Knock-out mutations in *MKK9* resulted in reduced leaf primordial sizes, while *mpk3* and *mpk6* were larger. *mkk7* was unaffected. Up: microscopic images of leaf primordias of seedlings that were collected at various time points. Scale bar: 20 µm. Down: graphs shows the surface area of leaf primordias was determined by ImageJ analyses of microscopic images. Red arrows show leaf primordias. Error bars represent standard deviation. Statistical analysis shows significant differences performed by One-way ANOVA Tukey's test. $p<0.05$.

I applied One-way ANOVA Tukey's multiple comparison test confirmed that both at 1d and 2d in light the *mpk6-KO* mutant leaf primordia was significantly larger than wild type, while *mkk9-KO* and the *mkk7/9-N3* double mutant was significantly smaller than wild type as indicated by blue arrows in fig 4.2 up. However the size of leaf primordia of *mkk7-KO* was not significantly different. This shows that MPK6 is the major components that have a role to restrain leaf growth both during dark arrest, as well as during leaf outgrowth in the light. MKK7 might work together with MPK6 (possibly with direct phosphorylation) at the very early phase (0 d and 1 d), but not at later time points 2 d.

4.3.2 MKK7::GUS & MKK9::GUS promoter studies under dark-light transition

The publicly available microarray data show that *MKK7* is ethylene induced, while *MKK9* is induced by hypoxia (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The expression domains of MKK7 and MKK9 were studied using GUS reporter under the control of around 2 kB MKK7 and MKK9 promoter regions. The construct was made and provided me by Dr Doczi (Martonvasar, Hungary). The effects of exogenously added ethylene, auxin (2,4-D), and auxin transport inhibitors (NPA) were tested previously in our laboratory by E. Hatzimasoura on seedlings of the MKK7-GUS and MKK9-GUS reporter lines. These experiments showed upregulation of both MKK7 and MKK9 by ethylene and auxin (E. Hatzimasoura PhD thesis). I aimed to study the GUS expression on seedlings during the dark to light transition. Two independent lines of MKK7; MKK7::GUS (1), MKK7::GUS (2) and MKK9; MKK9::GUS (5), MKK9::GUS (6) were used. Seedlings were grown on ½ GM *in vitro* in continuous dark and transferred into light (samples: 3 d dark, 3 d dark+1 d light, 3 d dark +2 d light, 5 d dark and 5 d light). Seedlings were harvested and fixed for GUS staining.

MKK7::GUS seedlings in the dark for 3 d or 5 d never showed GUS staining at any parts of the seedlings (Fig 4.3 up, Table A4.1). When dark grown seedlings were transferred to continuous light for 1 and 2 d or grown 5 d in light, GUS staining appeared in the cotyledon specifically in the stomata. This staining was transient and showed the highest expression at 1-2 d, and disappeared in continuous light at 5 d. GUS activity in plants grown in the light diffusely appeared in the emerging leaf primordia but only transiently at 2 days in light. Strongest signal developed in

continuous light at late time points of 2 and 5 d in stipules, which are small appendices at the base of the developing leaves and thought to be a place for auxin biosynthesis. Additionally, I observed MKK7-GUS staining in the root which was in the region of the quiescent centre (QC) but that again only in the light conditions (Fig 4.3 up).

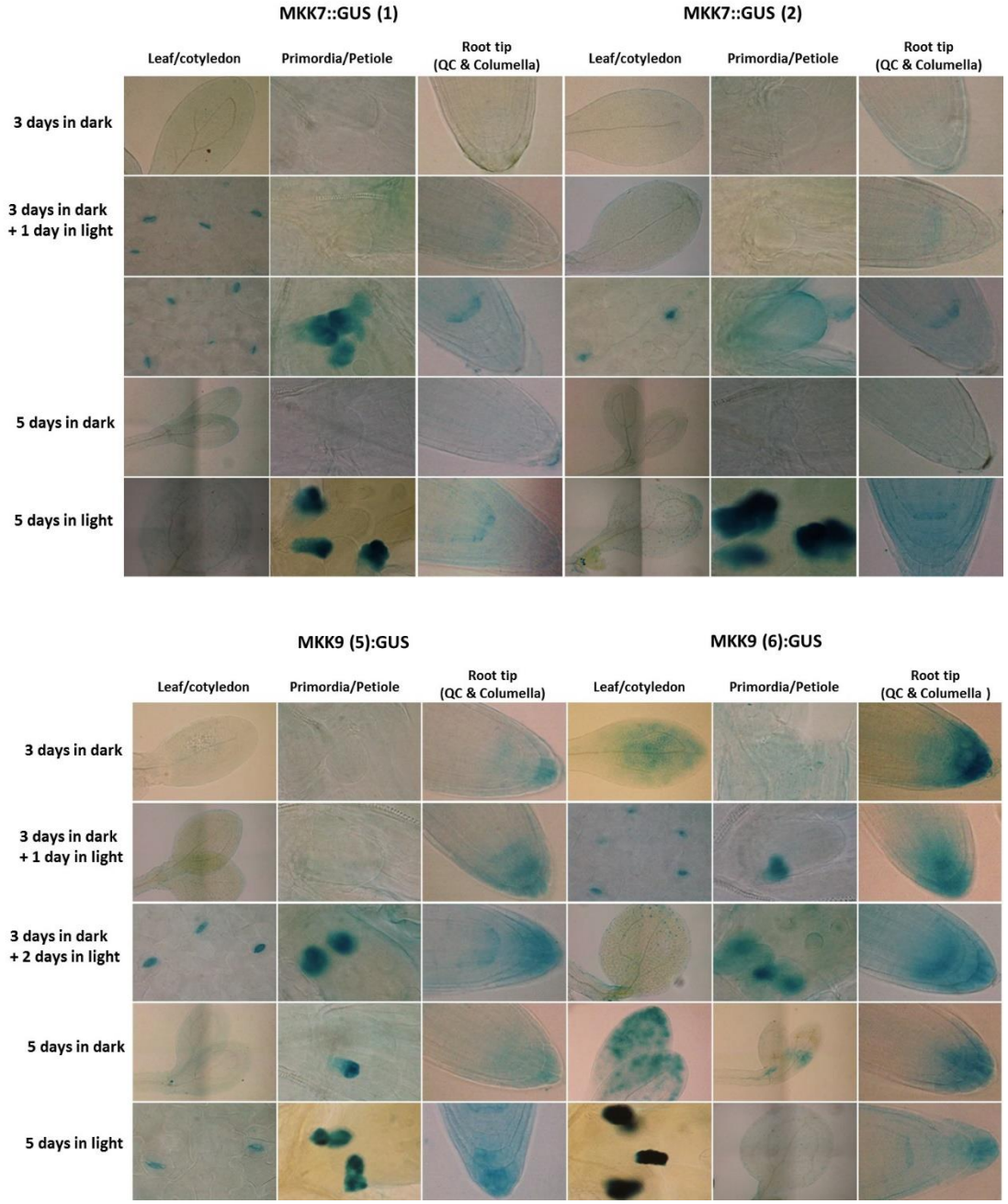

Figure 4.3 *MKKs::GUS* expressions in the dark to light transition experiment. Up: two independent lines expressing the GUS reporter gene under the control of the *MKK7* promoter used to monitor *MKK7* tissue specific transcription. Down: two independent lines carrying the *MKK9* promoter fused to the GUS reporter gene were used to follow *MKK9* tissue specific activity.

MKK9::GUS activity was generally higher than MKK7::GUS, and was already present in dark-grown seedlings as a diffuse staining in the root tip, where it mostly confined to the columella region, as well as a diffuse staining in the cotyledon. Similarly to MKK7, there was an upregulation of MKK9-GUS staining upon light transfer and the staining became confined to the stomata on cotyledon and to stipules. QC staining was also apparent with MKK9::GUS staining (Fig 4.3 down, Table A4.2).

4.3.3 Leaf initiation and flowering time under continuous light condition

MPK10 was reported to regulate leaf initiation and flowering time specifically under continuous light conditions (Stanko *et al.*, 2014). MPK10 was proposed to oppositely regulate auxin transport with MKK7. Therefore I decided to test the growth of knock out mutants in the MKK7/9 and MKK3/6 MAPK components. In this experiment *mkk7-KO*, *mkk9-KO*, *mkk7/9-N3*, *mkk7/9-N5*, *mpk3-KO*, *mpk6-KO* and WT, initially were grown in ½ GM media after 8 dag seedlings of each line were transferred into soil in 10 replicates and grown further in continuous light till 18 days. The growing performance and pattern recorded such as bolting, number of rosettes leaf, flowering time. The *mpk3-KO* and *mpk6-KO* bolted early and had grown somewhat faster than wild type, while the double mutant *mkk7/9-N3* was delayed in flowering time. However, the number of rosette leaves at which these lines flowered were not different from wild type. This indicated that not the flowering time, but the speed of leaf initiation that has changed (accelerated) in the *mpk3-KO* and *mpk6-KO* mutant lines. This is consistent with the faster leaf outgrowth during the dark to light transition.

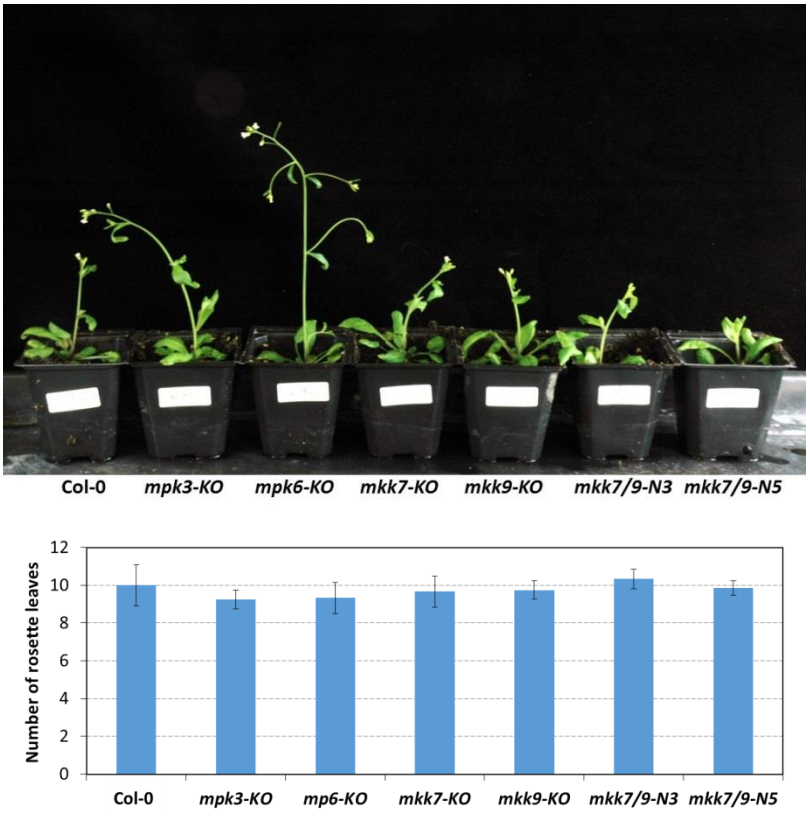

Figure 4.4 Inactivation of *MKK* and *MPK* genes affects flowering time. Wild type and mutant plants were grown in continuous light Number of rosettes leaves of wild type and mutant plants were grown in continuous light n>4.

4.4 Chapter discussion

Plant development flexibly responds to changes in the environment. Growth initiation itself may be conditional to a suitable environment, while the most common response of plants to adverse conditions is growth inhibition (Bray *et al.*, 2000). Most of our understanding about environmental growth inhibition comes from studies on various plant hormones, while little is known about signalling mechanisms involved. The mitogen-activated protein (MAP) kinase cascades are central signal transduction pathways in all eukaryotes and their roles in plant stress responses is well-established while increasing evidence points to their involvement in hormonal and developmental processes as well.

MKK7 has been shown to be a negative regulator of polar auxin transport, while the dark grown shoot apex is under a state of high auxin response (Dai *et al.*, 2006). Furthermore, promoter analysis of *MKK7* and *MKK9* using GUS reporter revealed that these kinases are themselves under tight transcriptional regulation.

These results underpin the role of stress-activated MAP kinase signalling in regulating growth responses at the whole plant level.

Organogenesis in plants differs from the process in vertebrates, being mainly postembryonic and continuing throughout the life of the plant (Perianez-Rodriguez *et al.*, 2014). All newly formed organs are derived from meristems, the source and organising tissue of plant growth: above-ground organs initiate from the shoot apical meristem (SAM) while roots from the root apical meristem (RAM) (Machida *et al.*, 2013). Furthermore, organ growth and morphogenesis in sessile plants show a remarkable plasticity to allow environmental adaptation, including changes in light, temperature, nutrient and water status and biotic interactions. The most common developmental response of plants to stress is pausing growth (Skirycz and Inze, 2010). On the other hand initiation of the entire meristem developmental programme may be contingent upon the environment.

Hormones are central to growth regulation and meristem activation or repression. Abscisic acid, jasmonates and salicylic acid are traditionally categorised as 'defence hormones' and act mainly as growth repressors (Noir *et al.*, 2013). Besides, the gaseous hormone ethylene regulates both biotic and abiotic stress responses as well as various developmental processes such as seed germination, seedling growth, leaf and petal abscission and organ senescence (Bleecker and Kende, 2000). Furthermore, it is increasingly clear that there is intense crosstalk between environmental signals and the long-established growth factor auxin (Potters *et al.*, 2007; Kazan and Manners, 2009).

The mitogen-activated protein (MAP) kinase phosphorylation cascades are conserved signalling modules in all eukaryotes and known to have pivotal roles to regulate cell division, cell growth and stress responses in animals. In plants MAP kinase pathways have been shown to play a major role in stress signalling (Pitzschke *et al.*, 2009). The best studied plant MAP kinase is MPK6 of Arabidopsis, which regulates various stress responses such as pathogen defence (Asai *et al.*, 2002; Meszaros *et al.*, 2006), cold and salt stress (Teige *et al.*, 2004), as well as developmental processes, such as inflorescence and embryo development (Bush and Krysan, 2007; Wang *et al.*, 2008) and stomatal patterning (Wang *et al.*, 2007). MPK6 has been implicated in the regulation of ethylene biosynthesis and signalling (reviewed by (Hahn and Harter, 2009)). In addition to this apparent functional complexity MPK6 has been assigned as being downstream of at least five of the ten

Arabidopsis MAP kinase kinases, belonging to all four phylogenetic MKK groups (Asai *et al.*, 2002) (Teige *et al.*, 2004; Meszaros *et al.*, 2006; Takahashi *et al.*, 2007; Yoo *et al.*, 2008; Zhou *et al.*, 2009), suggesting an elaborate regulatory network.

MPK6 has been associated with MKK9 (Xu *et al.*, 2008; Yoo and Sheen, 2008). MKK9 participates in salt signalling, genetic evidence supports both positive and negative regulation of salt tolerance by MKK9 (Alzwiy and Morris, 2007; Xu *et al.*, 2008). Furthermore, similarly to its downstream partner MPK6, MKK9 has been also functionally associated with both ethylene biosynthesis and signalling (Xu *et al.*, 2008; Yoo *et al.*, 2008). A dual role in inhibiting polar auxin transport and promoting pathogen defence has been unravelled for MKK7, the closely related MKK (Dai *et al.*, 2006; Zhang *et al.*, 2007). Furthermore, both MKK7 and MKK9 have negative as well as positive functions in stomatal cell fate regulation (Lampard *et al.*, 2009). In light of these diverse findings a novel, complex regulatory pathway is emerging at the cross-roads of plant developmental and stress responses, which minimally comprises of MKK7/9 and MPK6.

A good example of environmentally-induced developmental response is that of the shoot meristem to light. In flowering plants, seedlings which germinate in darkness undertake a developmental programme called skotomorphogenesis, in which the embryonic stem elongates but leaf growth at the SAM is arrested. The rapid and synchronous induction of growth in shoot apices when dark-grown seedlings are transferred to light (photomorphogenesis) offers an excellent experimental system to assess the state of shoot meristem activity, and it was recently used to unravel the underlying multi-layered gene expression program (Lopez-Juez *et al.*, 2008). It was observed, for example, that shoot apex deetiolation is characterised in the first few hours by the transient downregulation of auxin and ethylene and the upregulation of cytokinin and gibberellin hormonal responses. This was coupled with the induction of genes involved in protein translation and cell proliferation (Lopez-Juez *et al.*, 2008). Later on, leaf primordial expansion and chloroplast biogenesis programmes became apparent. Interestingly, this study found a number of MAP kinase signalling genes, including MPK6, with high dark expression and rapid light downregulation. Both hormonal and MAP kinase signalling responses were exclusive to the shoot apex.

Here, I have utilised this experimental approach and uncovered a negative regulatory function of the MKK7/9-MPK6 module in meristem function. I show that

meristem de-repression in response to exposure to light be reduced in *mkk7/9* and accelerated in *mpk6-KO* mutant seedlings.

Chapter 5: Involvement of MKK7/MKK9 and the downstream MPK3/MPK6 signalling in the drought-induced leaf growth arrest

5.1 Introduction

Drought is known to affect plant growth to reduce surface area as well as to conserve energy during stress conditions. The molecular basis of drought response was studied in *Arabidopsis* growing leaves administering mild osmotic stress that reduced leaf growth by around 50%. It was found that such a mannitol treatment arrested cell proliferation transiently, which was then resumed with an altered growth rate (Skirycz *et al.*, 2011a). What might be the mechanism for this growth adaptation upon osmotic stress was studied by microarray experiments (Skirycz *et al.*, 2011a; Baerenfaller *et al.*, 2012). The microarray data allowed building co-expression gene regulatory network and uncovering processes such as protein translation and cell cycle regulation that are impacted on by the mild drought treatment. Hormone rebalancing involving jasmonic acid, gibberellins and ethylene was an unexpected discovery through these microarrays, as so far mostly ABA that was connected with drought response. What is becoming clear that the regulation of stress survival upon serious stress and the regulation of growth adaptation to mild stress involve very distinct processes (Skirycz and Inze, 2010). Importantly in this microarray experiment it was found that MKK7 and MPK6 are transcriptionally induced upon mild drought stress in growing leaves. This led to the hypothesis that MKK7 might connect to the modulation of protein translation and cell cycle regulation to adapt leaf growth to drought.

MKK7/MKK9 were also connected to cell death phenotypes and ethylene signalling. MKK7 and MKK9-induced cell death phenotypes were strongly suppressed in NbSgt1b-silenced plants, indicating a possible connection to the cell

death induced during innate immune response. Cell death is also induced by MKK4/5 and their tomato homologues (Ren *et al.*, 2002; Pedley and Martin, 2005). In addition, MKK9, was found to promote constitutive ethylene signalling (Yoo *et al.*, 2008). Among the downstream MAPKs, MPK3, MPK4 and MPK6 had been widely studied and are activated by diverse set of stresses, including pathogens, osmotic, cold, and oxidative stress (Kovtun *et al.*, 2000; Nuhse *et al.*, 2000; Asai *et al.*, 2002; Desikan *et al.*, 2001; Droillard *et al.*, 2002; Colcombet and Hirt, 2008).

MKK4/5 and MKK7/9 were also found to inhibit meristemoid self-renewal in the stomata developmental linage. Recently it was shown that leaf growth is reduced through the stomata regulatory gene SPEACHLESS (Kumari *et al.*, 2014).

Plant development is depends on the coordination between growth and cell proliferation. The nutrient sensing (Target of rapamycin) TOR kinase and its downstream target 40S ribosomal S6 Kinases (S6Ks), are central controllers of cell growth (Fig 5.1) that tunes the translational capacity of cells through the phosphorylation of ribosomal protein S6 (RPS6) (Meyuhas, 2008). This pathway also have roles to determine cell size by inhibiting the onset of mitosis in yeast and animal cells (Wullschleger *et al.*, 2006; Diaz-Troya *et al.*, 2008; Ma and Blenis, 2009; Montagne *et al.*, 1999; Pende *et al.*, 2004). S6Ks are belong to AGC family (PKA, PKG, PKC) of serine/threonine kinases and are also present in plants (Bogre *et al.*, 2003). The mutations of the S6K phosphorylation sites of RPS6 affected cell size, but not protein synthesis (Ruvinsky *et al.*, 2005). S6K1 is expressed in meristematic and actively elongating cells within the root regions in *Arabidopsis* and lily (Zhang *et al.*, 1994; Tzeng *et al.*, 2009).

The *Arabidopsis* S6 Kinase1 inhibits cell proliferation via association with the RBR-E2FB complex. S6K1 interacts with RBR via its N-terminal RBR LVxCxE binding motif, promotes its nuclear localization (Henriques *et al.*, 2010), and this complex through E2FB potentiates the repression of cell cycle genes such as the plant specific mitotic cyclin dependent kinase *CDKB1;1* gene and the S-phase specific ribonucleotide reductase *RNR2* gene (Henriques *et al.*, 2013).They also demonstrated that depletion of RBR1 resulting higher expression of E2FA can lead to increased ploidy level and promotes aneuploidy, similarly to depletion of S6K1 and S6K2 in the *s6k1s6k2/++* and *s6k1* (XVE-RNAi) plants (Henriques *et al.*, 2010). The outcomes strongly suggest that S6K negatively regulates cell division in plants. A microarray experiment with inducible MKK7 line showed a rapid downregulation

of genes involved in protein translation and recently we collaborated with Dr. Gerold Beckers (Achen University) to do phosphoproteomics with the inducible MKK7 line and found that RPS6A get phosphorylated by MKK7 on the C-terminal S6K site. This indicates a crosstalk between the TOR-S6K and MKK7 MAPK pathway to regulate cell growth and cell proliferation.

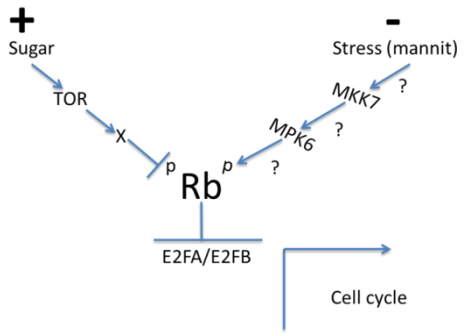

Figure 5.1 E2Fs/RB model regulated by upstream MKK proteins under drought stress and availability of nutrients**.**

5.2 Aims and objectives

1. To investigate the mechanisms underlying cell proliferation inhibition in young proliferating leaves of the *Arabidopsis* thaliana when subjected mannitol stress.
2. To decipher the connections between MAPKs regulatory network and growth, cell cycle inhibition via E2F/RB regulatory module.

5.3 Results

5.3.1 Characterisation of leaf growth in *mkk7/9* and *mpk3/6* mutants upon osmotic stress

Mannitol is a non-metabolisable sugar that can be used to increase turgor pressure in the cell resulting in water deficiency and therefore provides an experimental condition to mimic drought condition. It was shown that upon mild osmotic stress growth initially decreases rapidly but within one day plants adapt and the leaf growth recover at a reset rate (Skirycz and Inze, 2010; Skirycz *et al.*, 2011a). MAPKs are known to be induced by osmotic stress, and therefore I set out to test, whether MAPK pathways also involved in growth adaptation of leaf growth.

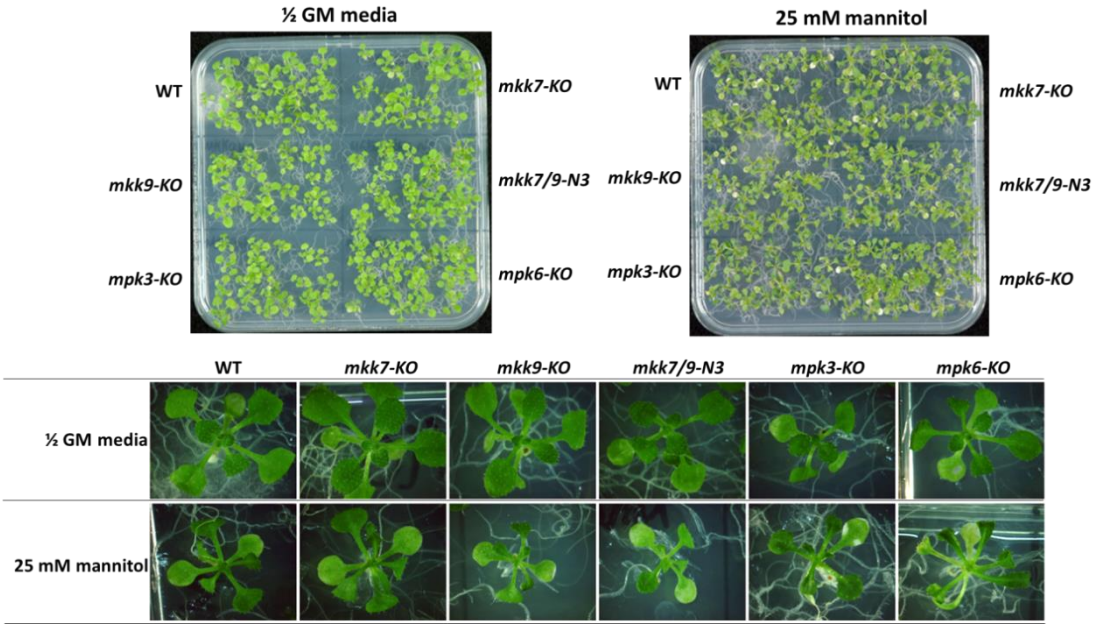

Figure 5.2 Physiological effects of mild osmotic stress. WT and mutant lines were grown in the presence and absence of 25 mM mannitol. Images of culturing plates (**top panel**) and typical plants (**bottom panel**) were taken 8 DAT. Scale bar: 10 mm.

In this experiment *mkk7-KO*, *mkk9-KO*, *mpk3-KO* and *mpk6-KO* single mutants and the *mkk7/9-N3* double mutant was studied alongside with WT Columbia-0. Seedlings were grown on ½ GM media and after 8 days (8 DAG) seedlings were individually transferred onto media containing 25 mM mannitol and without mannitol and seedling growth was followed. Phenotypic data are shown 8 days after transfer, the seedlings transferred onto 25 mM became visibly smaller,

leaves curling down, as compared to control seedlings in absence of mannitol (Fig 5.2 up). The *mpk6-KO* and, to a lesser extent *mpk9-KO* mutants, were somewhat more sensitive to 25 mM mannitol compared to wild type, while *mkk-7-KO* mutant was less effected (Fig 5.2 down) and quantitative data are shown in Fig 5.3.

5.3.1.1 3rd pair of leaves size characterization by methanol clearing

To quantitate the leaf growth arrest upon transfer to 25 mM mannitol transfer, the 3rd leaves were dissected and cleared in absolute methanol (to remove chlorophyll completely) and images were taken under light microscope. Using these images I measured and calculated leaf surface area as described in Section 2.4 (Fig 5.3).

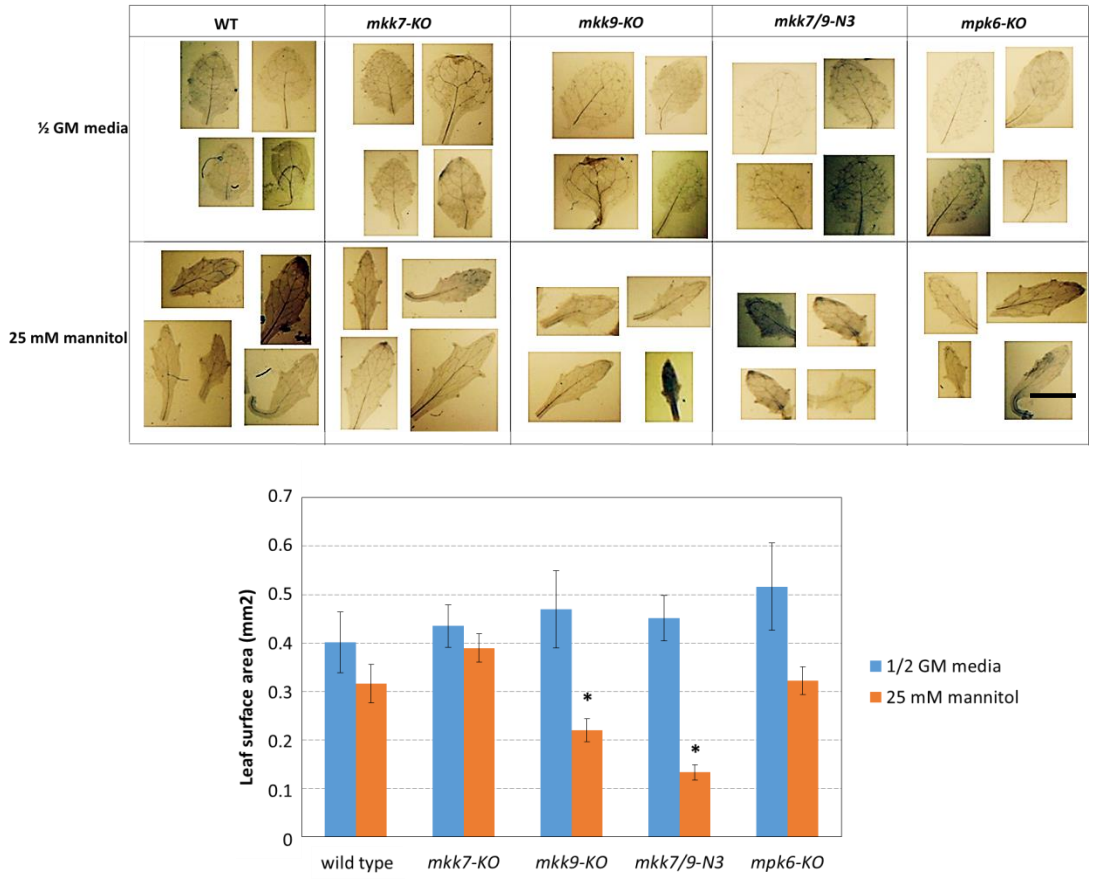

Figure 5.3 Inactivation of *MKK9* and *MPK6* results in reduced leaf size during mild osmotic stress. Lines were grown in the presence and absence of 25 mM mannitol in ½ GM media, leaves were collected 8 DAT and cleared in methanol (up) Down: ImageJ quantification of leaf sizes. Erros bars show SE. Statistical analysis: One-way ANOVA, Dunnett's test, $p<0.05$) Scale bar: 5 mm.

After 8 days on mannitol both the size and shape were noticeably different becoming longitudinal and serrated. All the mutant lines as well as the WT showed leaf growth inhibition in presence of mannitol compared to the control without mannitol. The *mkk7/9-N3* mutant and the *mkk9* double mutant showed the largest growth inhibition followed by *mpk6*. On the other hand, leaf growth inhibition was lower for *mkk7* compared to wild type (Fig 5.3).

The germination rate was also recorded on ½ GM medium till 5 days to rule out that any growth difference is due to delayed germination on the control media before transfer. All the lines geminated similarly at the same time (Fig 5.4). Hence I confirmed the variation in seedling growth were not due to delayed germination or any other growth conditions.

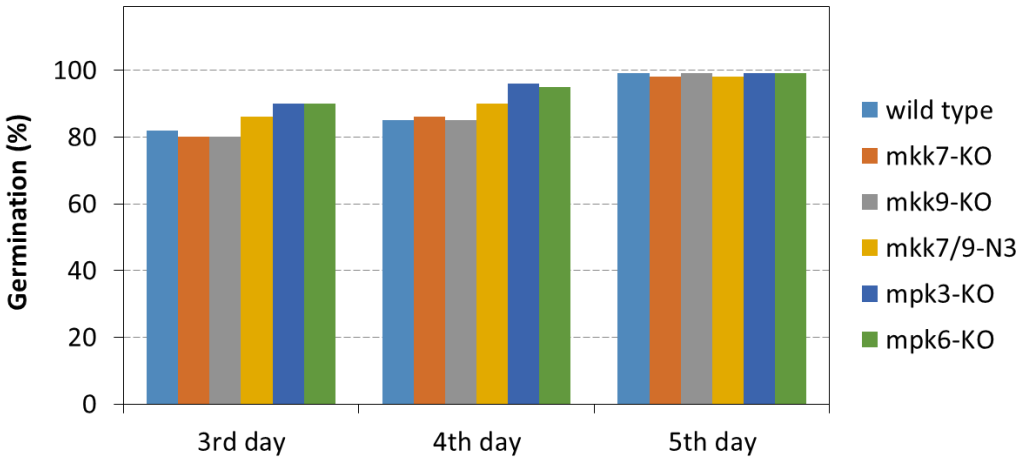

Figure 5.4 Seed germination rates in ½ GM media. The seed germination of mutants was recorded till 3 days under stereomicroscope.

5.3.2 Ploidy analysis of 3rd leaves of mkk7/9 and mpk3/6 mutants upon osmotic stress

Leaf cells when they begin to expand can enter endoreduplication (Edgar and Orr-Weaver, 2001). Because of the reduced leaf sizes and changed morphology that I observed upon 25 mM mannitol treatment, I asked whether the reduced growth is reflected by altered ploidy distribution. 8 DAG old seedlings on ½ GM media were individually transferred onto media supplemented with 25 mM mannitol and without mannitol observation were taken 4, 5, 6, 7 and 8 days after transferred. The ploidy distribution was analysed by flow cytometer. Upon mannitol treatment the wild type leaf showed an increased entry into endoreduplication having 8n cells. The entry into

endoreduplication was blocked in the *mkk9-KO* and *mpk6-KO* mutants and was significantly increased in the *mkk7-KO* mutant. This corresponded well with the higher sensitivities of leaf growth arrest in the *mkk9-KO* and mpk6-*KO* mutants and the relative resistance to growth inhibition in the mkk7 mutant compared to wild type. On the control plate without mannitol all mutant lines were similar to wild type except *mpk3* which showed a higher proportion of 2n (Fig 5.5). Our results are consistent with earlier report that showed that the ploidy increases in response to mannitol. There is an arrest at the G2/M transition that leads to the entry into endoreduplication (Skirycz *et al.*, 2011).

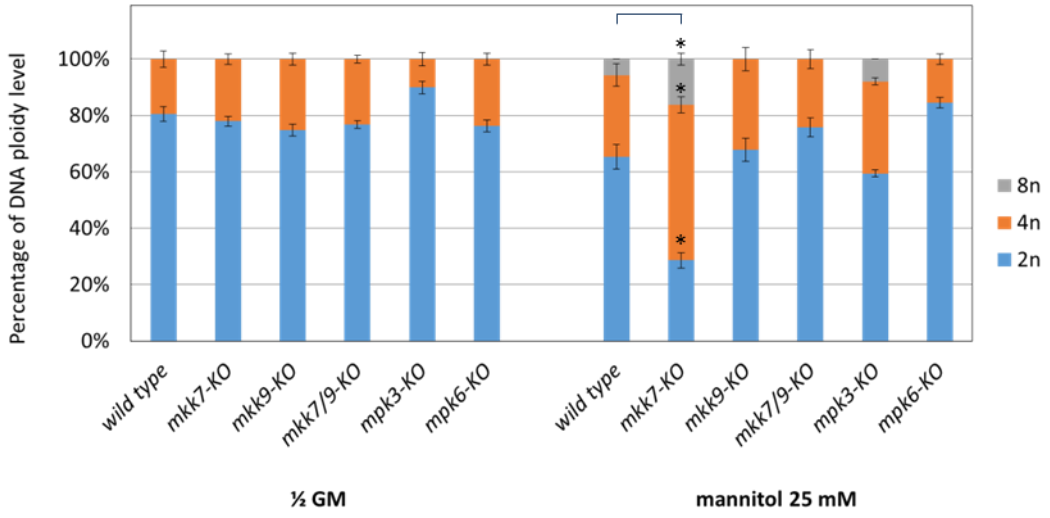

Figure 5.5 DNA ploidy analyses of *mkk* and *mpk* mutants. DNA content of WT and mutant lines in the presence and absence of 25 mM mannitol on ½ GM media (8DAT). Error bars show SE. Statistical differences were found between WT and *mkk7-KO* using One-way ANOVA Dunnett's test ($p<0.05$).

5.3.3 Mitotic reporter gene (CycB1;1::GUS) expression under mannitol stress

CycB1;1::GUS is a mitotic reporter construct having the promoter of *CYCB1;1* gene as well as the N-terminal coding region of CYCB1;1 that contain the mitotic destruction box and this is fused in frame with the GUS reporter gene (Colon-Carmona *et al.*, 1999). Therefore this GUS marker is expressed from late G2 phase until the meta- to anaphase transition when it is destroyed by proteolysis through the anaphase promoting complex. I transferred seedlings 7 DAG onto 25 mM mannitol and stained the 1st leaves for GUS activity at 0, 3, 6, 12 and 24 time points after transfer. The most noticeable difference in GUS staining was present 24

hrs after mannitol treatment (Fig 5.6). The GUS staining was restricted to the base of the leaves and there was less blue staining at the base of leaf in presence of mannitol compared to control (Fig 5.6).

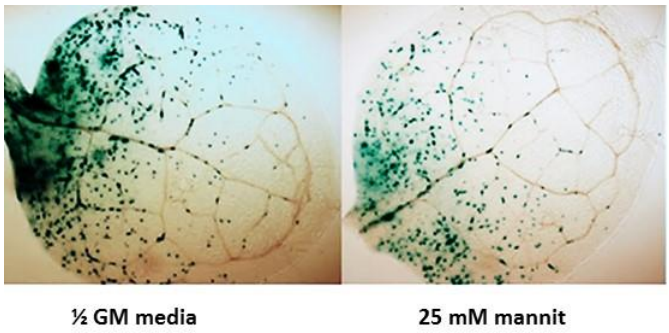

Figure 5.6 The *CYCB1;1::GUS* reporter showed reduced activity during mild osmotic stress. Activity of the mitotic marker *CYCB1;1::GUS* was detected at the leaf base in 7 days old plants. Mild osmotic stress treatment (25 mM mannitol) for 24 hours resulted in reduced *CycB1;1* promoter activity compared to the untreated leaf.

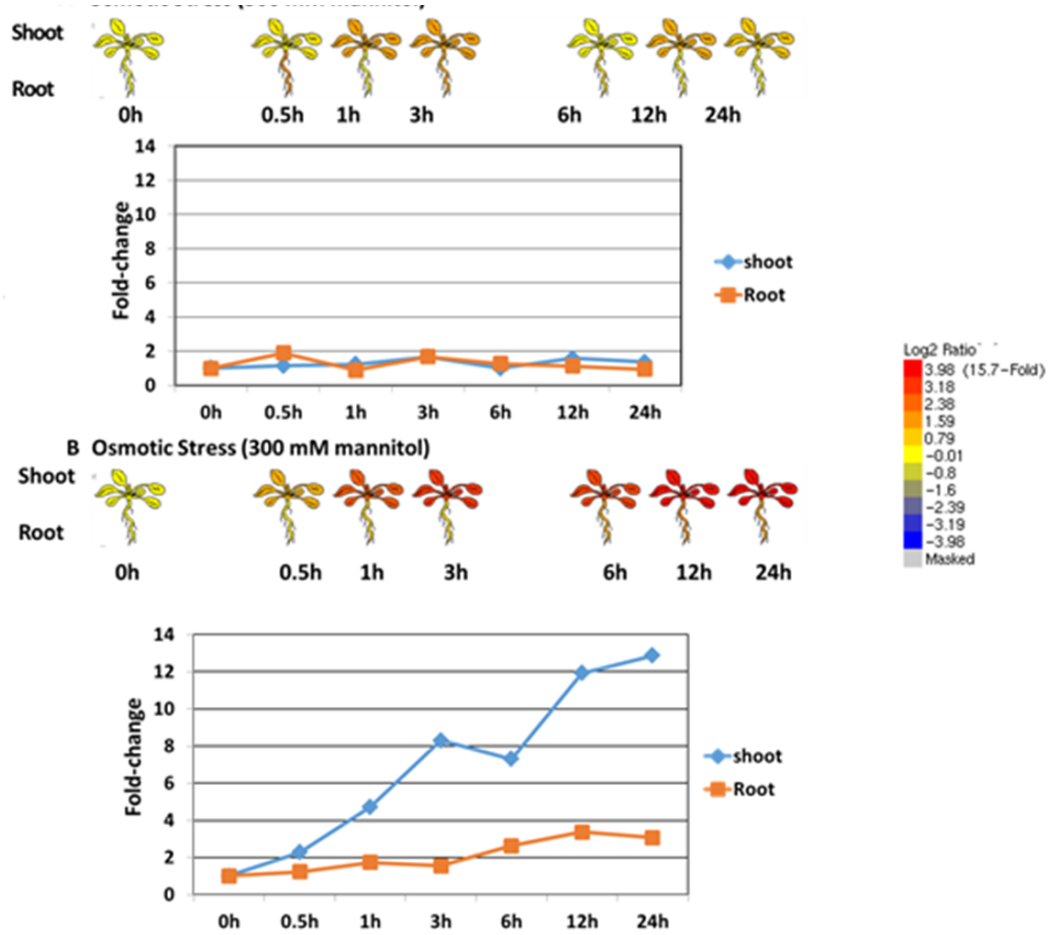

Figure 5.7 *MKK9* is induced by osmotic stress. Fold changes in gene activity at different time points for shoot and root in MKK7 (**A**) and MKK9 (**B**) in 300 mM mannitol stress treatment. Data collected from http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi.

5.3.4 Developmental regulation of MKK7 and MKK9 expression upon osmotic stress by mannitol in shoot and root

There are online resources for visualisation of microarray data that can give information on the tissue specific responses of various stresses. The transcriptomics data for gene-fold changes in MKK7 and MKK9 under 300 mM mannitol for several hrs of induction, were retrieved from the *Arabidopsis's* TAIR website searching through e-FP browser. In case of MKK7 there is no significant change neither in the shoot or root (Fig 5.7 up) while for MKK9 the gene-fold changed is pronounced, especially in the shoot but also in the root under 300 mM mannitol across the time points (Fig 5.7 down). This well corresponds to the increased sensitivity of leaf growth inhibition of *mkk9-KO* mutant upon 25 mM mannitol in the previous experiment (Fig 5.3).

5.3.5 RBR phosphorylation and association with E2FB upon mannitol treatment

I showed that upon osmotic stress conditions both the cell proliferation and leaf growth are arrested. The central regulator of cell proliferation is the Rb protein, which in plants called RETINOBLASTOMARELATED (RBR). Under favourable conditions RBR becomes phosphorylated through the CYCD3;1/CDK complex and this disrupts RBR interaction preferentially with the E2FB transcription factor, that promote cell proliferation (Magyar *et al.,* 2012). E2FB and RBR also form a complex with S6K, and this complex functions to restrain cell division (Henriques *et al.,* 2010). Interestingly, S6K1 overexpression leads to osmotic sensitivity that is relieved by the co-overexpression of RAPTOR (Mahfouz *et al.*, 2006). To begin to understand the molecular basis how osmotic stress might restrain leaf growth I set out to examine the changes in RBR, E2FB protein levels, the phosphorylation status of RBR and complex formation between RBR and E2FB upon 25mM mannitol treatment. In this experiment, 7 days old seedlings of RBR-GFP grown on ½ GM, were transferred into media containing 25 mM mannitol and without mannitol and leaf 1-2 were harvested on different time points. Western blots were performed to investigate the levels of RBR-GFP, P- RBR-GFP and E2FB with corresponding antibodies. RBR-GFP protein levels showed a mild transient increase in control condition that might reflect the transfer to fresh medium. It is known that RBR levels

follow the extent of proliferation in leaves (Magyar *et al.*, 2012). P-RBR level also increased when seedlings were transferred to fresh medium without mannitol (Fig 5.8A). Opposite to control conditions, samples transferred to 25 mM mannitol showed a steady decrease in RBR-GFP levels that might reflect the drop in the extent of cell proliferation. Surprisingly, the P-RBR levels did not drop upon osmotic stress of leaves in response to 25 mM mannitol at 10, 24, 48 and 72 hrs as compare to control but P-RBR levels was even higher in mannitol as compare to without. E2FB levels largely mirrored P-RBR levels. Stained membrane shows the loading control (Fig 5.8A). This unexpected result showed us that in spite of cell cycle arrest upon osmotic stress condition RBR does get phosphorylated.

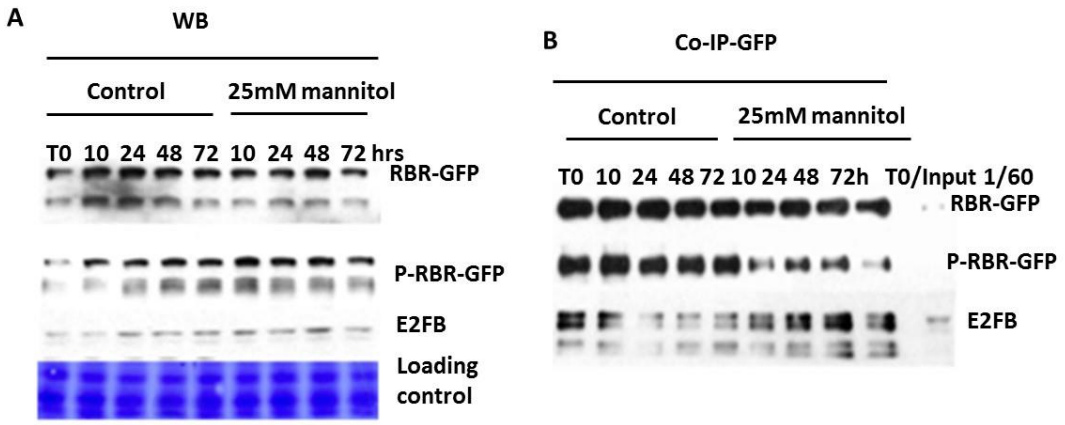

Figure 5.8 Western blot for RBR, P-RBR and E2FB and co-immunoprecipitation of E2FB with RBR1-GFP upon 25 mM mannitol treatment. (A) Western blot detection of RBR-GFP, P-RBR and E2FB in crude cell extract of leaves taken from one week old seedlings treated or not with mannitol for the indicated time points. Staining of the gel is shown for loading control. **(B)** Co-immunoprecipitation of E2FB with RBR1-GFP pulled down by RBR-GFP antibody beads at different time points of leaves collected from a week old seedlings treated with mannitol or without mannitol. Inputs are shown on the right for T0.

It is known that the phosphorylated RBR cannot associate with E2FB. To address this question upon osmotic stress we performed co-immunoprecipitation experiments with RBR-GFP using the GFP antibody-conjugated beads (ChromoTek) and detected E2FB with E2FB-specific antibody. In this experiment the immunoprecipitated RBR and P-RBR levels were different than what we detected in

crude extract. The reason for this is not clear. We found that P-RBR levels did decrease gradually upon treatment with 25 mM mannitol. In parallel there was an increasing amount of E2FB that associated with RBR during the time course of 10, 24, 48 and 72 hrs of 25 mM mannitol treatment (Fig 5.8B).

5.3.5.1 Generation of genetic crosses between pER8::MKK7 and E2FA-GFP or E2FB-GFP

Our hypothesis is that osmotic stress such as administered by the 25 mM mannitol treatment induces the MKK7 or MKK9 MAPK pathway and through that it impacts on the cell proliferation possibly through the regulation of RBR/E2F association. To address this question, I made genetic crosses between the inducible pER8-MKK7 and pER8-MKK9 lines and the E2FA and E2FB lines tagged with GFP and expressed under the control of their own promoters. In this genetic crosses I aim to induce the MKK7/9 MAPK pathway through the estradiol-induced expression of MKK7 and MKK9 and study the association of E2FA or E2FB with RBR. I also look to see whether the drastic halt of growth could be overcome by the elevated E2FA or E2FB expression. The transgenic lines pER8::MKK7 (1), E2FA-GFP82/1 and E2FB-GFP72/5 (these lines have elevated expression) were grown to perform crosses, pER8::MKK7 (1) was used as pollen while E2FA-GFP82/1 and E2FB-GFP72/5 were used as the female. F1 seeds were collected and tested for the transmission of the Hyg resistance marker of the pER8-MKK7 construct. The successful crosses were used to produce F2 generation and F3 generation and lines were selected that were homozygous both for the pER8-MKK7 (100% hygromycin resistant) and for the E2FA- or E2FB-GFP (100% norflurazone resistant). The homozygous plants for both parents were chosen for further studies such as root growth analysis of mutants.

5.3.5.2 Root growth studies of pER8-MKK7/E2FA and pER8-MKK7/E2FB

As I showed before in Capter 3, when MKK7 expression is induced by β-estradiol the root growth is immediately arrested in the pER8::MKK7 line. I decided to test whether the root growth arrest is retained in the crosses with the E2FA-GFP andE2FB-GFP lines. I set up an experiment with pER8::GUS as a negative control, the MKK7 (pER8::MKK7(1)), E2FA-GFP81/1, E2FB-GFP72/5 parental lines and the MKK7 X E2FA-GFP81/1, the MKK7 X E2FB-GFP72/5 double homozygous

lines. Seeds were plated on stripes of Whatman paper on ½ GM media and after 4 dag seedlings were transferred onto media containing 0.1 µM estradiol and without β-estradiol. The observations were taken from 1-4 days after induction, but data only shown at the end point of 4 days induction (Fig 5.9). As expected, root growth of MKK7 line was inhibited upon the β-estradiol treatment compared to the empty vector, the E2FA-GFP and E2FB-GFP parental lines were not affected. Surprisingly, the double MKK7 X E2FA-GFP82/1 and MKK7 X E2Fb-GFP72/5 showed a much reduced reduction in root growth than the MKK7 alone, even though I expected that the MKK7 became induced in the same extent in these crosses than in the parental line (Fig 5.9). To be certain of this assumption, the MKK7 levels will need to be tested in the crosses. If this is the case, it might indicate that the increased E2FA and E2FB levels can overcome the MKK7-induced root growth arrest. This would mean that MKK7 indeed act on the regulation of RBR-E2F complex.

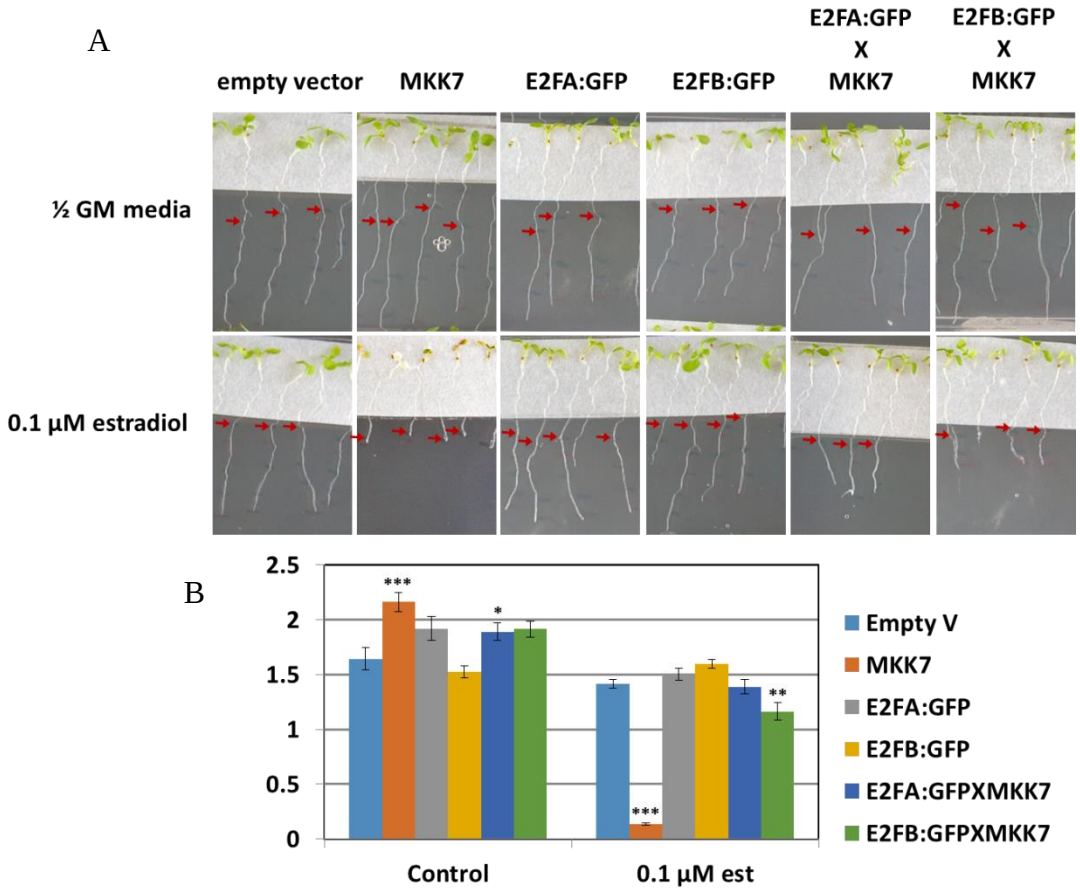

Figure 5.9 Root length measurements of MKK7 mutants induced by β-estradiol 4 days after induction with positive and negative controls. (A) Root length of mutants grown on ½ GM media and ½ GM supplemented with 0.1 µM β-estradiol. Red arrows indicate the root length of plants transferred onto β-estradiol containing media. (B) The chart shows mean of root length of mutants. Error bar represent SE, One-way ANOVA Dunnett's multiple comparison test was performed comparing each line to the empty vector line.

5.4 Chapter discussion

Drought is one of the most important environmental stress that arrest plant growth (Dubois *et al.*, 2013; Skirycz *et al.*, 2011b; Avramova *et al.*, 2015).This occurs in developing organs, such as developing young leaves by altering the meristematic activities (Skirycz and Inze, 2010). I showed here that mimicked drought conditions using low concentration of mannitol can reduce the mitotic activity of developing leaves as measured by the *CYCB1-GUS* marker line. This is consistent with the results published earlier (Skirycz *et al.*, 2011a). The inducible overexpression of MKK7 and MKK9 similarly arrest the mitotic activity of young leaves. I found using flow cytometry that cells arrest in the cell cycle at G1/S transition and do not enter into the modified cell cycle, called endocycle. Interestingly, these cells with lower ploidy still were larger in size (Kalve *et al.*, 2014). Thus the correlation of ploidy level and cell size did not hold.

In plants growth is restricted to meristems, and thus a key factor is the duration of cell proliferation and the timing of the exit from proliferation to cell expansion and differentiation (Doonan and Sablowski, 2010). The current view is that these events are controlled by an evolutionary conserved transcriptional regulatory switch, the E2F-RB pathway (Inze and De Veylder, 2006). The Retinoblastoma (RB) was the first tumour suppressor gene cloned from mammalian cells, while the first adenovirus E2 binding transcription factor (E2F) was identified based on its ability to form a complex with the RB protein (van den Heuvel and Dyson, 2008).

In principle E2F works as heterodimeric transcription factor forming complex with dimerization partner (DP) protein prior to binding to the DNA and activate the expression of target genes required for entering into the cell cycle (van den Heuvel and Dyson, 2008). RB binds to E2F in cells leaving mitosis at their carboxyl terminal RB binding motif and inhibits their activities. This is converted upon mitogen stimulation by hyperphosphorylation of RB by specific CDK-Cyclin D complexes, leading to the activation of genes required for DNA synthesis. Animal E2Fs were classified as activators and repressors based on whether they transactivate

genes which are repressed by RB or form a co-repressor complex with RB, respectively (van den Heuvel and Dyson, 2008).

The RB-E2F network is remarkably conserved between the plant and animal kingdoms, E2F and RB homologues have been identified from the unicellular green alga *Chlamydomonas reinhardtii*, to the higher plants including the model plant *Arabidopsis thaliana* (Berckmans and De Veylder, 2009). E2FA, E2FB and E2FC are structurally related to the canonical animal E2Fs since they have all the conserved domains characteristic for the animal E2F1-3, including the DNA-binding, dimerization, transactivation and RB binding domains. Functional characterization of the individual members of Arabidopsis E2Fs have already revealed differences among them: ectopic expression of E2FA with DPA resulted in strong activation of both mitotic cell cycle and endocycle (Magyar *et al.*, 2012; Berckmans *et al.*, 2011b). E2FB activate mitosis but it repressed the endocycle, whereas reduction in the level of E2FC confirmed its negative regulatory function in mitosis but a positive one in endoreduplication. I showed that the growth arrest of leaves might work through the modulation of E2F-RBR complexes. Upon drought I found a decreased RBR phosphorylation and a more stable RBR complex with E2FB. Importanely, elevated expression of E2FA and E2FB could overcome the root growth arrest induced by MKK7 overexpression.

Chapter 6: Discussion

6.1 Effects of drought on plant growth and development

The effects of drought on plant development depend upon species, native habitat and the species-specific strategies used to control plant water status and tolerate drought. One of the earliest effects of drought is the production of the phytohormone abscisic acid (ABA) that triggers stomatal closure to reduce evapotranspiration (Bray *et al.*, 2000). Initially, leaf stomatal conductance may decreases faster than carbon assimilation, leading to increased water use efficiency. However, even under moderate drought, stomatal closure leads to decreased intercellular CO_2 levels causing a reduction in photosynthesis (Chaves and Oliveira, 2004). In addition, drought directly inhibits cell division and expansion thereby reducing leaf area for photosynthesis (Claeys and Inze, 2013). This reduction in growth may be an adaptive response allowing diversion of energy and assimilates to drive the various drought tolerance mechanisms. It also limits the leaf area available for water loss. However, since leaf growth and photosynthesis are the primary drivers of biomass accumulation, the reduction in leaf area and photosynthesis has major negative consequences for crop yield. A secondary effect of drought is oxidative stress. In a field situation, plants are usually subjected to other stresses accompanying drought such as heat. Under these conditions, the rate of reducing power production can overcome the rate of its use by the Calvin cycle leading to the production of reactive oxygen species (ROS), which are highly destructive to lipids, nucleic acids and proteins (Skirycz and Inze, 2010).

6.1.1 Mechanisms of drought tolerance

In order to tolerate drought, plants deploy a plethora of mechanisms to (i) prevent or alleviate damage; (ii) re-establish homeostatic conditions in the new stressful environment; (iii) resume growth, albeit at a reduced rate. In addition to attempting to maintain water status by stomatal closure, plants accumulate compatible osmolytes including sugars, sugar alcohols, amino acids and amines, thus lowering the cellular osmotic potential and thereby sustaining water absorption from the soil (Szabados and Savoure, 2010). In addition, regulation of water channel membrane proteins might be involved in controlling water flow across cellular membranes. Prevention of cellular damage by elimination of ROS is an important drought-tolerance trait and is mainly undertaken by antioxidant compounds and ROS scavenging enzymes, whose activity increases under drought conditions (Chaves *et al.*, 2003). Repair and prevention of damage to cellular proteins is also an important tolerance mechanism and some compatible osmolytes may function in protecting and stabilizing proteins under drought conditions. Heat shock proteins (HSPs) and hydrophilins/late embryogenesis abundant (LEA) proteins are also synthesized in response to drought. HSPs bind to structurally unstable proteins preventing their aggregation and perform important functions as molecular chaperones. The role of the hydrophilins/LEAs is less clear but they may stabilize proteins and membranes during dehydration.

6.1.2 Regulation of the drought response

Underlying the developmental and physiological responses of plants to drought are changes in the expression of thousands of genes (Claeys and Inze, 2013). These genes encode proteins involved in numerous biological processes, including drought tolerance mechanisms as well as a large number of proteins of unknown function. Importantly, drought alters the expression of many genes with regulatory functions such as transcription factors, RNA-binding proteins, calcium-binding proteins, kinases, phosphatases etc. These genes not only regulate downstream drought responses, but also function in drought perception and signalling. Many drought-responsive genes are regulated by ABA and there exists both ABA-dependent and ABA-independent transcriptional factors controlling the drought response (Cramer *et al.*, 2011). Post-transcriptional and post-translational control

such as phosphorylation/dephosphorylation, RNA stability, and selective protein degradation also play a role in regulating plant drought signalling and downstream responses.

6.2 The impact of drought on agriculture

In virtually all agricultural regions, crop yields are periodically reduced by drought. Indeed, abiotic stresses such as drought reduce average yields for most major crop plants by more than 50%, representing a huge economic loss (Claeys and Inze, 2013). Countries at most regions have been facing changes in the spatial and temporal distribution of rainfall and consequently more frequent and intense periods of drought. Agriculture is the major user of water with a staggering amount of 3000 litre of water required to produce food for a single person per day. To produce more drought-resistant crops, it is vital to have a comprehensive understanding of: (i) how drought affects plant growth and development; (ii) what physiological mechanisms the plant employs to tolerate drought and (iii) how and what genes regulate these mechanisms. Furthermore, identification and characterization of gene regulatory networks governing plant development under drought will in turn facilitate identification of candidate genes for improving crop drought tolerance.

6.2.1 Understanding plant responses to drought requires a large-scale systems biology approach

Plant responses to drought are complex and involve the activation of a variety of mechanisms to enable the plant to survive. Furthermore, as part of its adaptive strategy, plant growth and development slows with the consequent deleterious effects on yield. High-throughput “omics” technologies have begun to catalogue transcripts, proteins and metabolites whose abundance changes in response to stresses such as drought and the next essential step is an integrated analysis of whole plant responses to drought. The challenge now, is to connect drought signalling and gene regulatory networks to plant developmental and physiological drought adaptation phenotypes. This requires a “systems biology” strategy that marries together phenotypic and physiological observations with analysis of signalling components, transcriptional and transcript regulation, the dynamic behaviour of

proteins, protein complexes, and metabolite abundance and flux thereby creating a bridge between genomics and whole plant physiology, agronomy and improvement of crop resistance to drought (Chory and Wu, 2001). The major obstacle, however, to obtaining meaningful, integrated comparisons between all the regulatory levels controlling the response of plant development to drought is that this goal cannot be achieved by any single laboratory. It requires the large-scale integrated efforts of experts in many areas of plant and computational biology to comprehensively characterize drought regulatory networks.

6.3 The evolution of MAPK signalling in land plants became central to drought response in many aspects

MAPKs cascades are fundamental signalling pathways controlling growth regulatory processes in response to environmental stimuli in eukaryotes (Saito, 2010). The genome analysis of flowering plants has uncovered the existence of a highly complex MAPK signalling network in flowering plants (Doczi *et al.*, 2012). The *Arabidopsis* genome encodes 10 MKKs and 20 MAPKs (Ichimura, 2002). The MKKs and MAPKs were categorised into four phylogenetic groups A–D based on their sequence similarities, (Ichimura, 2002; Doczi *et al.*, 2012). However, it is unclear that how plant MAPK signalling components are organised into functionally distinct modules and signalling pathways (Colcombet and Hirt, 2008). The emerging genome sequences in diverse plant species and crops are excellent sources to unravel the evolutionary history of gene families such as the MAPK signalling pathways in plant (Doczi *et al.*, 2012). Moreover, comparisons of plant species to far branches of evolutionary eukaryotic lineages of fungi and animals and the common ancestor at the base of the tree, *Naegleria gruberi* allow tracing MAPK evolution and such ancestral analysis MAPK signalling pathways allow recognising unique evolutionary signatures. MKKs are activated through phosphorylation by their cognate upstream kinases, and when active, they phosphorylate and activate specific downstream MAPKs at both threonine and tyrosine residues of the TxY motif in their activation loops. In all plant MAPKs and human the activation loop motif is either TEY, or TDY. Interestingly, *Naegleria* has only a single TEY motif containing conventional MAPK, therefore it was suggested that the complexity in MAPK signalling in plants all evolved from a common ancestor and branched into the distinct classes. The

different levels of kinases (MKKKs, MKKs, MPKs) and the substrates must have co-evolved to be organised into specific signalling pathways. Furthermore, the expansion of number and functional diversification of MAPK signalling components in multicellular land plants suggests that MAPK signalling is a highly evolvable system.

MAPKs proteins are comprise with a kinase domain and their interaction specificities they are relatively small, compact globular shape encoded within small surface patches. Therefore it is through that the MAPK pathways evolved on the basis of tight regulation surfaces around the kinase active sites and target phosphorylation sequences to provide MKK-MPK interaction as well as kinase-substrate recognition specificities. In this process, protein interaction specificities remain highly evolvable (Doczi *et al.*, 2012). The MAPK interaction motif specification characterized by the studied common docking (CD) site motif; recognize the complementary binding motif, the D-site, present in both MKKs, MAPK regulatory proteins, such as phosphatases and MAPK substrates. In the evolution of MAPK signalling pathways the coevolution of D- and CD site sequences are of paramount importance. The rewiring of MAPKs pathways might have been generated along the modification of these interaction surfaces. The MAPKs signalling networks in flowering plants are highly connected and probably even more complex in comparison to yeast and animal (Popescu *et al.*, 2009). MAPK signalling networks including up and down regulated phosphatase, substrates was studied (Ellis, 2012). But how the signalling specificity, involving molecules and substrates in such a highly complex and connected network achieved, is unclear yet.

6.3.1 Functional characterisation of MAPK signalling pathways in the *Arabidopsis* model plant

To link MAPK signalling pathways to specific functions it is important to genetically alter the pathways in a way that allow experimental switch on of specific pathways and study their effect. To do this I utilised gain of function mutant forms that are turned constitutively active my mimicking the activation phosphorylation. The switchable GOF-MKKs under the chemically inducible promoter system were produced in our laboratory by E. Hatzimasoura. Ectopic expression of a transgene

might affect the transcription of endogenous genes, moreover, an overexpressed protein can cause perturbations in the formation of protein complexes, and these malfunctions which can result in unexpected physiological effects (Gibson *et al.*, 2013). In *Arabidopsis* the so called XVE chimeric promoter system was developed that allow highly regulated transcriptional activation of target genes in specific spatial and temporal domains by adding β-estradiol without causing toxicity and unspecific effects to the *Arabidopsis* plant (Zuo *et al.*, 2000). This approach immensely applied to investigates the function of genes whose constitutive overexpression has lethal effects.

The inducible overexpression of the 10 different GOF-MKKs under control of the estradiol-inducible promoter system should activate distinct MAP kinases signalling cascades in *Arabidopsis* and therefore identify novel roles for MAP kinase modules in diverse biological processes (Fig 3.7). Indeed, inducible overexpression of MKK7 and MKK9 had very specific effect on meristem growth, leading to a rapid arrest and collapse both in shoot and root meristem (Fig 3.9, Fig 3.11).

Plants exposed to either abiotic or biotic stress conditions respond by actively altering their growth pattern as part of the overall defence response, which serves to minimise exposure to stress and to divert limited resources to defence mechanisms at the expense of growth (Claeys and Inze, 2013). When MKK7 and MKK9 expression was induced by different concentrations of estradiol I found a dose dependent arrest of root and leaf growth and meristem collapse (Fig 3.9-11). This was stronger for MKK7 than for MKK9. The confocal images analysis of root meristem I saw ectopic divisions in the quiescent centre (Fig 3.11). Similarity ethylene overexpressor mutants showed ectopic division of QC cells (Ortega-Martinez *et al.*, 2007). MKK9 was also shown to be part of ethylene signalling as well as ethylene biosynthesis (Yoo *et al.*, 2009). Diverse types of stresses are regulated by common pathways that target morphogenesis resulting in the inhibition of cell cycle, cell elongation, localized stimulation of cell division and alterations in cell differentiation status. ROS-production might provide a common signalling mechanism for all these responses that might also be through altered phytohormone transport and metabolism (Potters *et al.*, 2007).

Overexpression of MKK7 and MKK9 inhibit cell proliferation and entry into G2/M phase as revealed by the reduced CYCB1;1 reporter activity and flow cytometry measurements (Fig 3.13). Interestingly, in spite of reduced

endoreduplication, leaf cells expanded more when MKK7 and MKK9 were inducibly overexpressed. This might represent a compensation mechanism for reduced cell proliferation.

6.4 MKK7 and MKK9 restrain leaf outgrowth upon light

Plants are sessile in nature and thus flexibly respond to changing environmental conditions unlike animal where organogenesis happens in a constant environment. Light is one of the most fundamental environmental signal plants perceive and adopt their growth and development to. I utilised the light response of rapid leaf outgrowth after a dark arrest to study the role of MKK7/9 and MPK3/6 MAPK signalling components in regulating growth and cell proliferation. We used T-DNA insertional single mutants; *mkk7, mkk9, mpk3 mpk6* and double mutants*; mkk7/9-N5, mkk7/9-N3.* The results suggest that MKK7 and MPK6 have roles to restrain leaf outgrowth in the shoot meristem. For MKK7 this was only evident during the dark arrest while for MPK6 also during the growth phase upon transfer to light (Fig 4.2). Thus MAPK signalling might have roles maintaining the dark repressed shoot meristem and preventing leaf initiation prior to first light exposure. In accordance, the microarray study of dark repression and leaf growth upon light suggested a role for MPK6 in dark repression (Lopez-Juez *et al.*, 2008). In the same microarray experiment of leaf growth it was found that light stimulates a coordinated increase in the expression of cell cycle genes, whereas MKK7 and MKK9 clearly suppress this functional class. Similarly, exposure to light strongly simulates genes involved in photosynthesis, while this functional class is suppressed by MKK7 and MKK9. Light on the other hand negatively regulates auxin- and ethylene-"signature" genes in the shoot apex.

One question that can be legitimately asked is whether the faster leaf initiation rates of mutants defective in *mpk6* translate into plants with an enhanced growth rate, for example an accelerated plastochron, or larger leaves. This appears not to be the case; the phenotype of this KO mutant is barely noticeable under normal conditions. One possible difference between the two sets of conditions is the fact that leaf initiation during de-etiolation takes place under heterotrophic conditions, during which the shoot apical meristem produces leaves while the reserves stored in the embryonic cotyledons are mobilised.

The transgenic *Arabidopsis* seedlings harbouring the promoter regions of *MKK7* and *MKK9* fused to the GUS reporter gene were used to study their expression patterns. Both genes are under strict spatial regulation that was found here to be light-dependent. *MKK9::GUS* showed more extensive expression in roots while *MKK7::GUS* showed expression QC centre of root meristem (Fig 4.3). A prominent expression domain both for MKK7 and MKK9 is stipules, leaf appendices which are thought to be reservoirs of auxin biosynthesis.

6.5 Regulation of cell proliferation through MAPK signalling

Cell proliferation is modulated by stresses. MAPKs responds to number of stresses but whether this relates to the control of cell division during stresses are largely unknown. I found that osmotic stress blocks leaf growth and impacts both on mitotic transition based on *CYCB1;1-GUS* reporter and endoreduplication (Fig 5.5-6). Using T-DNA mutants I showed that *mkk9* and *mpk6* might be involved in maintaining growth upon stress, as these mutants were more sensitive for growth arrest upon osmotic stress (Fig 5.3). On the other hand I found that *mkk7* 3rd pair of leave growth was less inhibited by osmotic stress suggesting a role for growth arrest for MKK7. Earlier studies in *Arabidospsis* ANP1-activated MPK3 and MPK6 shown a dual role, in stress responses and also inhibited auxin response (Kovtun *et al.*, 2000).

In plants MAPK functions were mostly connected to the regulation of mitotic events. Two MAPKs structurally similar proteins the *Medicago* MAP kinase 3 (MMK3) and the *Nicotiana tabacum* FUS3-related kinase (NTF6) showed a possible involvement in G2 control, based on mRNA abundance in G2-phase and M-phases, protein localisation to the cell plate and transient activation during mitosis (Jonak *et al.*, 2002). Later it was shown that the *Medicago* and *tobacco* pathways are conserved in *Arabidopsis* and constitute a cytokinesis regulatory signalling module (Kosetsu *et al.*, 2010b).

The growth response to osmotic stress is dynamic, the acute stress effect on cell division rates in leaves rapidly recover (Skirycz and Inze, 2010). Similar stress response also has been reported in the case of roots, and other plants such as sunflower (Helianthus annuus) leaves, monocot leaves (Skirycz and Inze, 2010). It was also found that the down regulation of stress related cell cycle genes recover

after 24 h of stress onset (Skirycz *et al.*, 2011a). Osmotic stress also accelerates mitotic cycle exit and entry into endoreduplication in Arabidopsis leaf (Skirycz *et al.*, 2011a).

Mild osmotic stress evokes the ethylene signalling pathway and through this pathway it is connected to cell cycle progression and inhibition of CDKA activity (Skirycz *et al.*, 2011a). The MKK9-MPK3/MPK6 is also integral to the ethylene signalling and biosynthesis pathways (Xu *et al.*, 2008; Yoo *et al.*, 2008; Hahn and Harter, 2009). Thus osmotic stress might act through the ethylene pathway to activate MAPK signalling and restrain leaf growth. This is further supported by the fact that *mkk9* mutant was shown to be partially resistant with ACC and mannitol for growth inhibition. Another hormonal signalling pathway that was connected to osmotic stress and leaf growth is gibberellin through the DELLA proteins (Claeys *et al.*, 2012). The DELLAs are also important regulators of cell cycle progression through the CDK inhibitors, called KRPs.

The main target of CDK regulation is the RBR phosphorylation. Thus the induction of KRPs would lead to unphopshorylated RBR that becomes active as a repressor of cell proliferation. RBR represses E2F activity (Magyar, 2008). E2FB was found to be the main activator E2F in plants that RBR represses to halt cell proliferation (Magyar *et al.*, 2012). We have found here that osmotic stress reduces RBR phosphorylation and promotes the formation of the RBR-E2FB repressor complex (Fig 5.8). I also show that elevation of E2FB level overcomes the root growth arrest imposed by induced MKK7 expression (Fig 5.9). It remains to be seen whether elevated E2FB expression also can overcome the restrain of growth upon osmotic stress.

Chapter 7: Conclusion

One of the largest challenge in the 21st century is to provide food security for the fast growing population that is predicted to reach over 9 billion by 2050. Water is by large the major limiting factor for crop production. Most abiotic stress research has focussed on mature organs and survival responses under severe drought stress. Modifying regulatory pathways in stress responses however did not yield better performing crops. The focus is shifting now to the mechanisms how crops adopt their growth to mild but limiting water shortages. My research identified a MAPK pathway that may play important roles in this process. The MKK7/9 and MPK3/6 components of a MAPK module regulate meristem growth and connected to drought stress responses. These pathways appear to regulate cell division by impacting on the RBR/E2F transcriptional repressor complex. Future experiments will be necessary to investigate how the MKK7/9 –MPK3/6 signalling is interconnected with hormonal pathways (ABA, ethylene, gibberellins, jasmonate or auxin). In the future it might be possible to tune this regulatory pathway for better adaptation of plants to abiotic stresses, therefore increase crop yield.

Chapter 8: References

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Appendices

Table A2.1 Details of materials used

Relevant Section	Equipment/Instrument	Company and Brand Name
2.1.1	Seed collection bags	Homebase
2.1.1	Sieve/Filter	Homebase
2.2.1	Laminar flow hood	Bassaire
2.2.1	Bleach	Kleen Off, Original thick bleach
2.2.1	Vacuum	Polaris Instruments Ltd. Vacuumed
2.2.1	Rotator	Grant Bio
2.2.2	Weighing machine	Sartorius
2.2.2	Murshinge & Skoog (MS Media)	Duchefa Biochemie
2.2.2	Phyto-agar	Duchefa Biochemie
2.2.2	Sucrose	Fluka
2.2.2	MES	Sigma Aldrich: MES hydrate
2.2.2	Oven	Genlab windne England
2.2.2	Mixer with heating	Ikamag reo
2.2.2	pH meter	Hanna instruments
2.2.2	Mannitol sucrose	BDH analaR
2.2.2	Square petri dishes	12.5cm x 12.5cm1.5cm deep
2.2.2	Circular cell culture petri dishes	Cellstar: cell culture dishes 100 x 20 mm with vents
2.2.3	Laminar flow hood	Bassaire Model: A6HB
2.2.3	Filter/mess	Sefar
2.2.3	Micro-porous tape	3M Micropore™ 1.25cm width
2.4	Camera D70	Nikon digital, Thailand
2.4, 2.4.1	Storeroom Microscope	Nikon SM21500, Japan
2.4.1	Additional lighting source	Photonic PL2000

2.4.1, 2.7,2.8	DIC	Nikon Japan DIC-cam DXM12 NIS Freeware 2.10
2.4.2	Gratitude	SGI
2.5	LMS cooled incubator	LMS Ltd
2.5	Percival3	Percival scientific since 1886
2.5	Procema 3	Procema environment
2.6	Flow cytometer PIII	Partech
2.6.1 2.7.1 2.8.1 2.9 2.10 2.11	Micro fine tweezers	Brand: Biologie (Number 5) Rustless, Dumoxel, Nun-magnetic
2.6.2	DAPI	Partech
2.6.2	Petri dish	Greiner bio-one
2.6.2	Plastic cuvettes	Brand: Sarstedt, Length/Ømm 51/12
2.6.2	Filter	30µm non-sterile CellTrics®filters
2.7.1	Lactic acid	Sigma-Aldrich
2.7.1, 2.8, 2.9	Microscope Slide and Cover slip 22x55, 22x22mm	Menzel Glaser (Fisher)
2.8, 2.10	Deep well microtiter plate	Corning incorporate castor
2.8	X-gluc	5-bromo-4-chloro-3-indoly beta-D-glucuronide sodium salt, Brand: Slater and Frith Ltd
2.8	Vacuum	Polaris instruments
2.8	Gum arabic	Sigma
2.8	Incubator 37ºC	Memmert
2.8	Stereo microscope/binocular	SLS – Scientific Laboratory Supplies, Pyser-SGI
2.8.1	Nikon Optiphot	Nikon Optiphot 2 Dig-cam DXM1200 NIS Elements AR
2.9	Confocal microscope	Olympus
2.9	Propidium Iodide	Sigma
2.10	Tough tag	Diversified biotech
2.10	Tissue raptor	Qiagen
2.10	Centrifuge	Eppendorf

2.10	Whirl mixer	Fisons scientific equipment
2.10	Bradford	Bio-red
2.10	Gel-electrophoresis Tank	Life Technologies™ GIBCO BRL Horizontal Gel Electrophoresis Apparatus
2.10	Ladder	Page ruler
2.10	Gel assembly and casting trays	AA Hoefer
2.10	SDS	National Diagnostic
2.10	Corning tube	Sarstedt
2.10	Speed vac	Heto-DNA mini speed vac
2.10	Butanol	Sigma
2.10	Acrylamide	Sigma
2.10	Tween 20	Fisher Bio reagent
2.10	Power supply	Pharmacia Biotech
2.10	Blotting buffer	National diagnostic
2.10	Shaker	Platform Rocker STR6
2.10	Chemilunescent HRP substrate	Millipore
2.10	Bag sealing machine	Russell hobbs
2.10	Sodium chloride	Fisher
2.10	Heater/hot plate	Techne
2.10	Chemilunminescent substrate	Thermo scientific
2.10	X-ray film	CL-^{X} posure ^{TM} film
2.10	Polybags	Sapphire

Table A2.2 Preparation MES solution

- 25 g MES hydrate (Sigma-Aldrich)
- 500 ml distilled water
- Set pH to 5.8 using 1N KOH (pre-made buffer)
- Autoclave at 110°C for 15 mins
- Keep at room temperature

Table A2.3 GUS assay, sodium phosphate preparation

100 mM sodium Phosphate buffer

Phosphate Buffer (Sorensens)

Stock solutions (0.2M)

X Na2HPO4. 2H2O 3.561 g Dibasic
or Na2HPO4. 7H2O 5.365 g
or Na2HPO4. 12H2O 7.164 g

distilled water to make 100 ml

Y NaH2PO4. H2O 2.760 g Monobasic
or NaH2PO4. 2H2O 3.121 g
distilled water to make 100 ml

Prepare the 0.1M phosphate buffer by mixing x ml of solution X with y ml of solution Y depending on the acquired pH. Make the resulting solution up to 100ml with distilled water.

pH (at 25oC)	X ml	Y ml
6.8	24.5	25.5
7.0	30.5	19.5
7.2	36.0	14.0
7.4	40.5	9.4

Other pH values from 5.8 to 8.0 can be obtained but are not usually used with this buffer. Adjust the osmolarity of the buffer by variation of the phosphate molarities or the addition of sucrose, glucose of NaCl. Other electrolytes such as Ca salts cannot be used as they will precipitate with the phosphate.

Table A2.4 GUS assay, modified Murray's protocol

x-gluc solution based on:	Chemical Reagent	Calculations	Final Conc needed	µl needed Per 50ml
J Murray's Modified protocol	**X-Gluc**	(0.3 mg/ml x 50 ml) /50 mg/ml =0.3 ml 0.3x1000=300 ul	0.3 mg/ml	300
	Potassium Ferricyanide	(0.005M x 50 ml)/ 0.1M = 0.25 ml x1000 = 250	0.5 mM	250
	Potassium Ferrocyanide	As above	0.5 mM	250
	Tween 20	((50 mlx1000)/100) x 0.1 = 50 ul	0.1%	50
	Sodium Phosphate Buffer	Make up to 50 ml	100 mM	49150

Table A2.5 Hoyer's solution preparation

Hoyer's solution	
Chemical/Components	**Amount**
Distilled water	30 ml
Choral hydrate	100 g
Arabic gum	2.5 g
Glycerol	15 ml

Table A2.6 Preparation of western blot solutions

Extraction buffer: 2000 µl	
Lacus buffer*	1900 µl
(1M) para-Nitrophenylphosphate (pNPP, Sigma)	20 µl
Protease Inhibitor Coctail (PIC, Roche)	20 µl
0.1M Phenylmethanesulfonylfluoride (PMSF, Sigma)	20 µl
Phos-STOP (Roche)	40 µl
Dithiothreitol (DTT, Sigma)	2 µ1
10 % Running Gel: 10 ml	
water	4.0 ml
30% Acrylamide/bis-Acrylamide mix(Sigma-Aldrich)	3.3 ml
1.5 M Tris-HCl pH8.8	2.5 ml
10% Sodium-dodecyl-sulphate (SDS, Sigma)	0.1 ml
10% Ammonium persulphate (APS, Sigma)	0.1 0.06 ml
Tetramethylethylenediamine (TEMED, Sigma)	0.006 ml
Stacking Gel: 5 ml	
water	3.4 ml
30% Acrylamide/bis-Acrylamide mix(Sigma-Aldrich)	0.83 ml
1 M Tris-HCl pH6.8	0.63 ml
10% Sodium-dodecyl-sulphate (SDS, Sigma)	0.05 ml
10% Ammonium persulphate (APS, Sigma)	0.05 ml
Tetramethylethylenediamine (TEMED, Sigma)	0.005 ml

***Lacus buffer**: 25 mM Tris-HCl, 10 mM $MgCl_2$, 15 mM Ethylene glycol tetraacetic acid (EGTA, Sigma), 75 mM NaCl, 1mM NaF, 0.5 mM NaVO3, 15 mM β-glycerolphosphate, 0.1% Tween 20 (Sigma-Aldrich).

Figure A3.1 A phylogenetic tree shows the relationship of Arabidopsis, rice and poplar MKKs.

Protein kinase domains of three species MKKs were aligned (pairwise alignment) with 100 bootstrap replicates using the human MEK1 (HsMek1) as an outgroup. Species acronyms: At, Arabidopsis thaliana; Hs, Homo sapiens; Os, Oryza (The image was taken from Hamel *et al.*, 2006).

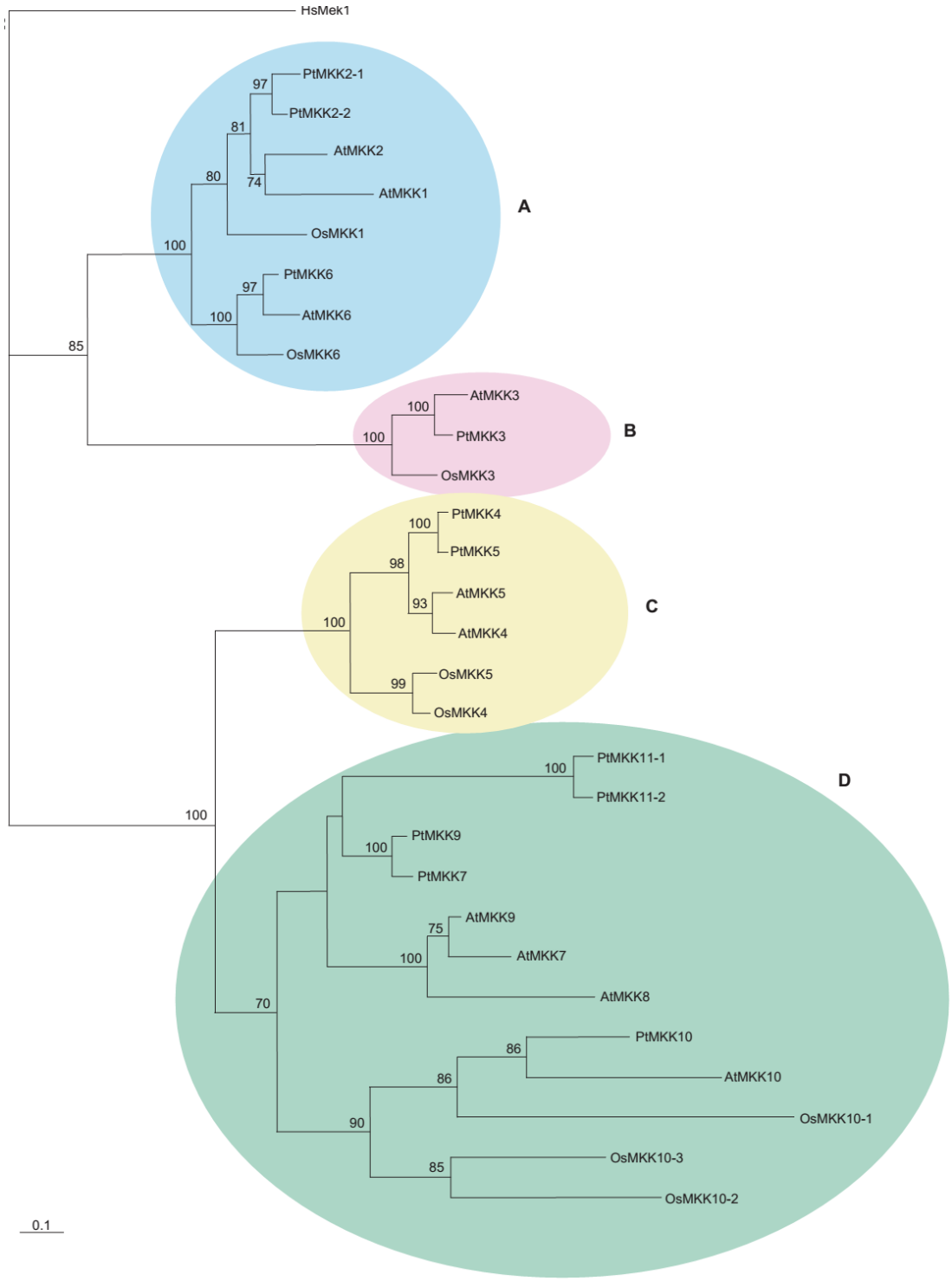

Table A4.1 MKK7::GUS expression under dark-light transition

	MKK7 (1):GUS				MKK7 (2):GUS			
Light Conditions	**Leaf/ cotyledon**	**Primordia/ Petiole/stipules**	**Hypocotypl/ Root**	**Root tip/QC/ Colembella**	**Leaf/ cotyledon**	**Primordia/ Petiole/stipules**	**Hypocotypl/ Root**	**Root tip/QC/ Colembella**
3dd+0dl	Not stained well, very less in the leaf edge	Not stained	Very weaker	No expression	Not at all Gus expression	Not at all Gus expression	Not at all Gus expression	Very-2 weaker at over the root tip
3dd+1dl	Dots are all over the leaf	Not very good staining	Not at all	weaker only in QC	No expression (very weaker)	Not at all Gus expression	----	Only in QC but weakerer
3dd+2dl	Dots are all over the leaf	Both sides of primordia, the petioles are stained	Very faint in the midrib	Only in QC & above region of QC of root tip	Nicely dotted in all over the leaf	Primordia & petiole are fully stained	Very weaker in midrib	Only in QC & above region of QC of root tip
5dd+0dl	Not considerable (very weak expression)							weaker in QC & above region bit good
5dl+0dd	Nicely dotted in all over the leaf	Nicely expressed in petioles of primodia	Expression weaker in the midrib	All over the root tip & clear visual in QC as well	Nicely dotted in all over the leaf	Nicely expressed in petioles of primodia	Expression weaker in the midrib	All over in the root tip & clearly in QC

Table A4.2 MKK9::GUS expression under dark-light transition

	MKK9 (5/2):GUS				MKK9 (6/1):GUS			
Light Conditions	Leaf/ cotyledon	Primordia/ Petiole/stipules	Hypocotypl/ Root	Root tip/QC/ Colembella	Leaf/ cotyledon	Primordia/ Petiole/stipules	Hypocotypl/ Root	Root tip/QC/ Colembella
3dd+0dl	Not stained	Not stained	Not stained	weaker in both QC&CC	Patches on ¾ Upper portion of leaf	Not stained	midrib	Stained both QC&CC
3dd+1dl	Not at all	Only on petioles very less	Stained well	Stained both QC&CC	Dots are all over the leaf& on edge	Expressed in both sides of petioles of primordia	Strongly expressed in midrib of root and hypo	Stained both QC&CC
3dd+2dl	Nicely dotted in all over the leaf	Both sides of primordia, the petioles are stained	Faint stained in the midrib	Stained both QC&CC (up/downward)	Nicely dotted in all over the leaf	Very weaker in midrib	Strongly expressed in midrib of root	Stained both QC&CC but much more in downward
5dd+0dl	Not good at all	In petioles	Very weaker in midrib	weaker in both QC&CC, downward	Patches & dots on all over the leaf	Little bit on the petioles	Highly expressed in midrib	Stained both QC&CC but much more in downward
5dl+0dd	Nicely dotted in all over the leaf	Very strong in petioles	Expressed well in the midrib	All over the root tip & clear visual in QC&CCas well	Nicely dotted in all over the leaf	Very strong in petioles	Strongly expressed in midrib of root and hypo	All over in the root tip & QC is less than CC