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EXTENDING THE ABA SIGNALING NETWORK:

THE PROTEIN KINASE AtWNK8 IS A NEGATIVE REGULATOR

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Heidelberg, 22. Mai 2012

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LIST OF ABBREVIATIONS

µM	Micromolar
3-AT	3-amino-1,2,4-triazole
A	Adenine
aa	amino acid
ABA	Abscisic acid
AD	activation domain
ABI	ABA-insensitive
Arabidopsis	*Arabidopsis thaliana*
BD	Binding domain
BIFC	Bimolecular fluorescence complementation
bp	Base pair
C°	Celsius
CLSM	Confocal laser scanning microscopy
CSM	Amino acid supplement mixture
CT	C-terminus
d	Day(s)
DAG	Day(s) after germination
DAS	Day(s) after stratification
DM	Double mutant
DNA	Desoxyribonucleic acid
EtOH	Ethanol
Fig.	Figure
g	Gramm
G	Glycine

LIST OF ABBREVIATIONS

GFP	Green fluorescent protein
GUS	ß-Glucuronidase
H	Histidine
h	Hours
HS	Heat shock
IPTG	Isopropyl-β-D-thiogalactopyranosid
kD	Kilodalton
KD	Kinase domain
L	Leucine
LB	Luria Bertani
LD	Long day
MBq	Megabecquerel
MCS	Multiple cloning site
mM	Millimolar
mRFP	Monomeric red fluorescent protein
MS	Murashige and Skoog
ORF	Open reading frame
P	Proline
PAGE	Polyacrylamide gel electrophoresis
pH	Negative logarithm of proton concentration
PP2CA	Protein phosphatase 2C, group A
PYL8	Pyrabactin resistance-like 8
PYR1	Pyrabactin resistance 1
RNA	Ribonucleic acid
RT	Room temperature
S	Serine

LIST OF ABBREVIATIONS

SDS	Sodium dodecylsulfate
SE	Standard error
SD	Standard deviation
SnRK2	Snf1-related protein kinase 2
T	Threonine
T-DNA	Transfer DNA
TF	Transcription factor
UBQ10	Ubiquitin-10 promoter
W	Tryptophan
WNK8	With No Lysine (K)
Y2H	Yeast-two-hybrid

SUMMARY

The phytohormone abscisic acid (ABA) plays a major role in plant development and plant adaptation to both biotic and abiotic stressors. Key processes regulated by ABA include dormancy, seed germination, early seedling development and stomatal closure. To coordinate these functions an extensive network of signal transducers is necessary. In recent years knowledge of the ABA signal transduction pathway in *Arabidopsis* has advanced rapidly and provided a core signaling module which forms the basis for early ABA signaling events. Three major protein classes are involved: ABA binding proteins of the PYR/PYL family, Protein Phosphatases 2Cs (PP2Cs) and SNF1-related protein kinase 2s (SnRK2s). Specific recognition of the hormone is mediated by PYR/PYL receptors. Once ABA is bound to the receptor, PYR/PYLs inhibit the central negative regulators, PP2Cs, thus allowing the activation of downstream SnRK2s which positively regulate ABA responses.

Reversible post-translational modification as a result of the antagonistic action of protein kinases and protein phosphatases is crucial to the ABA core signaling pathway. However, except for SnRK2s information on additional protein kinases is limited. Our work provides evidence that, opposite SnRK2s, a kinase of the WNK family is a new component of the ABA signaling pathway. The *Arabidopsis* WNK family consists of 11 serine/threonine protein kinases exhibiting a characteristic replacement of a unique lysine residue in the catalytic domain, hence the name WNK [With No K (Lysine)]. Phenotypic analysis of *wnk8* mutants, exhibiting altered postgermination growth in response to ABA, indicated that AtWNK8 could be involved in ABA signaling. Genetic as well as direct interaction studies proved that AtWNK8 and a member of the PP2C family of protein phosphatases (AtPP2CA) interact. Biochemical data suggested that AtWNK8 activity is directly regulated by AtPP2CA, and, in turn, the ABA receptor AtPYR1 is phosphorylated by AtWNK8.

In summary, our data support a role for the protein kinase AtWNK8 in ABA signaling. Opposite the main positive regulators SnRK2, we propose AtWNK8 as a new negative regulator closely associated with the members of the core ABA signaling module.

ZUSAMMENFASSUNG

Das Phytohormone Abscisinsäure (ABA) spielt eine entscheidene Rolle währed der pflanzlichen Entwicklung und bei der Anpassung an biotischen und abiotischen Stress. Zu den wichtigsten Prozessen, die durch ABA reguliert werden, zählen Samenruhe (Dormanz), Keimung, frühste Keimlingsentwicklung und das Schließen der Stomata. Ein aufwändiges Zussammenspiel von Signalüberträgern ist notwendig, um die verschiedenen Aufgaben auf einander abzustimmen. Mit der Entdeckung der Kernkomponenten, die an der frühen ABA Signaltransduktion beteiligt sind, hat die Forschung in den letzten Jahren einen entscheidenen Beitrag zum Verständnis des ABA Signalweges geleistet. Folgende Proteingruppen sind an der frühen ABA Signaltransduktion beteiligt: Mitglieder der ABA-Rezeptoren aus der PYR/PYL Familie, Proteinphosphatasen der PP2C Familie und SNF1-verwandte Proteinkinasen 2, SnRK2s. Sobald ABA an die Rezeptoren gebunden hat, blockieren diese die zentralen negativen Regulatoren, die PP2C Phosphatasen. Inhibition der Phosphatasen erlaubt dann wiederum die Aktivierung der SnRK2 Kinasen, die als positive Regulatoren der ABA Antwort beschrieben sind. Reversible post-translationale Modifikation von Proteinen durch das antagonistische Zusammenspiel von Phosphatasen und Kinasen ist ein wesentlicher Bestandteil der zentralen ABA Signaltransduktion. Mit Ausnahme der SnRK2 Kinasen sind andere Kinasen im ABA Signalweg nur wenig beschrieben. Diese Arbeit liefert deutliche Hinweise darauf, dass eine Kinase der WNK Familie eine weitere Komponente im ABA Singalweg darstellt. Die *Arabidopsis* WNK Familie besteht aus 11 Serin/Threonin Proteinkinasen und zeichnet sich durch ein fehlendes Lysin (K) in der katalytischen Domäne aus; WNK steht für ‘With no K (Lysine)‘.
Durch die phenotypsiche Untersuchung von *wnk8* Mutanten, die in ihrer frühen Keimlingsentwicklung gestört sind, erhielten wir erste Hinweise auf eine Beteiligung von AtWNK8 an der ABA Signaltransduktion. Interaktionsstudien bestätigten, dass AtWNK8 mit einem Mitglied der PP2C Phosphatasen interagiert, AtPP2CA. Biochemische Daten deuteten darauf hin, dass AtPP2CA die Aktivität von AtWNK8 direkt reguliert und dass AtWNK8 den ABA Rezeptor AtPYR1 phosphoryliert. Zusammenfassend beschreiben wir einen neuen, negativen Regulator des ABA Signalweges, die Proteinkinase AtWNK8, die eng mit den Kernkomponenten der ABA Signaltransduktion verbunden ist.

CONTRIBUTION TO THE THESIS

Apart from the author and the supervisor, the people listed below contributed to this work as indicated.

Anne Hong-Hermesdorf	Construct design and cloning of HS:GFP-WNK8; generation of *wnk8-1* recapitulation lines
Till Franz	Support transcript levels in *wnk8-1* recapitulation lines
Angela Brüx	Construct design and cloning of AD-WNK8KD and WNK8p-GUS
Kenji Hashimoto	Construct design and cloning of pIVEX PP2CA; *in vitro* phosphorylation assay
Martin Scholz	Mass spectrometry and identification of phosphorylation sites
Leonie Steinhorst	Yeast-two-hybrid WNK8 and CIPKs
Maik Böhmer	SnRK2 in-gel kinase assay

1 INTRODUCTION

1.1 The protein kinase AtWNK8

AtWNK8 is a serine/threonine protein kinase

Plants as sessile organisms continuously sense their environment for external and internal stimuli, such as light, nutrients, water status, pH, pathogens or hormones. Sensing is followed by transfer of information in order to continually alter physiology, morphology and developmental stages. Protein phosphorylation is a key mechanism for intracellular signal transduction in eukaryotic as well as prokaryotic cells. It can affect protein activity, conformation, stability or localization (CHAMPION *et al.* 2004a). Protein phosphorylation is mediated by protein kinases whose activity is counterbalanced by the action of specific phosphatases. Today, genes encoding for protein kinases are estimated 4 % of the *Arabidopsis* genome (CHAMPION *et al.* 2004a). In general, the catalytic domain of kinases consists of approximately 230 to 250 amino acids and can be divided into 12 subdomains. Each subdomain contains characteristic stretches of conserved residues, for example the most important invariant lysine in subdomain II for ATP binding. After ATP binding the protein kinases catalyze the transfer of the γ-phosphoryl group from ATP to a specific residue of the target protein. The attachment of the γ-phosphoryl group directly to the kinase is known as autophosphorylation and can be observed for many kinases. Phosphorylation is found on hydroxylated amino acids such as serine (Ser), threonine (Thr), tyrosine (Tyr) but also on histidine (His). Accordingly, kinases have been classified into four major groups: Ser/Thr kinases and Tyr kinases which strictly phosphorylate Ser/Thr and Tyr residues, respectively and histidine kinases. Tyr kinases with dual-specificity act on Ser, Thr and Tyr residues. In *Arabidopsis* most of the protein kinase are Ser/Thr. Based on their sequence relationship they can be divided into various families and subfamilies (HARDIE 1999). Tyrosine phosphorylation is clearly minor compared to Ser and Thr and much better known from the animal system (OLSEN *et al.* 2006). His kinases are part of the 'two-component-system' which is a signal transduction system relying on a phosphotransfer between two types of signal transducers (reviewed by GREFENandHARTER 2004).

WNK kinases are a subfamily of Ser/Thr kinases related to the Ste20p family (XU *et al.* 2002). Until now they have been identified in numerous organisms, including *A.thaliana*, *Oryza*, *C.elegans*, *Phycomyces*, rodents and humans (HISAMOTO *et al.* 2008, MURAKAMI-KOJIMA *et al.* 2002, RUIZ-PEREZ *et al.* 1995, VERÍSSIMOandJORDAN 2001, XU *et al.* 2000). However, no WNKs have been found in yeast, although the genomes of unicellular eukaryotes, for example *Chlamydomonas reinhardtii* or *Phytophtora sojae*, do encode WNK kinases (HONG-HERMESDORF *et al.* 2006). The rat WNK1 was the first protein of this family to be cloned and characterized (XU *et al.* 2000). The most striking feature of this new family of kinases is a missing lysine residue in subdomain II of the catalytic domain which is conserved in all other protein kinases, hence the name WNK (With No K (=lysine)). Instead, the invariant lysine, responsible for ATP binding, is replaced by a cysteine (KNIGHTON *et al.* 1991, XU *et al.* 2000). Kinase activity is mediated by an alternative lysine residue in subdomain I located within the glycine string (XU *et al.* 2000). RnWNK1 was identified as an active Ser/Thr kinase which displays autophosphorylation (XU *et al.* 2000). While activation requires the phosphorylation of at least one serine residue within the activation loop of the kinase domain, inactivation is regulated via the autoinhibitory domain. This domain is localized C-terminally of the catalytic domain and contains two phenylalanine residues essential for autoinhibition. Additional domains include two potential coiled-coil and two proline-rich regions (XU *et al.* 2002).

The human WNK family (HsWNK1 – HsWNK4) has been extensively studied because of the role of the kinases in the human disease familial hyperkalemic hypertension, also known as Gordon's syndrome (MCCORMICKandELLISON 2011). In particular, WNK kinases are mainly investigated for their role in renal regulation and ion transport. For example, WNK4 negatively regulates the activity of a Na-Cl cotransporter (NCC) by reducing NCC abundance at the plasma membrane (YANG *et al.* 2007). Human WNKs are considered potential targets for the treatment of human disease.

Information on plant WNKs is rather limited. The *Arabidopsis* genome encodes for 11 WNK kinases (HONG-HERMESDORF *et al.* 2006) which all share the highly conserved N-terminal kinase domain. With the exception of AtWNK11, which consist only of the conserved N-terminus, all other plant WNKs have a very divergent C-terminus. The only conserved region in the C-terminus is the autoinhibitory domain. Just a subset of the

Arabidopsis WNKs (including AtWNK8) contains a coiled-coil domain that could be identified for mammalian WNKs. Figure 1 shows a schematic illustration of AtWNK8.

Figure 1. AtWNK8 structure
Schematic illustrating showing the domain structure of AtWNK8. The kinase domain is indicated in blue, including the activation loop (AL). In the C-terminus the autoinhibitory domain (ID) and the predicted coiled coil domain (CC) are highlighted.

Only few members of the Arabidopsis WNK family have been described so far. AtWNKs were first mentioned in 2002 to be involved in the regulation of circadian rhythms (MURAKAMI-KOJIMA *et al.* 2002, NAKAMICHI *et al.* 2002). AtWNK1 was shown to interact with the ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 3 (APRR3), a member of the circadian clock components. While the expression of *AtWNK1* as well as *AtWNK2*, *AtWNK4* and *AtWNK6* is under the control of a free-running circadian rhythm, *AtWNK8* appears to be constitutively expressed. AtWNK8, AtWNK2 and AtWNK5 were subsequently shown to play a role in the regulation of flowering time (WANG *et al.* 2008). T-DNA knockout mutants presented with an early flowering phenotype and transcript levels of several genes in the photoperiodic pathway of flowering were shown to be altered in these mutants.

Over the last years different interaction partners for AtWNK8 have been suggested. First, AtWNK8 was shown to be an interacting partner of the C-subunit of the vacuolar H^+-ATPase, AtVHA-C (HONG-HERMESDORF *et al.* 2006). *In vitro* data indicated that AtWNK8 interacts via the C-terminus and phosphorylates several residues within AtVHA-C. Furthermore, AtWNK8 was shown to display autophosphorylation. Recently, AtWNK8 was reported to interact with AtEDM2 (ENHANCED DOWNY MILDEW 2) and AtEMF1 (EMBRYONIC FLOWER 1) (PARK *et al.* 2011, TSUCHIYAandEULGEM 2010). AtEDM2, originally identified to play a role in plant immunity, was shown to be phosphorylated by AtWNK8 and, together with the kinase, have an additional function in controlling floral transition (TSUCHIYAandEULGEM 2010). Flowering time appears to be further regulated by the interaction of AtWNK8 and AtEMF1 which was previously reported to act as a floral repressor (MOON *et al.* 2003).

Lately, studies in rice and soybean have started to investigate the role of WNKs in organisms other than the model plant *Arabidopsis* (KUMAR *et al.* 2011, WANG *et al.* 2010).

1.2 The phytohormone abscisic acid (ABA)

ABA controls various developmental and physiological processes

The plant hormone ABA was initially discovered in the 1960s when it was isolated from cotton and sycamore leaves as an abscission-promoting substance and as an endogenous substance to induce dormancy, respectively (ADDICOTT *et al.* 1968, OHKUMA *et al.* 1963). Since then the sesquiterpenoid molecule has been identified in various organisms across kingdoms, including fungi, plants and metazoa (LE PAGE-DEGIVRY *et al.* 1986, NAMBARAandMARION-POLL 2005, WASILEWSKA *et al.* 2008). ABA biosynthesis predominantly occurs in the vascular tissue. Apart from passive diffusion, which contributes to the distribution of protonated ABA across membranes, different ABC-transporters are required for active transport of the hormone (KANG *et al.* 2010, KUROMORI *et al.* 2010).

ABA is known to regulate diverse processes acting as an endogenous messenger. For plants which are sessile organisms and face frequent environmental changes, ABA is the key hormone mediating the response to abiotic as well as biotic stresses, thus ensuring plant performance and productivity (CAO *et al.* 2011, FUJITA *et al.* 2006, ZHU 2002).

The effect on plant growth by abiotic stresses including drought, salinity or extreme temperatures can be both general and specific. Whereas drought reduces plant growth due to a decrease in photosynthetic activity and limits nutrient availability from the soil, salinity causes physiological drought and ion toxicity (ZHU 2002). Cold temperatures directly affect metabolism and cause additional osmotic stress (THOMASHOW 1999). To cope with different stresses ABA-induced expression of genes that encode for proteins associated with stress responses and tolerance is crucial (FUJITA *et al.* 2006, NEMHAUSER *et al.* 2006, SEKI *et al.* 2002). Among those are many regulatory proteins, such as transcription factors (TFs), protein kinases and phosphatases, a variety of transporters and enzymes involved in the synthesis of osmolytes, for example proline and different sugars. In contrast, ABA-mediated down regulation of genes can be observed for those encoding for proteins involved in growth and development, for example ribosomal, chloroplast or cell wall proteins (reviewed in FUJITA *et al.* 2011). One of the immediate

events upon water stress is the regulation of stomatal aperture which is mediated by raising concentrations of ABA in guard cells. This event involves the redistribution of anions across membranes facilitated by different channels that are well characterized to date (reviewed by KIM *et al.* 2010). Stomatal closure is essential to the plant as it allows it to conserve water.

Under non-stress conditions ABA is the key player in modulating plant growth, including seed development, dormancy and germination (FINKELSTEIN *et al.* 2002). While ABA content is typically low early in embryogenesis, it accumulates in seeds during mid- and late embryogenesis allowing the induction and maintenance of seed dormancy, the accumulation of nutrients and the acquisition of desiccation tolerance. Dormancy release and promotion of germination are induced by a decrease in ABA concentration and sensitivity and an increase in gibberellic acid (GA) (FINCH-SAVAGEandLEUBNER-METZGER 2006, FINKELSTEIN *et al.* 2007). If conditions become unfavorable during early seedling development, for example drought stress, ABA is able to induce postgermination growth arrest by effectively delaying vegetative growth (LOPEZ-MOLINA *et al.* 2001). This provides an efficient, adaptive mechanism to increase the survival rate of plants under stress conditions.

Identification of ABA signaling components: ABA-insensitive mutants

As a key regulator of growth, development and adaptive responses to a variety of environmental stimuli, ABA influences almost all aspects of a plant's life. To coordinate these different functions ABA perception and an extensive network of signal transducers are necessary. ABA-insensitive screens have been widely used to identify molecular, genetic components of the ABA signaling pathway. In *Arabidopsis* the first ABA-insensitive mutants (*abi1*, *abi2*, *abi3*) were initially selected based on their altered sensitivity towards exogenous ABA (KOORNNEEF *et al.* 1984). The *abi1-1* and *abi2-1* mutations are dominant and cause pleiotropic phenotypes including ABA-resistant seed germination and seedling growth, reduced seed dormancy and abnormal stomatal regulation (ALLEN *et al.* 1999, KOORNNEEF *et al.* 1984, LEUNG *et al.* 1997). They result from the same amino acid substitution at equivalent positions in the homologous proteins AtABI1 and AtABI2 (LEUNG *et al.* 1997) Intragenic revertants later showed the opposite phenotypes indicating that AtABI1 and AtABI2 are redundant, negative regulators of ABA signaling (GOSTI *et al.* 1999).

AtABI1 and AtABI2 belong to the PP2C family of protein phosphatases which are monomeric enzymes and fall into seven groups (A-J). Group A of PP2C phosphatases is the one associated with ABA signal transduction (reviewed by SCHWEIGHOFER *et al.* 2004) and besides AtABI1 and AtABI2, four other members of this group have been described to negatively regulate ABA signaling: HYPERSENSITIVE TO ABA 1/2 (HAB1/HAB2) and ABA HYPERSENSITIVE GERMINATION 1/3 (AHG1/AHG3, also known as PP2CA) (GOSTI *et al.* 1999, KUHN *et al.* 2006, NISHIMURA *et al.* 2007, RODRIGUEZ *et al.* 1997, SAEZ *et al.* 2004, YOSHIDA *et al.* 2006). AtHAB1 and AtHAB2 were originally identified on the basis of sequence homology to AtABI1 and AtABI2 (RODRIGUEZ *et al.* 1998). Expression level of *AtHAB1* and *AtHAB2* are upregulated by ABA and both proteins are broadly expressed within the plant at key sites of ABA action, such as seeds and guard cells (SAEZ *et al.* 2004). However, ABA responses in seeds and during early seedling development are most likely mediated by AtAHG3/PP2CA. The *AtAHG3* gene was shown to be identical to *AtPP2CA* (YOSHIDA *et al.* 2006). Two independent groups analyzing mutants which lack total phosphatase activity *(ahg3-1* and *pp2ca-1*) could show strong hypersensitive responses to ABA during germination and early seedling growth (KUHN *et al.* 2006, YOSHIDA *et al.* 2006). In addition, AtAHG3/PP2CA also seems to have a role during vegetative development, since ectopic expression of AtPP2CA causes ABA-insensitivity in ABA-induced stomatal closure (KUHN *et al.* 2006). In contrast, AHG1 is active in seeds and seedlings but seems to have no function in adult plants (NISHIMURA *et al.* 2007).

To date, many of the members of the group A protein phosphatases have been genetically well established to be negative regulators of ABA signaling (LEUNG *et al.* 1994, LEUNG *et al.* 1997, MEYER *et al.* 1994, RODRIGUEZ *et al.* 1998, SAEZ *et al.* 2004).

Apart from phosphatases ABA-insensitive screens revealed three important ABA-responsive transcription factors. The *abi3*, *abi4* and *abi5* mutants were selected on the basis of ABA-resistant germination (FINKELSTEIN 1994). All three mutations cause defects in seed ABA sensitivity and seed-specific gene expression, but do not alter vegetative growth. *ABI3*, *ABI4* and *ABI5* encode for transcription factors of the B3-, AP2- and bZIP-domain families, respectively (FINKELSTEINandLYNCH 2000, FINKELSTEIN *et al.* 1998, GIRAUDAT *et al.* 1992). AtABI5 is the main player regulating ABA-induced postgermination growth arrest of seedlings under conditions of water deficit (LOPEZ-MOLINA *et al.* 2001). Within a

limited developmental time window following germination *AtABI5* expression is induced by ABA and protein levels stabilize. In addition, AtABI5 phosphorylation can be observed which is, at least *in vitro*, mediated by members of the SNF1-RELATED PROTEIN KINASE 2 family (LOPEZ-MOLINA *et al.* 2001, NAKASHIMA *et al.* 2009). Execution of growth arrest involves the direct interaction with AtABI3 which acts upstream of AtABI5 and is known to be required for the expression of a large number of late embryogenesis genes thought to be essential for the acquisition of desiccation tolerance (LOPEZ-MOLINA *et al.* 2002, NAKAMURA *et al.* 2001, PARCY *et al.* 1994). To allow growth arrest of germinated embryos and promotion of osmotolerance ABI5 re-induces the expression of some LATE EMBRYOGENESIS ABUNDANT genes (*LEA*) genes through binding to ABA-responsive elements (ABRE) in their promoters (CARLES *et al.* 2002, LOPEZ-MOLINA *et al.* 2002, NAKAMURA *et al.* 2001). Some additional direct transcriptional targets of AtABI5 have been identified recently but have not been characterized in detail, yet (REEVES *et al.* 2011). Several negative regulators of ABI5 have been reported to date. Among those that mediate AtABI5 degradation via the 26S proteasome are the ABI5-BINDING PROTEINS (AFP), and a RING finger ubiquitin E3 ligase, KEEP ON GOING (KEG) (LIUandSTONE 2010, LOPEZ-MOLINA *et al.* 2003, STONE *et al.* 2006).

Kinases involved in ABA signaling

The existence of phosphatases in a signaling pathway naturally implies the activity of kinases, since phosporylation and dephosphorylation usually counterbalance each other. One of the first kinases to play a role in ABA signal transduction was purified from *Vicia faba*. This kinase is activated by the plant hormone, hence the name ABA-activated protein kinase (AAPK) (LI and ASSMANN, 1996; LI et al., 2000). The orthologous kinase in *Arabidopsis* is SnRK2.6, a member of the SNF1-RELATED PROTEIN KINASE 2 family, and also known as OPEN STOMATA 1 (OST1). The SnRK2 family represents a group of Ser/Thr protein kinases and includes 10 genes in *Arabidopsis* (HRABAK *et al.* 2003).

AtOST1 was first described in 2002 when mutants that displayed reduced ability to close stomata in response to ABA were discovered (MUSTILLI *et al.* 2002). Simultaneously, expression of *AtOST1* was found to be restricted to guard cells. ABA-activated protein kinases that regulate responses other than stomatal closure are AtSnRK2.2 and AtSnRK2.3 which are widely expressed in various tissues (FUJII *et al.* 2007). While *snrk2.2* and *snrk2.3*

single mutants do not show any phenotypes, the double mutant is insensitive to ABA during germination and post germination development, indicating functional redundancy of the two kinases. However, the effect on water loss is much smaller than in the *ost1* mutants, suggesting distinct roles for AtSnRK2.2 and AtSnRK2.3. Extreme ABA-insensitivity could recently be described for the *snrk2.2/2.3/2.6* triple mutant which is affected in all ABA-mediated responses (FUJITA *et al.* 2009, NAKASHIMA *et al.* 2009).

All three SnRK2 kinases have been shown to localize to the cytosol as well as to the nucleus using GFP-fusion proteins (FUJITA *et al.* 2009). Besides being the main activators of ABA-responsive transcription factors (reviewed by FUJITA *et al.* 2011) some immediate responses to ABA are directly regulated by SnRK2s, in particular OST1. Among these is the regulation of anion channels in guard cells, where OST1 directly activates SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) by phosphorylation, and the production of reactive oxygen species (ROS) by phosphorylation of AtrbohF NADPH oxidase (GEIGER *et al.* 2009, SIRICHANDRA *et al.* 2009). Therefore, SnRK2 kinases so far represent the main positive regulators of ABA signaling

Another family of Ser/Thr protein kinases and closely related to SnRK2s are the group of SnRK3s that have previously been published as CBL-INTERACTING PROTEIN KINASES (CIPKs; reviewed in BATISTICandKUDLA 2009). The SnRK3 subfamily appears to be unique to plants and includes 26 genes in *Arabidopsis*. CIPKs specifically interact with the CALCINEURIN-B LIKE proteins (CBLs) which bind calcium and regulate the activity and localization of CIPKs (BATISTICandKUDLA 2009). CIPKs do not possess distinct localization signals; however CBL-CIPK interaction analyses showed that CIPKs are targeted to different membranes by their respective interacting CBL proteins. The CBL-CIPK complexes are best known for their function in salt stress signaling in the SOS (SALT OVERLY SENSITIVE) pathway (HALFTER *et al.* 2000, QIU *et al.* 2002). However, some CIPKs have been shown to be involved in ABA signaling (D'ANGELO *et al.* 2006, PANDEY *et al.* 2008) and to interact with the protein phosphatase AtPP2CA (LAN *et al.* 2011).

ABA receptors

Although knowledge of ABA metabolism and signal transduction has advanced rapidly, identification of protein receptors that directly bind the hormone and trigger signaling events leading to ABA's distinctive effects on plant physiology have been difficult.

Microinjection studies and treatments with impermeant ABA analogs in the 1990s suggested that ABA may have both intracellular and extracellular sites of perception (ANDERSON *et al.* 1994, JEANNETTE *et al.* 1999, SCHWARTZ *et al.* 1994). Multiple candidates, including the chloroplast-localized H SUBUNIT OF THE MG-CHELATASE (CHLH), the membrane-localized G PROTEIN-COUPLED RECEPTOR 2 (GCR2) and the GPCR-TYPE G PROTEIN 1/2 (GTG1/GTG2) have been proposed. However, some of the results are controversial and the molecular mechanism by which the ABA signal is transduced remains unclear (evaluated by KLINGLER *et al.* 2010). Identification of key receptors by straightforward genetic approaches was probably hindered by the high functional redundancy of these genes.

However, in 2009 two independent groups succeeded in the identification of the *Arabidopsis* PYR/RCAR proteins as ABA receptors (MA *et al.* 2009, PARK *et al.* 2009). In a yeast-two-hybrid screen searching for interaction partners of the protein phosphatase AtABI2 a protein called REGULATORY COMPONENT OF ABA RESPONSE 1 (RCAR1) was discovered (MA *et al.* 2009). In an alternative approach a chemical genetic screen with pyrabactin, an ABA agonist, identified the PYRABACTIN RESISTANCE 1 (PYR1) locus. Furthermore, 13 PYR1-LIKE (PYL) proteins were found in the *Arabidopsis* genome so that today the PYR/PYL/RCAR family of ABA receptors comprises 14 members.

Since the *pyr1* mutant responds normally to ABA it is likely that other family members are indeed functionally redundant. Only triple or quadruple mutants (*pyr1 pyl1 pyl4* or *pyr1 pyl1 pyl2 pyl4*) show distinct ABA phenotypes, such as ABA-insensitivity during germination (PARK *et al.* 2009).

All ABA receptor proteins are soluble and belong to the Bet v 1-fold superfamily, named for a major allergen in pollen of white birch (RADAUER *et al.* 2008). Structurally this superfamily is characterized by a helix-grip fold which creates a large binding cavity for hydrophobic ligands. The crystal structures of three proteins of the PYR/PYL/RCAR family could be determined and confirm the importance of the helix-grip fold for ABA binding (MELCHER *et al.* 2009, MIYAZONO *et al.* 2009, NISHIMURA *et al.* 2009b, SANTIAGO *et al.* 2009a, YIN *et al.* 2009). Once ABA is bound it is completely contained within the pocket. The receptor protein undergoes a conformational shift and two characteristic loops, ‘gate and latch’, fold over the ABA binding cavity (MELCHER *et al.* 2009). In addition, four of the five reported crystal structures indicate that the receptor proteins form homodimers in the

absence of ABA (MELCHER *et al.* 2009, NISHIMURA *et al.* 2009b, SANTIAGO *et al.* 2009a, YIN *et al.* 2009). Binding of the ABA molecule to only one of the two monomers results in dissociation of the homodimer and allows the binding surface for the interacting PP2C phosphatases.

The core ABA signaling pathway

With the identification and biochemical characterization of the ABA receptor proteins important progress has been made in elucidating early ABA signal transduction. Subsequent studies linking ABA receptors, protein phosphatases of the PP2C family and protein kinases of the SnRK2 family have provided a minimal but crucial set of factors representing the core ABA signaling pathway (Fig. 2) which has been reviewed extensively (CUTLER *et al.* 2010, HUBBARD *et al.* 2010, KLINGLER *et al.* 2010).

In the absence of ABA, PP2C phosphatases (such as AtABI1) inhibit SnRK2 kinase activity through the removal of activating phosphates (Fig. 2A). In the presence of ABA, the hormone is bound by intracellular PYR/PYL dimers which dissociate and form ABA receptor-PP2C complexes in the nucleus as well as in the cytosol (here shown in guard cells; 21B and 2C). Physical interaction of PYR/PYL receptors with PP2C phosphatases and subsequent inhibition of the latter has been shown for almost all members of the phosphatase family (MA *et al.* 2009, NISHIMURA *et al.* 2010, PARK *et al.* 2009, SANTIAGO *et al.* 2009b, SZOSTKIEWICZ *et al.* 2010). Interestingly there are biochemical differences in the PYR/PYL isoforms, indicating possible functional specialization among the family members. Some interacting receptor-phosphatase pairs interact also in the absence of the hormone but might still need ABA for inhibition of the phosphatase. Several specific residues on either receptor or phosphatase protein have been identified to be important for the interaction. For example, substitutions of the residues P88 and S152 of the AtPYR1 receptor protein, which severely reduce the capacity to interact with AtHAB1 (NISHIMURA *et al.* 2009b, SANTIAGO *et al.* 2009a), and residue S85 (S89 in AtPYL2) are crucial for the interaction with different PP2Cs (SANTIAGO *et al.* 2012). Conversely, the amino acid substitution G168D of AtABI2 impairs the inhibitory effect of AtPYR1 and blocks the interaction with AtPYL9 (MA *et al.* 2009, PARK *et al.* 2009).

Inhibition of the negative regulators then allows activation of the downstream SnRK2 kinases which in turn regulate the expression of ABA-responsive genes. Up to 10 % of

protein coding genes in *Arabidopsis* are likely to be regulated by ABA (NEMHAUSER *et al.* 2006). Several SnRK2 targets have been identified both in nucleus and at the plasma membrane. In the nucleus the ABA-activated SnRK2 kinases are well known to activate basic-domain leucine zipper (bZIP) transcription factors by phosphorylation (FUJII *et al.* 2007). Those transcription factors bind to a *cis*-acting regulatory element in the promoter of ABA-responsive genes, the ABA-responsive element (ABRE; YAMAGUCHI-SHINOZAKIandSHINOZAKI 2005).

Among the bZIP transcription factors controlling ABRE-mediated transcription are the ABRE-BINDING FACTORS (ABFs) and members of the ABI5 family (CHOI *et al.* 2000, FUJITA *et al.* 2011, UNO *et al.* 2000). Members of the ABF family are mainly expressed in vegetative tissue under abiotic stress where they require phosphorylation to be fully active. In contrast, ABI5 family members are mainly active in seeds (reviewed by FUJITA *et al.* 2011).

At the plasma membrane SnRK2 phosphorylation directly regulates the activity of ion channels, such as SLAC1 or K^+ CHANNEL IN *ARABIDOPSIS THALIANA* (KAT1) (GEIGER *et al.* 2009, SATO *et al.* 2009).

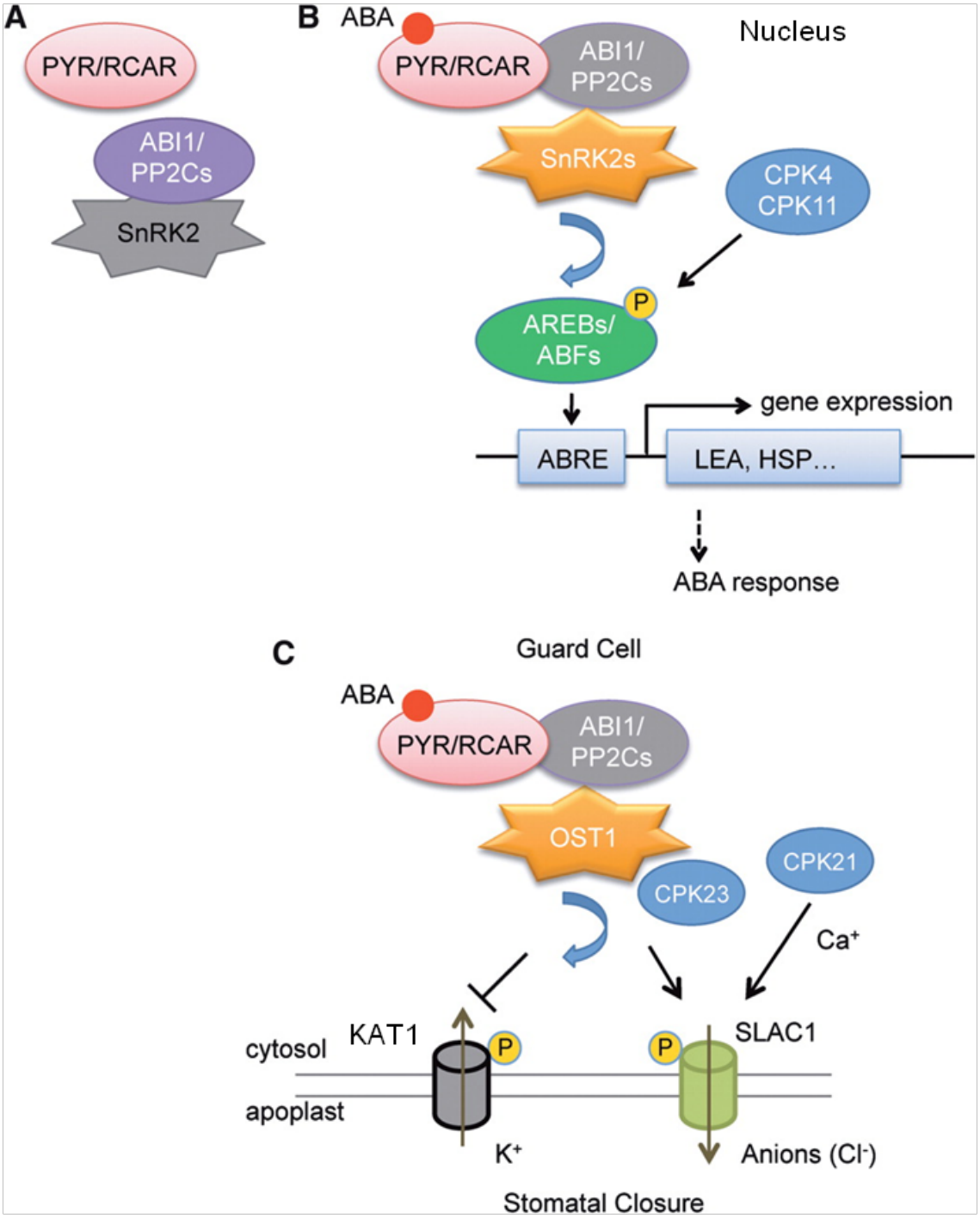

Figure 2. The core ABA signaling pathway (modified according to QIN *et al.* 2011)
The current model of ABA signaling including the PYR/RCAR ABA receptors, the PP2C phosphatases and the SnRK2 kinases. Some of the most common downstream transcription factors (ABFs), channels (KAT1 and SLAC1) and genes (*LEA*, *HSP*) are depicted in addition.
(A) In the absence of ABA, **(B)** In the presence of ABA in the nuclei, **(C)** In the presence of ABA in guard cells

1.3 Scientific goal of this work

The aim of this study was to characterize the function of the protein kinase AtWNK8 in more detailed. Previous studies in our lab had identified the kinase as an interaction partner of the C-subunit of the vacuolar H^+-ATPase (HONG-HERMESDORF *et al.* 2006). Our data showed that AtWNK8 is an active kinase which phosphorylates a specific subunit of the vacuolar proton pump, however, did not allow us to describe the *in vivo* function of AtWNK8. Surprisingly, phenotypic analysis of *wnk8* mutants, exhibiting altered postgermination growth in response to ABA, indicated that AtWNK8 could be involved in ABA signaling. Including key components of ABA signal transduction in interaction studies and biochemical analyses allowed us to place AtWNK8 within this complex network. This work suggests a new role for AtWNK8 as a negative regulator in ABA signaling.

2 MATERIAL AND METHODS

Construct design

Generation of basic plant binary vectors pUTkan, pHTkan and pUTbar^{+}

All basic plant binary vectors that confer kanamycin resistance in plants are based on the vector pTkan, described by M. Krebs in the supplement material (KREBS *et al.* 2011). For the following constructs vectors containing either a constitutive Ubiquitin-10 or an inducible hsp18.2 heat shock promoter were used. The generation of the pUTkan vector, carrying the Ubiquitin-10 promoter, has been described before (KREBS *et al.* 2011). This vector is based on the binary plant expression vector pJH212 *(nptII* under control of the *mas* promoter), a derivative of the pPZP212 vectors (HAJDUKIEWICZ *et al.* 1994).

For the generation of the pHTkan vector the *Arabidopsis* heat-shock-inducible promoter hsp18.2 was amplified from Col-0 genomic DNA using the primer pair HSP18.2_SacI.FWD and HSP18.2_KpnI.REV. Subsequently, a 895 bp fragment flanked by SacI/KpnI restriction sites was subjected to A-tailing and sub-cloned into pCR®2.1-TOPO® (Invitrogen) and for further sequence analysis. From there the SacI/KpnI digested hsp18.2 fragment was ligated into equally digested pTkan producing the pHTkan vector.

All basic plant binary vectors that confer Basta resistance in plants are based on the pTbar vector, a derivative of pPZP312 based on pPZP vectors described by Hajdukiewicz and colleagues (HAJDUKIEWICZ *et al.* 1994). First a new MCS was introduced into the pTbar vector. The oligos MCS_for and MCS_rev were annealed by stepwise lowering the temperarture from 99 °C to room temperature and phosphorylated at the 5'-ends using the T4 polynucleotide kinase (Fermentas). The double stranded oligo contained 5'-BamHI and 3'-SalI overhangs to be ligated into the BamHI/SalI digested pTbar, generating the pTbar^{+}. Next a 647 bp fragment of the Ubiquitin-10 promoter sequence was PCR-amplified using the primer pair UBIQ10_BamHI_for and UBIQ10_BamHI_rev adding BamHI restriction sites on both ends. pTbar^{+} was digested with BamHI to ligate the Ubiquitin-10 fragment and generate pUTbar^{+}.

Generation of GFP / RFP- vectors for localization studies

To produce N- or C-terminal GFP-fusion proteins two GFP-containing vectors were generated: pUGT1kan and pUGT2kan. For co-localization studies a third vector containing mRFP for N-terminal fusions was established in addition, designated pURT2kan. All three vectors are provided with the Ubiquitin-10 promoter and the rbcs terminator.

The pUGT1kan vector was generated based on pUTkan described previously. It contains the GFP5 variant GFP5 (S65T) for C-terminal fusion with the protein of interest. The GFP5 (S65T) was kindly provided by C. Fankhauser, Geneva.

For the pUGT2kan, the GFP5 (S65T) gene was PCR amplified with the primer pair GFP5_BglII_F and GFP5_BamHI_R. The 721 bp fragment was sub-cloned into the pJET1.2/blunt Cloning Vector (Fermentas) for sequencing. The GFP5 sequence was isolated using BglII and BamHI restriction enzymes and ligated into the BamHI-digested pUTkan described previously.

For the pURT2Kan vector a 678 bp DNA fragment encoding the ORF of the monomeric red fluorescent protein (mRFP) was generated by PCR amplification using Ara6-mRFP as a template (UEDA *et al.* 2004). BamHI restriction sites were introduced with the primers RFP_BamHI_F and RFP_BamHI_R. The fragment was sub-cloned into the pJET1.2/blunt Cloning Vector (Fermentas) for sequencing and then ligated into the BamHI/BamHI digested pUTkan. This construct was generated by Christoph Neubert.

To produce N-terminally GFP-tagged proteins under inducible conditions the pHGT2kan vector was generated. For this purpose a 746 bp fragment containing GFP5 (S65T) cDNA (without stop codon) was amplified by PCR using the primer pair GFP5_BglII_F and GFP5_BamHI_R, sub-cloned into pCR®2.1-TOPO® (Invitrogen) and subjected to sequence analysis. After digestion with BamHI the GFP5 fragment was ligated into the BglII/BamHI-digested pHTkan vector, designated pHGT2kan. This construct was generated by Zhao Xing Wang.

Generation of BIFC basic vectors

The generation of pYNx and pxYN for N- and C-terminal fusion proteins with the N-terminal half of eYFP was performed by Christoph Neubert. A 683 bp fragment containing the MCS, a c-myc tag and the N-terminal 155 aa of the eYFP was PCR-amplified using the 35S::SPYNE173 vector (Accession-No. EU796363) as a template. BglII and BamHI

restriction sites were introduced with YN173_BglII_F and YN173_BamHI_R. The amplified cassette was sub-cloned into the pJET1.2/blunt cloning vector (Fermentas) and verified by sequence analysis. Using the provided restriction sites the whole cassette was isolated from pJET1.2/blunt and ligated into the BamHI digested pUTkan vector to produce pYNx. For the pxYN vector a 575 bp PCR fragment was amplified from the 35S::SPYNE template (Accession-No. EU796373) including the MCS, a c-myc tag and the N-terminal half of the eYFP. The pimer pair used (cYN_SpeI_for and cYN_XbaI_rev) added SpeI and XbaI restriction sites to the end of the fragment. After sequence analysis in pJET1.2/blunt the cassette was ligated into the SpeI/XbaI digested pUTkan.

The vectors pYCx and pxYC were used to construct N- or C-terminal fusion proteins with the C-terminal half of the eYFP including an additional HA-tag. Both vectors were generated based on pUTbar^{+} described previously. For pYCx the primer pair nYC_AatII_For and nYC_AatII_Rev was used to PCR-amplify the eYFP-HA cassette from the SPYCE(MR) template (WAADT *et al.* 2008). Flanked by AatII restriction sites the fragment was sub-cloned into the pJET1.2/blunt vector and subjected to sequence analysis. Finally the eYPF-HA cassette was isolated from pJET and ligated into the AatII digested pUTbar^{+}. Correct orientation of the insert was confirmed by test digestion. For the generation of pxYC the HA-eYFP cassette was PCR-amplified from SPYCE(M) using cYC_SalI_For and cYC_SalI_Rev (WAADT *et al.* 2008). The fragment, flanked by SalI restriction sites was sub-cloned into the pJET1.2/blunt vector and sequenced. After digestion with SalI the HA-eYFP fragment was ligated into the SalI digested pUTbar^{+}, designated pxYC.

WNK8pGUS

For the generation of the WNK8pGUS construct the binary plant vector pCB308kan was used (XIANG *et al.* 1999). A 1875 bp *WNK8* promoter fragment was PCR- amplified from Col-0 genomic DNA using WNK8PromFW2 and WNK8PromRV2 primers. The fragment was sub-cloned into pCR®2.1-TOPO® (Invitrogen) and subjected to sequence analysis. With the provided XbaI/BamHI restriction sites the promoter was isolated and ligated into the equally digested pCB308. This construct was generated by Angela Brüx.

Recapitulating the *wnk8-1* phenotype

The UBQ10:WNK8KD construct was generated according to the transcript still present in the *wnk8-1* mutant. From *wnk8-1* cDNA a 984 bp fragment, including 966 bp of *WNK8* and 18 additional base pairs of the T-DNA insert, was PCR-amplified using WNK8Bam.fw and wnk8-1.rev2 primers. For sequence analysis the fragment was sub-cloned into the pJET1.2/blunt vector (Fermentas). Using the attached BamHI restriction sites the truncated WNK8 fragment was isolated from pJET and ligated into the BamHI digested pUTkan vector. This construct was generated by Anne Hong-Hermesdorf.

WNK8 and PP2CA fluorescent constructs

For heat shock (HS) inducible expression of GFP-WNK8 the *WNK8* cDNA was PCR-amplified from an existing plasmid using WNK8SacII_fwd and WNK8SalI_rv primers. The SacII/SalI flanked fragment was sub-cloned into pCR®2.1-TOPO® (Invitrogen) and sequenced. To yield HS::GFP-WNK8 the fragment was ligated into the SacII/SalI digested pHGT2kan. This construct was generated by Anne Hong-Hermesdorf.

The constitutive GFP-WNK8 expression construct was generated based on HS::GFP-WNK8 described previously. The GFP-WNK8 fragment was isolated using the provided SacII/SalI restriction sites and sub-cloned into the equally digested pUGT2kan, designated UBQ10::GFP-WNK8.

For the GFP-WNK8(K41M) construct the WNK8 fragment of UBQ10::GFP-WNK8 was isolated using flanking BamHI/SalI restrictions sites and sub-cloned into pBluescript (Fermentas). Site-directed mutagenesis was performed according to the QuikChange® Site-Directed Mutagenesis Kit protocol by Stratagene using the primer pair mutK41Mrev and mutK41Mfwd. Introduction of the mutation was verified by sequence analysis and the modified WNK8 was ligated back into pUGT2kan via BamHI/SalI restriction sites.

The WNK8KD(K41M)-GFP construct originates from the GFP-WNK8(K41M) construct. The latter was used as a template to PCR-amplify the first 860 bp of *WNK8* including the N-terminally localized kinase domain. The primer pair WNK8_BamHI.for and WNK8NTnoStop_BamHI.rev was used adding additional BamHI restriction sites to both ends. The PCR product was sub-cloned into the pJET1.2/blunt cloning vector (Fermentas). After sequence analysis the WNK8 kinase domain fragment was isolated from pJET using BamHI restricition sites and ligated into pUGT1kan.

To generate the UBQ10::RFP-PP2CA construct a 1230 bp fragment including the cDNA of PP2CA with a C-terminal cmyc-tag and final stop codon was PCR-amplified using the following primer pair: PP2CA-XmaI.FOR and PP2CA-cmyc-SpeI.REV. The PCR fragment was sub-cloned into the pJET1.2/blunt vector. After sequence analysis the PP2CA-cmyc fragment was isolated from pJET using the provided XmaI/SpeI restriction sites and ligated into the XmaI/SpeI digested pURT2kan vector, designated UBQ10:RFP-PP2CA.

Yeast-two-hybrid constructs

For the generation of the WNK8KD prey construct a 860 bp fragment containing the cDNA of the *WNK8* kinase domain (plus stop codon) was PCR-amplified using the primers WNK8-BamHI.Fwd and WNK8.NT-XhoI.Rev. The PCR fragment was subjected to A-tailing and sub-cloned into the pCR®2.1-TOPO® (Invitrogen). After sequence analysis and subsequent digestion with BamHI and XhoI the WNK8KD fragment was ligated into the BamHI/XhoI digested pACT2 AD vector (Clontech, GenBank Accession No.: U29899) providing the DNA activiation domain. This construct was generated by Angela Brüx.

The WNK8NdeFW and WNK8NT-BamHI-REV primer pair functioned to PCR-amplify the 860 bp WNK8 kinase domain fragment (plus Stop codon) to generate the WNK8KD(K41M) prey construct. The PCR fragment, amplified from the GFP-WNK8(K41M) template, was sub-cloned into the pJET1.2/blunt cloning vector (Fermentas), sequenced and isolated from the vector using the attached NdeI/BamHI restriction sites. The insert was cloned into the NdeI/BamHI digested pGADT vector (Clontech) containing the DNA activation domain.

For the third prey construct containing WNK8 fused to the DNA activation domain the complete *WNK8* cDNA was PCR amplified using the primers WNK8NdeFW and WNK8-BamHI-REV adding NdeI and BamHI restriction sites to the PCR fragment. Next the WNK8 fragment was sub cloned into pJET1.2/blunt from where it was isolated via NdeI/BamHI digestion and ligated into the pGADT vector.

The PP2CA bait construct contains the full length PP2CA cDNA that was PCR amplified from an existing plasmid using the primers PP2CA-NdeI For and PP2CA-SalI REV. The fragment was sub-cloned into pJET1.2/blunt and subjected to sequencing. Subsequently it was isolated from the vector using the attached NdeI/SalI restriction sites and ligated into the pGBKT vector (Clontech) containing the DNA binding domain.

The ABI1 and ABI2 bait constructs were kindly provided by the lab of Jörg Kudla, University of Münster, containing the full length ABI1 or ABI2 cDNA fused to the DNA binding domain.

All CIPKs were cloned in the lab of Jörg Kudla, University of Münster, into the pGBT9.BS vector.

BIFC constructs

Prior to ligation of the appropriate fragments into the final vectors, all of them were sub-cloned into the pJET1.2/blunt cloning vector (Fermentas) and analyzed for the correct sequences.

For YN-WNK8 the insert was cut out from the existing UBQ10::GFP-WNK8 construct using the available SacII/SalI restriction sites and ligated into the equally digested pYNx vector.

The WNK8 kinase domain fragment (WNK8KD) was PCR-amplified from the UBQ10::GFP-WNK8 construct using the primer pair WNK8_BamHI.for and WNK8NT-BamHI-REV and ultimately ligated into the BamHI digested pYNx vector. For YN-WNK8(K41M) the same primer pair was used for PCR but the GFP-WNK8(K41M) template already carrying the mutation.

PP2CA and mCherry were cloned into the complementary pxYC vector. For this purpose the cDNA of PP2CA was PCR-amplified (no stop codon) from the existing PP2CA bait construct using the primer pair PP2CA_PacI.for and PP2CAnoStop_SpeI.rev. The PacI/SpeI restriction sites introduced by PCR were used to ligate the PP2CA fragment into pxYC.

A 710 bp mCherry fragment was PCR-amplified with AatII/SpeI flanking restriction sites from an existing plasmid using mCherry-AatII.for and mCherry-SpeI.rev primers. For the final mCherry-YC construct the mCherry fragment was ligated into the AatII/SpeI digested pxYC vector.

Constructs for protein expression

WNK8 and PP2CA expression constructs were generated based on the pIVEX1.3 vector from 5 PRIME. In the lab of Jörg Kudla, University Münster the vector was modified as follows: for StrepII-tagged fusion proteins the 6xHis tag was removed and replaced by a strepII tag. The existing MCS was modified by PCR mutagenesis. The full length WNK8 cDNA was PCR-amplified from the existing EG21 construct using WNK8_BamHI.for and

WNK8_XmaI.rev primers. The BamHI/XmaI flanked fragment was sub-cloned into the pJET1.2/blunt cloning vector (Fermentas) and sequenced. Subsequently, the WNK8 fragment was ligated into the BamHI/XmaI digested pIVEX to form the pIVEX WNK8 construct. The pIVEX PP2CA construct was provided from the lab of Jörg Kudla, University Münster and was generated from a PP2CA cDNA fragment amplified with additional SpeI/XmaI restriction sites. This fragment was ligated into the SpeI/XmaI digested pIVEX vector.

For expression of PYR1 protein E. Grill, TU Munich kindly provided the pQE70 PYR1(RCAR11) vector published previously (KEPKA *et al.* 2011). The PYL8 expression construct was generated in the lab of P. Rodriguez, IBMCP Valencia. It contains the cDNA of PYL8 in the pET28a vector (Novagen).

Accession numbers

WNK8: At5g41990; PP2CA: At3g11410; PYR1: At4g17870; PYL8: At5g53160; ABI1: At4g26080; ABI2: At5g57050

List of T-DNA mutants used in this work

wnk8-1 SALK_024887 (Arabidopsis Biological Resource Centre, Ohio, USA)

wnk8-4 SALK_103318 (Arabidopsis Biological Resource Centre, Ohio, USA)

pp2ca-1 SALK_028132 (KUHN *et al.* 2006)

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 seeds were used in all experiments described. For plate experiments seeds were surface-sterilized with EtOH. Prior to light exposure all seeds were kept at 4°C for 2 days for stratification.

Plants were grown at 22°C under long day conditions with 16 h light and 8 h darkness.

For post germination growth arrest analysis, characterization of *wnk8-1* recapitulation lines and analysis of *wnk8-1 x Col-0* double mutants seeds were grown on 1 x MS medium containing 1 % sucrose and 0.6% phyto agar, pH 5.7-5.8 and additional 1 µM ABA.

For analysis of the *pp2ca-1 wnk8* double mutants seeds were grown on 0.25 x MS solidified with 0.6% phyto agar, pH5.7-5.8 and additional 0.75 µM ABA.

Agar and MS basal salt mixture were purchased from Duchefa (Haarlem, Netherlands). (+/-)-Abscisic acid was purchased from Wako Chemicals or Sigma, dissolved in EtOH and

stored as 10 mM aliquots at -20°C. It was diluted freshly in sterile H_2O before preparation of the plates.

Transgenic plants were selected on 1 x MS plates containing 50 µg / ml kanamycin.

GUS assays

5 d old seedlings were analyzed for WNK8p-GUS activity. Staining solution, including 50 mM phosphate buffer, 0.1 % Triton X-100, 20 mM X-Gluc and 2 mM K_3FeCN_6 and K_4FeCN_6, was vacuum infiltrated into whole seedlings using a standard water jet pump until all air was removed from the tissue. The samples were incubated at 37 °C in darkness for several hours. To stop the reaction the staining solution was replaced with 70 % EtOH.

Generation of double mutants

Homozygous *pp2ca-1 wnk8-1* and *pp2ca-1 wnk8-4* double mutants were verified among 96 F_2 individuals by genotyping. The absence of the wild type allele was confirmed using the following primer pairs in a standard PCR reaction with genomic DNA as template: pp2ca-1.fwd / pp2ca-1.rev (*PP2CA*), wnk8-4.fw / wnk8-4.rv and wnk8-1.fw / wnk8-4.rv (*WNK8*). The T-DNA insertion was verified using the TL2 T-DNA specific primer in combination with pp2ca-1.fwd, wnk8-1.fw or wnk8-4.rv.

Root length measurements

For measurements of root length seedlings were sandwiched between two layers of overhead transparency and scanned. The digitized images were analyzed using the program ImageJ 1.41 (NIH, USA).

Imaging

Pictures of whole plants were taken using a Nikon D60 digital camera.

For documentation of yeast-two-hybrid assay results plates were scanned in a flatbed scanner. Expression of *WNK8* in *Arabidopsis* seedlings was recorded using either the Zeiss microscope AxioImager M1 or the Zeiss stereomicroscope SteREO Discovery V20. Image processing was performed with Adobe Photoshop CS4.

Plant transformation

The binary constructs were introduced into *Agrobacterium tumefaciens* using chemical competent cells of the strain GV3101:pMP90. Successfully transformed cells were selected on 5 µg / ml rifampicin, 10 µg / ml gentamycin and 100 µg / ml spectinomycin.

Arabidopsis thaliana ecotype Col-0 was used for transformation using standard procedures.

Arabidopsis protoplasts transfection

For transient expression of recombinant protein in *Arabidopsis thaliana* protoplasts ecotype Ler suspension cultured cells were transformed using the appropriate constructs. Transformation was performed according to the polyethylene glycole-mediated method (NEGRUTIU *et al.* 1987). Cells were analyzed by confocal microscopy 24 to 72 hours after transformation.

Tobacco leave infiltration

For transient expression of recombinant proteins in tobacco 3 – 4 week old *Nicotiana benthamiana* leaves were infiltrated with a suspension of *Agrobacterium tumefaciens* carrying the appropriate constructs. Prior to infiltration bacterial cell density was measured and set to OD_{600} = 0.75. For co-expression of different constructs the corresponding cell suspensions were mixed at a ratio of 1:1. In addition, the same amount of the silencing inhibitor 19K was added to the mix that was then incubated at room temperature for 2 – 4 h (TE *et al.* 2005). Using a 1 ml syringe, leaves were infiltrated on the lower leaf side within the intercellular space. Protein expression in the lower epidermis was analyzed 3 - 5 days after infiltration by confocal microscopy.

RNA Isolation and cDNA synthesis

For the analysis of *WNK8* transcript levels in Col-0 wild type, *wnk8-1* and *wnk8-4* mutants RNA was isolated from 5-d-old seedlings using the RNeasy Plant Mini Kit (Qiagen) according to the protocol provided. cDNA was generated from 2 µg of total RNA using MMLV-RTase (Fermentas) and an oligo dT primer.

RT-PCR, qRT-PCR

The presence of the *WNK8* transcript in Col-0, *wnk8-1* and *wnk8-4* was tested by RT-PCR with the amplification of *ACTIN2* as a cDNA loading control. To test for the transcript of the N-terminal kinase domain a 366 bp fragment (KD) was amplified using the primer combination AtWNK8.fwd5 / WNK8RT.rev2 (29 cycles). For the amplification of a 533 bp fragment in the C-terminal region (CT), indicating the presence of the full length *WNK8* transcript, the primer pair vcip1160.fwd and WNK8SalI_rv was used (29 cycles).

Quantitative RT-PCR (qRT-PCR) was performed in the presence of the double-stranded DNA binding dye SYBR Green (Absolute QPCR SYBR Green Mix, Thermo Scientific). The reaction was monitored using the DNA Engine Opticon 2 Two-Color Real-Time PCR Detection System (BioRad). The *WNK8 KD* transcript in the recapitulation lines was amplified using the primer combination qRT1021_FOR / WNK8RT.rev2, the *WNK8 CT* transcript was amplified with the primer pair qRT311.1_FOR / WNK8SalI_rv.

CLSM

Confocal microscopy was performed using either a Zeiss Axiovert LSM510 Meta microscope with a Plan-Neofluar 25×/0.8 Imm corr DIC or a C-Apochromat 63×/1.2 W corr water immersion objective or a Leica SP5II system equipped with an inverted DMI6000 microscope stand. Images were recorded using a HCX PL APO lambda blue 63.0 × 1.20 WATER UV objective. For image acquisition the appropriate Leica- or LSM software was used. Detection of RFP was performed by excitation at 543 nm and emission at 593-635 nm. GFP was excited using the 488 laser line and the emission was detected between 510 and 540 nm. YFP was detected at 529–550 nm, after excitation with a 514 nm laser beam. Image processing was performed with Adobe Photoshop CS4 software.

Yeast-two-hybrid

A yeast-two-hybrid screen was performed by Hybrigenics (Paris, France). The DNA coding sequence for WNK8 amino acids 1 – 449 was PCR-amplified and cloned into pB27 vector as a N-LexA-bait-C fusion. The construct was checked by sequencing the entire insert and used as a bait to screen a library of one-week-old *Arabidopsis* seedlings (*Arabidopsis thaliana* seedlings_RP2). 64.2 million interactions were analyzed in the presence of 50 mM 3-AT and subsequently 121 clones were processed and sequenced to identify the corresponding interacting proteins.

Direct yeast-two-hybrid analysis were performed using the yeast strain pJ69-4A (JAMES *et al.* 1996). Yeast cells were transformed according to the small-scale LiAc yeast transformation procedure published by Clontech (Yeast Protocol Handbook). Successfully transformed cells were selected on selective dropout (SD) medium containing 6.7 g yeast nitrogen base (YNB), CSM -LW (according to the manufacturer), 2 % glucose, 0.003 % adenine hemisulfate and 20 g agar per liter. The pH of SD plates was set to 5.8. For subsequent spot tests transformants were grown in liquid culture (SD medium), diluted to

OD_{600} = 1 and 10-fold serial dilutions were spotted on SD plates (6.7 g YNB, CSM -LWAH (according to the manufacturer), 2 % glucose and 20 g agar) to identify interactions. Plates were incubated at 28 – 30 °C for 2 – 4 days. YNB, CSM –LW and CSM –LWAH were purchased from MP Biomedicals.

To test the interaction between CIPKs and WNK8 yeast cells were transformed similarly as described above. SD medium used for spot tests contained 6.7 g YNB, CSM -LWH, 2 % glucose and 20 g agar per liter. Plates were incubated at 30 °C for up to 14 days and contained additional 0.5 mM 3-AT to competitively inhibit the product of the yeast *HIS3* gene.

Expression and purification of recombinant proteins

His-tagged PYR1 and PYL8 proteins were expressed in M15 pREP4 and BL21(DE3) *E. coli* cells, respectively. Cells were grown in LB medium at 37 °C shaking until an OD_{600} of 0.5 was reached. Protein expression was induced by adding IPTG to a final concentration of 0.75 mM (PYR1) or 1 mM (PYL8). Cells were harvested at 4 °C and 4500 x g for 15 minutes 4 h after induction. Preparation of clear lysate under native conditions and protein purification was performed according to the Macherey & Nagel manual for Protino® Ni-TED 2000 Packed Columns. After treatment with lysozyme as indicated in the protocol cells were subsequently disrupted by sonification on ice with 6 x 15 s bursts with 15 s cooling period between each burst. The crude lysate was centrifuged twice at 4 °C and 10,000 x g for 30 min and loaded on the columns provided. Wash and elution fractions were eventually analyzed by SDS-PAGE. To keep proteins under optimized buffer conditions 500 µl of fraction 2 was subjected to buffer exchange using Amicon Ultra-0.5 mL Centrifugal Filters (Millipore). Buffer A (100 mM Tris HCl pH 7.6, 100 mM NaCl, 0.3 mM MnCl2, 4 mM DTT) was used to store PYR1 protein, PYL8 was stored in the following buffer: 50 mM Tris HCl 7.5, 0.1 % Tween20, 10 mM ß-ME.

StrepII-tagged PP2CA and WNK8 proteins were expressed in a eukaryotic cell-free protein synthesis system based on wheat germ lysate using the RTS™ 500 Wheat Germ CECF Kit (5PRIME, Hamburg). All reaction components were reconstituted according to the protocol. 60 µg of each pIVEX PP2CA and pIVEX WNK8 plasmid in a final volume of 300 µl DNase- and RNase-free water were used as a template. The filled reaction devices were incubated in the Eppendorf Thermomixer Comfort using the RTS 500 Adapter (5 PRIME)

at 24 °C shaking and 850 rpm. After incubation for 24 h the reaction solution was removed from the reaction device and the chamber was cleaned with 1 ml of washing buffer (100 mM Tris/HCl pH8, 150 mM NaCl) to remove all residual solution. The 2 ml reaction solution was mixed with 20 µl of 10 mg / ml avidin stock to block biotinylated proteins. Prior to adding 800 µl of resin material (*Strep*-Tactin® Superflow®, 50 % suspension, IBA BioTAGnology) to 1 ml of reaction solution equilibration of the resin was performed by washing 10 x with 1 ml of washing buffer. Incubation of resin and reaction solution was performed at 4 °C for 1 h with end-over-end rotation. For protein purification the mixture (2 x 800 µl) was transferred to a chromatography column (PolyPrep®, BioRad) and allowed to flow through by gravity. Washing was performed 5 x using 1 column volume (CV) of washing buffer and proteins were finally eluted with 0.5 CV of elution buffer (washing buffer + 2.5 mM desthiobiotin). Small aliquots of each wash and elution fraction were subjected to SDS-PAGE to examine protein concentration (in comparison with BSA standards) and purity.

In vitro phosphorylation assay

Purified proteins were mixed as indicated in the experiment in equal amounts and adjusted to a final volume of 16 µl with water. PP2CA and ABA receptor proteins (PYR1 or PYL8) were incubated on ice for 20 min with or without 10 µM (+/-) ABA before adding the WNK8 protein to the mix. After incubation 4 µl of 6 x reaction buffer (30 mM $MnSO_4$, 3 mM $CaCl_2$, 12 mM DTT) were added to all samples. To start the reaction 4 µl of 6 x ATP mixture (60 µM ATP magnesium salt, 1 µCi / µl [gamma-^{32}P] ATP) were added and the samples were incubated at 30 °C for 30 minutes to allow phosphorylation of the proteins. Radioactive-labeled adenosine 5'triphosphate (gamma-^{32}P ATP), at a concentration of 10 mCi / ml and an activity of 370 MBq was purchased from Hartmann Analytic, Braunschweig. The reaction was stopped by adding 1 µl of 0.5 M EDTA and 6 µl of 5 x Lämmli buffer (250 mM Tris HCl pH 6.8, 10 % SDS, 0.05 % Bromophenol blue, 10 % ß-Mercaptoethanol, 40 % Glycerol) followed by incubation at 95 °C for 5 minutes. Finally, samples were separated by SDS-PAGE (14 %) and stained with coomassie brilliant blue. The coomassie-stained gel was scanned for documentation of protein amounts and then exposed to x-ray film at room temperature for the times indicated.

In-gel digestion of gel-separated proteins for mass spectrometry

Coomassie stained protein bands were excised from the gel and subjected to overnight in-gel trypsin digestion according to established protocols (SHEVCHENKO *et al.* 2007). No reduction and alkylation steps were performed. Phosphopeptides were enriched by TiO_2 affinity chromatography (NuTips 10-200 µl, Glygen) according to the manufacturer's instructions. Samples were resuspended in eluent A (see below) prior to LC-MS/MS analysis.

LC-MS/MS, data analysis and MS-spectra interpretation

Analysis was performed on an Ultimate 3000 Nanoflow HPLC system (Dionex) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Finnigan). Eluents were composed of 5 % (v/v) acetonitrile (JT Baker)/0.1% (v/v) formic acid (Fluka) in Millipore water (A) and 80 % acetonitrile/0.1 % formic acid in Millipore water (B). The flow rate was set to 300 nl/min. The sample (1µl) was loaded on a trap column (C18 PepMap100, 300µM x 5mm, 5µm particle size, 100Å pore size; Dionex) and desalted using eluent A at 25µl/min for 4 min. Subsequently the trap column was switched in series with a capillary column (Atlantis dC18, 75µm x 10mm, 3µM particle size, 100 Å pore size) and the following gradient was applied for peptide separation: 0 to 15 % B over 10 min, 15-60 % B over 45 min, 60-100 % B over 2 min and 100% B for 5 min. The column was re-equilibrated with 100 % A for 10 min. Peptides eluted directly into the nanospray source of the mass spectrometer. The mass spectrometer was operated in positive ion mode. Survey scans were obtained by FT-MS (*m/z* 400-1600) at a resolution of 60.000 FWHM using internal lock mass calibration on *m/z* 445.120025. The five most intense ions were sequentially subjected to CID-fragmentation (35 % normalized collision energy) in the linear ion trap (MS2). Fragment ions exhibiting a neutral loss of 32.7, 49 or 98 Dalton were activated further while the remaining MS2 ions were stored in the ion trap. After the second step of fragmentation MS2 and MS3 ions were simultaneously analysed in the mass analyser of the ion trap resulting in composite MS2/MS3 spectra (*multistage activation*, MSA). Dynamic exclusion was enabled with a repeat duration of 90s, repeat count of 1 and exclusion mass width of +/- 25 ppm.

For the identification of peptides, MS spectra were matched against a protein sequence database composed of *Arabidopsis* RefSeq sequences and supplemented with client provided target sequences. Algorithms used for peptide spectra matching were Sequest

(Thermo Finnigan) and OMSSA 2.1.9 (GEER *et al.* 2004). OMSSA was integrated into the data processing pipeline Proteomatic (SPECHT *et al.* 2011). The maximum number of missed cleavages allowed was 2. Mass accuracy was set to 10 ppm for MS1 precursor ions and 0.8 Da for product ions. Serine, threonine and tyrosine phosphorylation and oxidation of methionine were used as variable parameters. Phosphopeptide identifications were validated by manual inspection of fragmentation spectra. At least three successive b- or y-ions and two neutral loss ions were required for positive identification. The occurrence of unique +80 or -98 Da mass shifts in the b- or y-ion series was used as criterion for phosphorylation site assignment.

SnRK2 activation assay

Arabidopsis thaliana seeds of genotypes Col-0, *snrk2.2;snrk2.3*, *wnk8-1* and *wnk8-4* were incubated on 0.5 x MS plates with 3% sucrose for about 5 weeks. Roots were then dissected and incubated for 24h in distilled water. Part of the root material was then further incubated with 10 µM ABA for 30 minutes. Roots were blotted dry between tissue paper and protein was extracted by homogenization in liquid nitrogen and addition of extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 0,5 % Triton X-100, 150 mM NaCl, 10 µM DTT, 10mM NaF, 2 mM AEBSF, 0.5 % (vol/vol) protease inhibitor (Sigma-Aldrich), 0.5 % (vol/vol) phosphatase inhibitor (Sigma-Aldrich), 5 mM Na_3VO_4). Soluble protein was run on a 10.5% SDS-PAGE containing 0.025 % Histone III-S. After electrophoresis, the gel was washed three times with washing buffer (25 mM Tris-HCl pH 8.0, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.05 % BSA, 0.1 % Triton X-100) for 30 min at room temperature, followed by two washes with renaturation buffer (25 mM Tris-HCl pH 8.0, 1 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF) at RT for 30 min and one wash at 4°C overnight. The next day the gel was washed with reaction buffer (25 mM HEPES pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$) for 30 min at room temperature and then incubated in 12.5 ml of reaction buffer with 50 µCi [γ-^{32}P]-ATP for 90 min at room temperature. The gel was washed four times with washing solution (5 % TCA, 1 % pyrophosphoric acid) for 30 min, and 2 times with washing solution for 15 min or until no radioactivity was detectable in the washing solution. The gel was then dried on Whatman 3MM paper and exposed to a storage phosphor screen (GE Life Sciences) for three days. Phosphorylation was visualized using a Typhoon imaging system (GE Life Sciences).

3 RESULTS

3.1 Expression and localization of AtWNK8

AtWNK8 promoter GUS activity

The expression pattern of *AtWNK8* (*At5g41990*) was first evaluated using the AtGenExpress Developmental data set indicating that the kinase is ubiquitously expressed in all tissues (SCHMID *et al.* 2005). Similar expression level could be detected in stems, leaves, flowers, apices and seeds. *AtWNK8* is slightly more abundant in roots than in aerial parts of the plant and particularly high expression could be detected in mature pollen (Fig. 1A).

To further examine the tissue specific expression of the *AtWNK8* gene the promoter (1878 bp upstream of the ATG) was fused to a ß-glucuronidase (GUS) reporter gene and stable transgenic *Arabidopsis* lines were generated (WNK8p-GUS). The AtWNK8p-GUS activity was studied in 5 d old seedlings showing a similar expression pattern as it had been observed in the AtGenExpress Developmental data set before (Fig. 1B). AtWNK8p-GUS activity was detected in shoots and roots, with clearly higher *AtWNK8* expression in the roots. In both organs expression of the *AtWNK8* is more pronounced in the vascular system than in the surrounding tissue. Furthermore, *AtWNK8* is expressed in the vascular system of leaves and in the stomata. In addition, AtWNK8p-GUS activity is present in flowers, with most of the GUS activity visible in the anthers' filaments and the stigma.

GFP-WNK8 localizes to the nucleus and the cytosol

To determine the subcellular localization of AtWNK8, translational fusions to the green fluorescent protein (GFP) driven by the promoter of the *Arabidopsis ubiquitin-10* (*UBQ10*) gene were generated. GFP-WNK8 was transiently expressed *in Nicotiana benthamiana* leaves or *Arabidopsis* protoplast. In both expression systems GFP-tagged WNK8 localized to the nucleus and the cytosol (Fig. 2A and B). However, several attempts to generate stable transgenic lines expressing GFP-WNK8 failed. Although kanamycin resistant individuals could be selected from the T1 population these seedlings did not show ubiquitous expression of GFP-WNK8 as expected. In fact, expression was restricted to single cells in the root tip, particularly within the central cylinder (Fig. 2C).

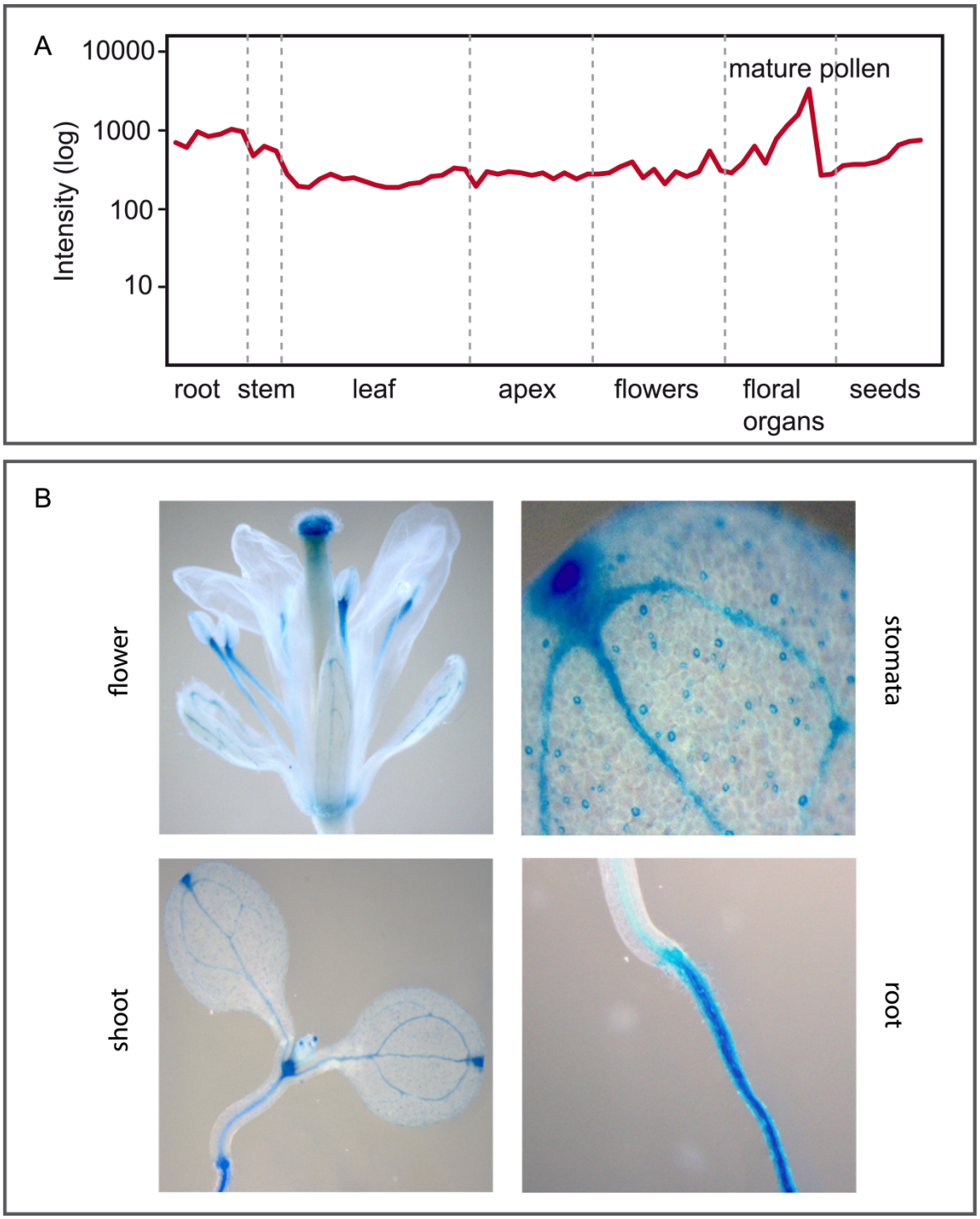

Figure 1. AtWNK8 is expressed throughout the plant

(A) The line graph shows raw intensity values with a logarithmic scale. Each tissue includes multiple values indicating different developmental time points. Expression data were obtained from the AtGenExpress Developmental series (SCHMID *et al.* 2005). **(B)** WNK8p-GUS activity depicted in flowers, leaves, stomata, shoots and roots of 5 d old *Arabidopsis* seedlings.

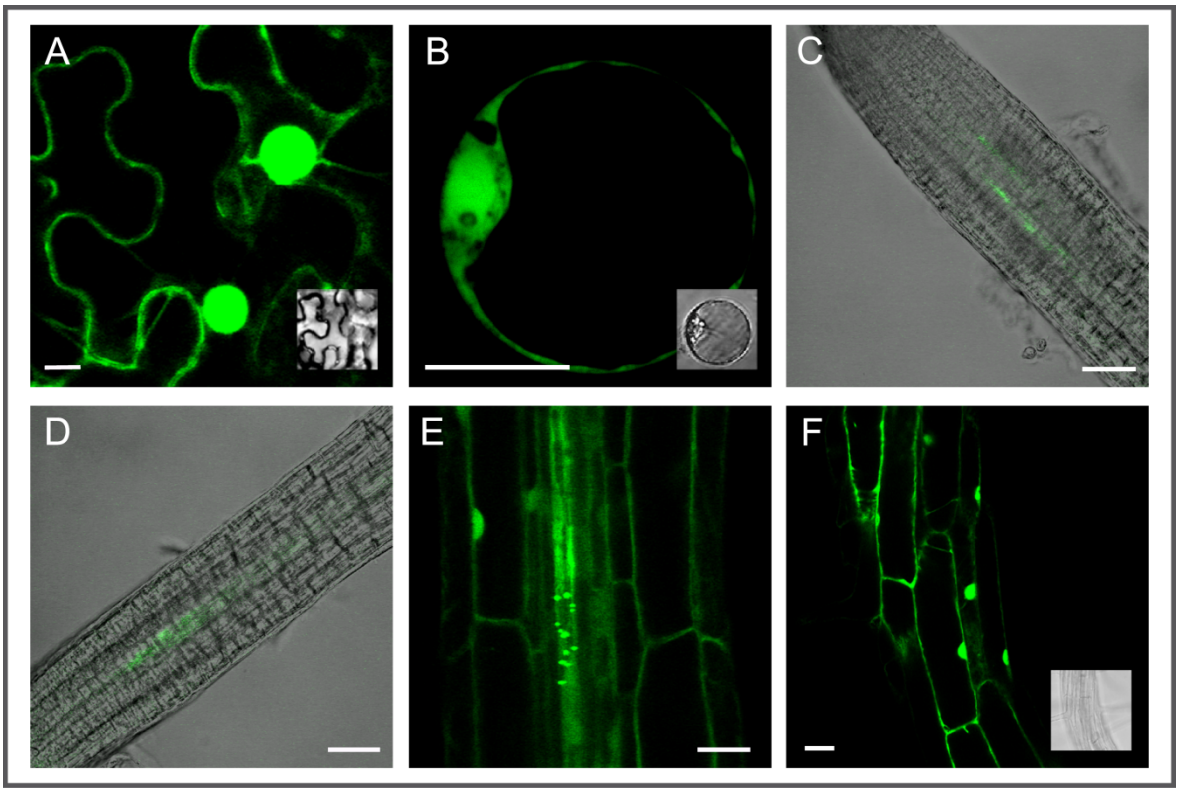

Figure 2. Transient and stable expression of GFP-WNK8
(A+B) Transient expression of UBQ10:GFP-WNK8 in tobacco leaves, 2 d after infiltration (bar = 10 µm) and *Arabidopsis* protoplasts, 36 h after transfection (bar = 20 µm). **(C-E)** T2 Arabidopsis plants expressing GFP-WNK8, GFP-WNK8$_{K41M}$ (D+E, bars = 50 µm) or WNK8KD$_{K41M}$-GFP (F, bar = 20 µm) under the control of the UBQ10 promoter. A mutation at position K41 renders the kinase. The WNK8 kinase domain (WNK8KD) includes the first 287 aa of the protein. **(F)** Heat shock-inducible expression of GFP-WNK8 in Col-0 root cells (bar = 20 µm). 5 d old seedlings were incubated at 37°C for 3 hours and analyzed 1 – 2 h after heat-shock.

In order to investigate whether expression of GFP-WNK8 was dependent on kinase activity, we introduced a single point mutation (K41M) to render AtWNK8 inactive (HONG-HERMESDORF *et al.* 2006). K41 corresponds to a conserved lysine residue which is responsible for binding ATP in the active center of the kinase. However, figure 2D shows that GFP-WNK8$_{K41M}$ expression was still limited to a few cells within the root tip.

Finally, stable transgenic lines could be established when either a truncated, inactive AtWNK8 fused to GFP was transformed or the GFP-WNK8 was driven by an inducible promoter. The WNK8KD$_{K41M}$-GFP construct contains the first 287 amino acids of WNK8 (including only the N-terminal kinase domain) carrying the K41 mutation fused to GFP.

Analysis of 5 d old T2 seedlings showed strong expression in all cells and localization to the nucleus and the cytosol as observed previously (Fig. 2E).

In addition, stable transgenic lines expressing GFP-WNK8 under the control of a heat shock promoter were generated. Expression of GFP-WNK8 was analyzed after incubation at 37 °C for 3 hours. Again, GFP-tagged AtWNK8 was present in the nucleus and the cytosol of *Arabidopsis root* cells but decreased rapidly several hours after induction (Fig. 2F).

3.2 Characterization of *wnk8* T-DNA insertion alleles

Isolation of T-DNA insertion lines for *AtWNK8*

To investigate the *in vivo* function of *AtWNK8* two T-DNA insertion lines were analyzed: *wnk8-1* (SALK_024887) and *wnk8-4* (SALK_103318). The T-DNA insertion in *wnk8-1* is located in exon VI (Fig. 3). Using a T-DNA specific primer the insertion site could be determined 1662 bp downstream of the start codon. The second T-DNA insertion line, *wnk8-4*, harbors the insertion in exon III, 767 bp downstream of the ATG (Fig. 3). For both SALK lines plants homozygous for the T-DNA insertion were recovered by PCR genotyping using gene-specific primer combinations.

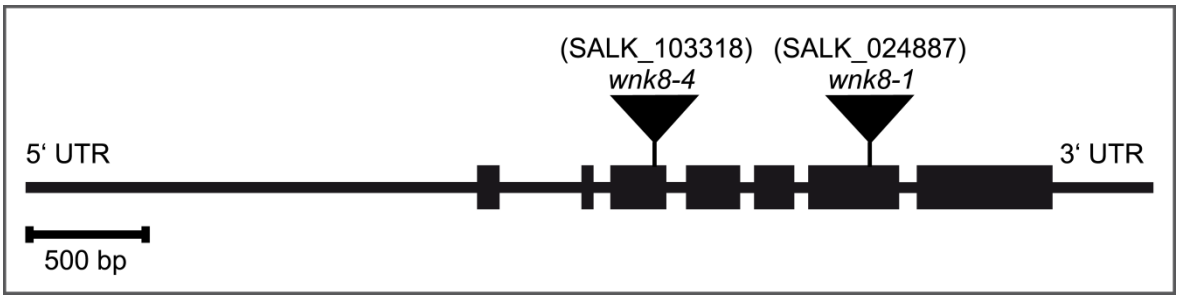

Figure 3. Identification of T-DNA insertion lines for *AtWNK8*
Genomic structure of *WNK8* indicating the position of the T-DNA (black triangle) for *wnk8-1* and *wnk8-4*. Exons are depicted as black boxes, introns as black bars. The 5’- and the 3’-UTR are labeled. In *wnk8-1* the T-DNA is located in exon VI, in *wnk8-4* the T-DNA inserts in exon III.

ABA-induced postgermination growth arrest is altered in *wnk8-1* seedlings

Under long day conditions soil-grown *wnk8-1* and *wnk8-4* homozygous mutants were indistinguishable from Col-0 wild type plants (supplementary material Fig. S1). For a more detailed phenotypic analysis both mutants were grown under various experimental conditions including growth in the presence of the plant hormone ABA. We monitored the development of young *Arabidopsis* seedlings on MS plates supplemented with 1 µM ABA. To investigate whether germination was altered in the mutants, emergence of the root tip from the seed coat was monitored in 2 d old seedlings. ABA-induced inhibition of germination was indistinguishable in wild type and the two mutants (Fig. 4A). To observe any changes during postgermination development seedling were grown in the presence of 1 µM ABA and analyzed 5 DAG. Two parameters were measured: formation of green, unfolded cotyledons (Fig. 4B and C) and length of the primary root (Fig. 4D). In the absence of exogenous ABA (control) there was no difference between the wild type and the two mutants. In the presence of the hormone the typical ABA-induced postgermination growth arrest is known to be detectable in wild type seedlings a few days after germination (LOPEZ-MOLINA *et al.* 2001). Indeed, formation and development of green cotyledons was inhibited in 5 d old Col-0 seedlings (Fig. 4B and C). In contrast, *wnk8-1* mutants were less sensitive to ABA than the wild type and postgermination growth arrest was reduced (Fig. 4B and C). The analysis of primary root elongation provided further evidence that *wnk8-1* mutants are ABA-hyposensitive during early seedling development. When grown in the presence of 1 µM ABA the majority of 7 d old *wnk8-1* seedlings formed a longer primary root than the wild type (Fig. 4D). Interestingly, *wnk8-4* seedlings did not show this ABA-dependent phenotype. They showed wild type-like development of the primary root and no change in ABA-induced growth arrest (Fig. 4B and D, respectively).

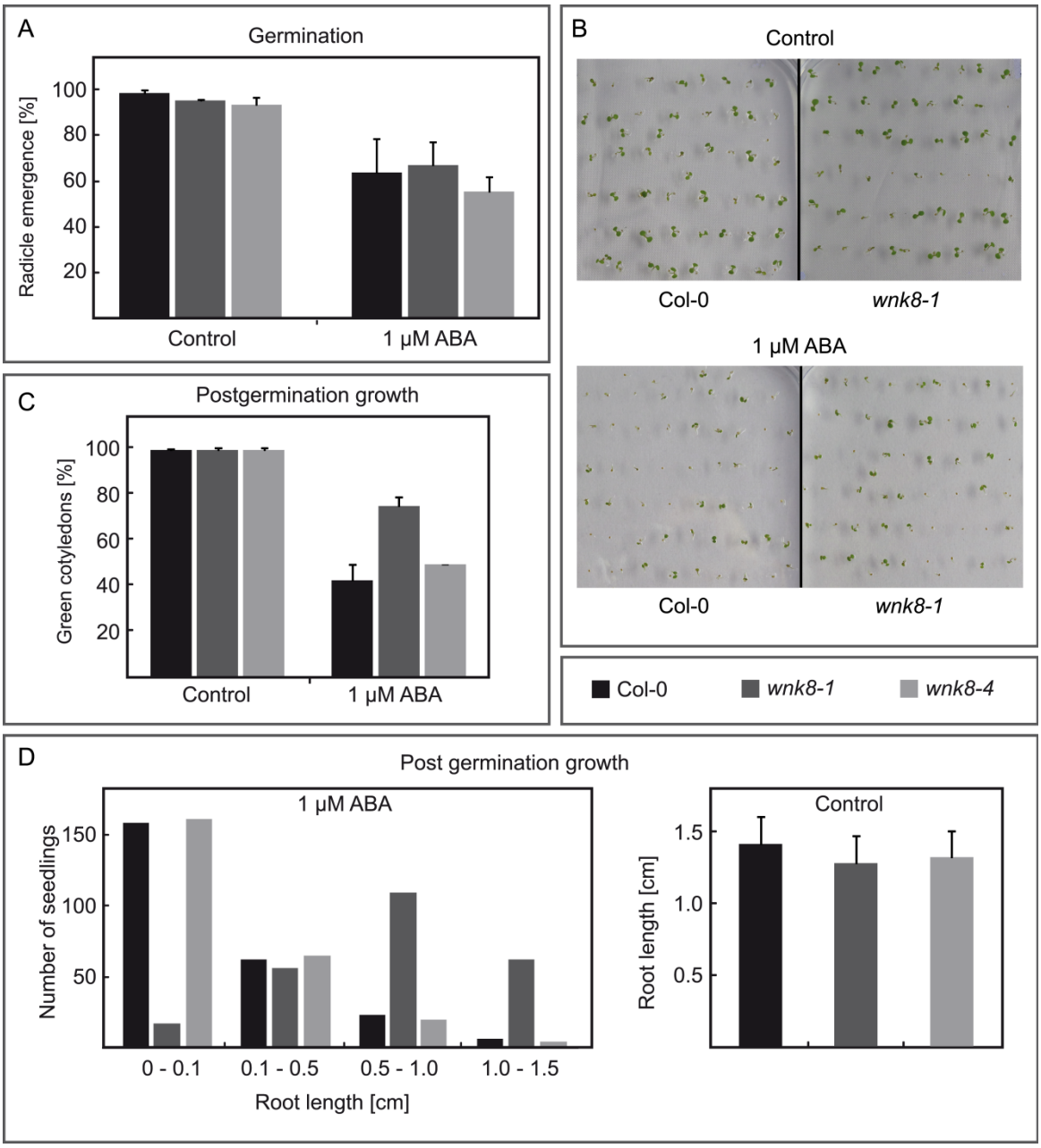

Figure 4. Phenotypic analysis of *wnk8* mutants

(A) Germination efficiency. Radicle emergence from the seed coat of 2 d old seeds in the absence (control) or the presence of 1 µM ABA. Error bars represent the SE of 3 independent experiments with n = 45 – 50 seeds. **(B)** Postgermination growth of Col-0 and *wnk8-1* seedlings in the absence (control) or presence of 1 µM ABA. Plants were grown under long day conditions (16 h light, 8 h darkness). Pictures were taken 4 DAG. **(C+D)** Postgermination efficiency. **(C)** To monitor postgermination development in the presence of 1 µM ABA, unfolded, green cotyledons of 5 d old Col-0 wild type and *wnk8* mutant seedlings were counted. Error bars represent the SE of 3 independent experiments with n = 45-50 seedlings. **(D)** Histogram showing primary root length of 7 d old Col-0 wild type and *wnk8* mutant seedlings in the presence of 1 µM ABA. Distribution of root length is shown for 250 roots measured for each genotype in 3 independent experiments. Average root length are shown for control conditions (no exogenous ABA), error bars represent the SE of 3 independent experiments with n = 35 seedlings.

Dominance of *wnk8-1* results from a constitutively active kinase

Phenotypic analysis of the *wnk8* mutants revealed an ABA hyposensitive phenotype for *wnk8-1* but not for the *wnk8-4* mutant. Both alleles carry the T-DNA within an exon of the *AtWNK8* gene and thus, likely are knockout mutants supposedly showing similar phenotypes. The apparently different phenotypes could on the one hand be explained by a second T-DNA insertion tightly linked to *WNK8*. On the other hand it is possible that truncated mRNAs upstream of the T-DNA are still produced, leading to partially functional polypeptides. In these cases a T-DNA insertion can lead to dominantly inherited phenotypes.

To investigate dominance of the *wnk8-1* allele it was introduced into the Col-0 wild type background. If the *wnk8-1* allele was dominant a single copy should cause ABA hyposensitivity similar to the phenotype of *wnk8-1* homozygous mutants. The F1 heterozygous progeny resulting from this cross was analyzed for their ability to perform postgermination growth arrest in the presence of ABA (Fig. 5A). As a quantifiable parameter the formation of green cotyledons was monitored in 4 d old Col-0, *wnk8-1* and heterozygous *wnk8-1*/+ seedlings**.** In the absence of ABA both mutants grew indistinguishable from wild type. ABA-induced growth arrest was visible for Col-0 but strongly decreased in *wnk8-1* mutants as expected from previous results. The heterozygous *wnk8-1*/+ seedlings showed an intermediate phenotype indicating that one copy of the mutant allele is sufficient to prevent the wild type phenotyp.

In order to explain the dominant effect of the *wnk8-1* allele we investigated the possibility of a truncated transcript still present in the *wnk8-1* mutant. RT-PCR using a gene-specific primer pair binding to a region in the 5'-end encoded kinase domain (→KD←) confirmed the presence of such a transcript in *wnk8-1* (Fig. 5B). The same transcript was not detected in the *wnk8-4* mutant. Sequence analysis further suggested that the open reading frame (ORF) of the *wnk8-1* transcript includes the first 966 base pairs (bp) of *AtWNK8* followed by a short stretch of T-DNA specific sequence. This transcript is likely to be translated into a truncated AtWNK8 protein of 327 aa including the kinase domain but lacking the autoinhibitory and the coiled-coil domain in the C-terminus (Fig. 6A). When a primer pair that binds to the 3'-end was used (→CT←) no transcript could be detected, indicating that the *wnk8-1* mutant lacks a full length transcript. The transcript detected in

the *wnk8-4* mutant likely initiates from a start codon on the T-DNA. However, the position of the T-DNA in exon III prevents full length transcription of *AtWNK8*.

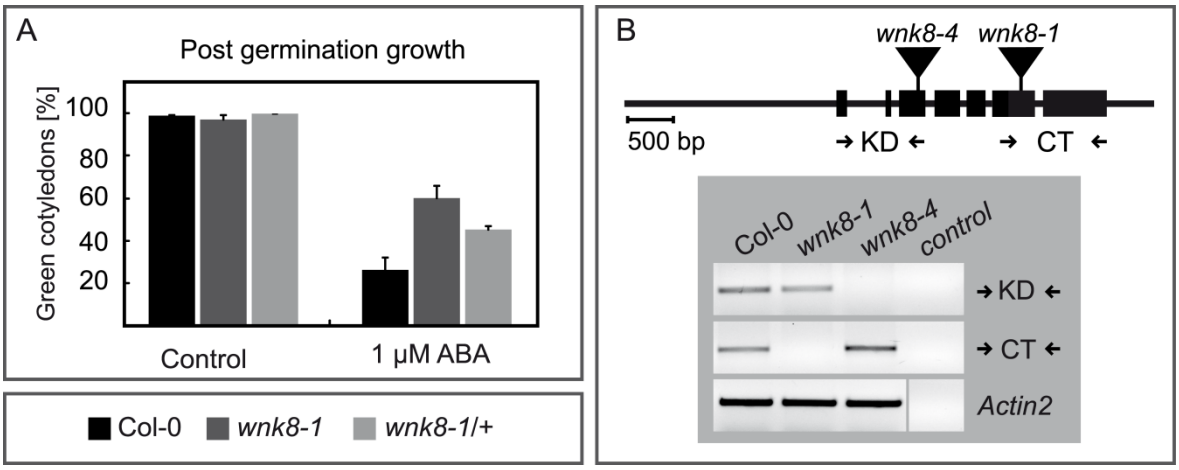

Figure 5. The *wnk8-1* allele is dominant

(A) Postgermination efficiency of F1 *wnk8-1*/+ seedlings. Formation of green cotyledons was analyzed in 4 d old Col-0, *wnk8-1* and *wnk8-1*/+ seedlings in the absence (control) or in the presence of 1 µM ABA to monitor postgermination growth. Error bars represent the SE of 4 independent experiments with n = 50 seedlings. (**B)** RT-PCR, using gene specific primers, to confirm the presence of a truncated transcript in *wnk8-1*. cDNA of Col-0, *wnk8-1* and *wnk8-4* seedlings was used as a template. To test for the kinase domain transcript a primer pair at the 5'-end of *AtWNK8* was used (→KD←). To test for the presence of the full length transcript a primer combination at the 3'-end of *AtWNK8* was used (→CT←). *Actin2* served as a cDNA loading control.

Expression of a constitutively active AtWNK8 kinase causes ABA-hyposensitivity

As described in the previous chapter *wnk8-1* was identified to be a dominant, gain-of-function allele. Still, we had to verify whether ABA-insensitivity was in fact caused by the constitutively active kinase or a second mutation inherited tightly linked to *AtWNK8* in a similar dominant fashion. Since dominance prevents complementation through the wild-type allele, we tried to recapitulate the mutant phenotype and thereby show *WNK8*-dependence. Recapitulation lines were generated in the Col-0 ecotype background.

From *WNK8* cDNA we amplified the first 966 bp adding the short T-DNA specific sequence and a Stop codon via an appropriate primer.

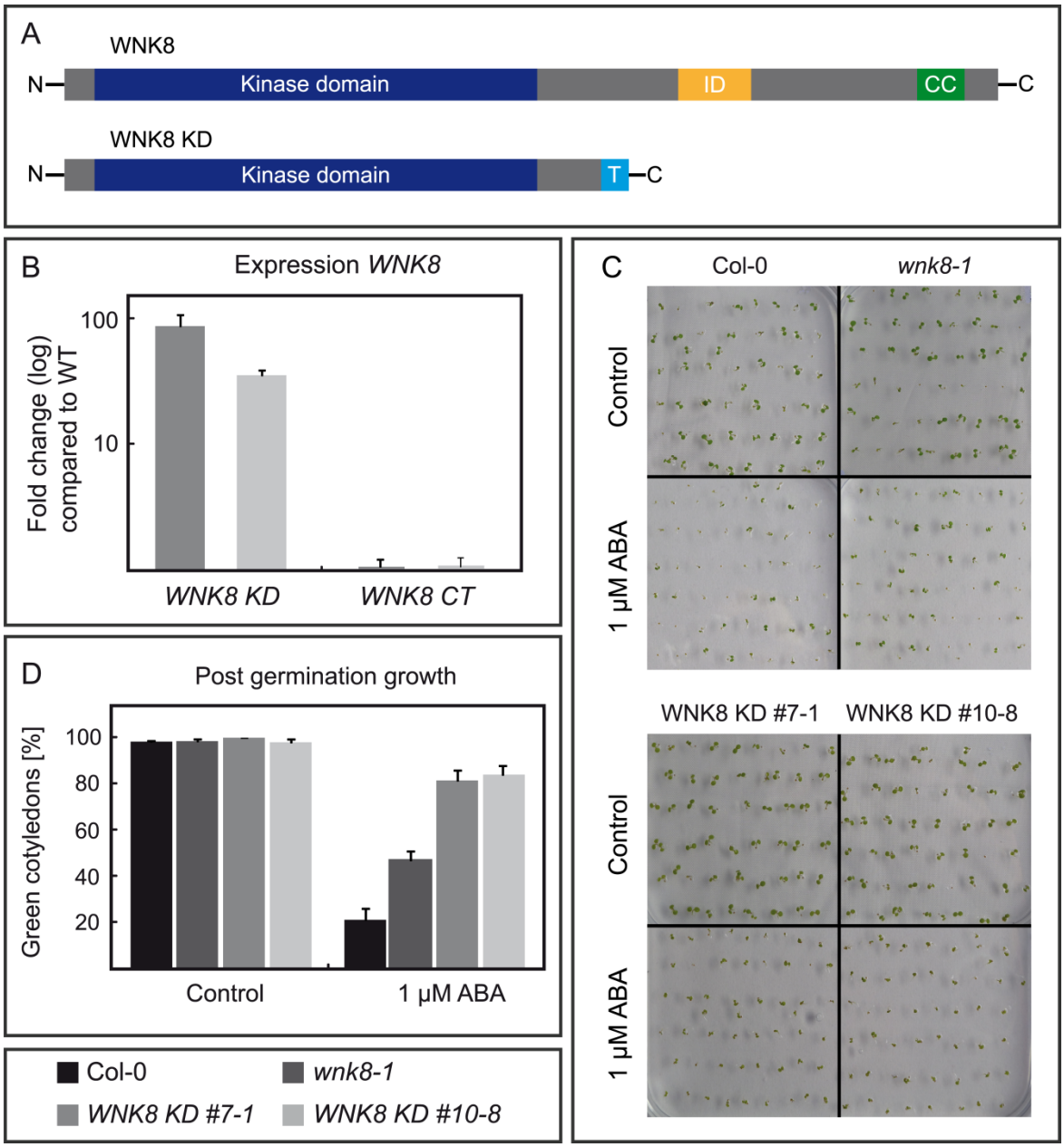

Figure 6. The *wnk8-1* ABA-insensitive phenotype is *WNK8* dependent

(A) To recapitulate the *wnk8-1* phenotype, transgenic plants constitutively expressing a truncated WNK8, missing the C-terminally localized autoinhibitory domain and the coiled-coil domain, were generated. The Col-0 wild type background was used. **(B)** qRT-PCR using gene specific primer was performed to detect transcript levels of the *WNK8* kinase domain. cDNAs of Col-0 and two independent transgenic lines (#7-1 and #10-8) were used in the PCR reaction. Results are presented as fold change compared to wild type. Error bars represent the SD of 3 technical replicates. **(C)** Postgermination growth of Col-0, *wnk8-1* and transgenic seedlings in the absence (control) or presence of 1 µM ABA. Plants were grown under long day conditions (16 h light, 8 h darkness). Pictures were taken 4 DAG. **(D)** Formation of green, unfolded cotyledons in 4 d old seedlings was monitored to study postgermination development. Plants were grown in the absence (control) or in the presence of 1 µM ABA. Error bars represent the SE of 3 independent experiments with n = 45 – 50 seedlings.

The complete cDNA corresponding to the identified ORF in the *wnk8-1* mutant was cloned under the control of the UBQ10 promoter (Fig. 6A). Two independent lines (#7-1 and #10-8) were subjected to RT-PCR analysis to verify the overexpression of the AtWNK8 kinase domain. The results showed that At*WNK8* kinase domain transcript levels (*WNK8 KD*) are significantly up regulated as compared to wild type (Fig. 6B). As a control the expression level of the *AtWNK8* 3'-end (*WNK8 CT*) was also monitored in the same experiment and, as expected, was not different to wild type expression. Postgermination development was analyzed to investigate the effect of WNK8 kinase domain overexpression on ABA-induced growth arrest. In comparison with Col-0 wild type and the *wnk8-1* mutant two independent transgenic lines were grown on 1 x MS plates containing 1 µM ABA (Fig. 6C and D). In the absence of exogenous ABA all mutants grew equally to wild type seedlings. As expected postgermination growth arrest of *wnk8-1* seedling was clearly reduced. However, 4 d old transgenic lines overexpressing the AtWNK8 kinase domain were even more insensitive to ABA than *wnk8-1* mutants. Quantifying the formation of cotyledons showed that significantly more seedlings produced green, unfolded cotyledons indicating that postgermination development was hardly influenced by ABA in these lines.

3.3 AtWNK8 and AtPP2CA interaction

Genetic interaction: The *wnk8* alleles modify the *pp2ca-1* phenotype

Phenotypes caused by dominant alleles require careful interpretation, since they might not represent the normal gene function. To classify the *wnk8-1* gain-of-function mutation as hypermorph (increase in normal gene function), antimorph (opposite to normal gene activity) or neomorph (different from normal gene function) further investigation of the *wnk8-4* allele was necessary (MULLER 1932).

Since *wnk8-4* did not show any phenotype we decided to generate double mutants using other available ABA response mutants. Amongst others we used the T-DNA insertion mutant *pp2ca-1* (SALK_028132) which lacks full-length *AtPP2CA* gene expression and is identical to *ahg3* (KUHN *et al.* 2006, YOSHIDA *et al.* 2006). Seedlings of both mutants show a severe ABA-hypersensitive phenotype. Compared to loss-of-function mutations in structurally related *PP2C* genes (*ABI1*, *ABI2*), the *ahg3/pp2ca* mutant shows the strongest

ABA-hypersensitive phenotype during early seedling development. Homozygous double mutants were isolated from the F_2 progeny of the single mutants, *pp2ca-1* and *wnk8-1* or *wnk8-4*.

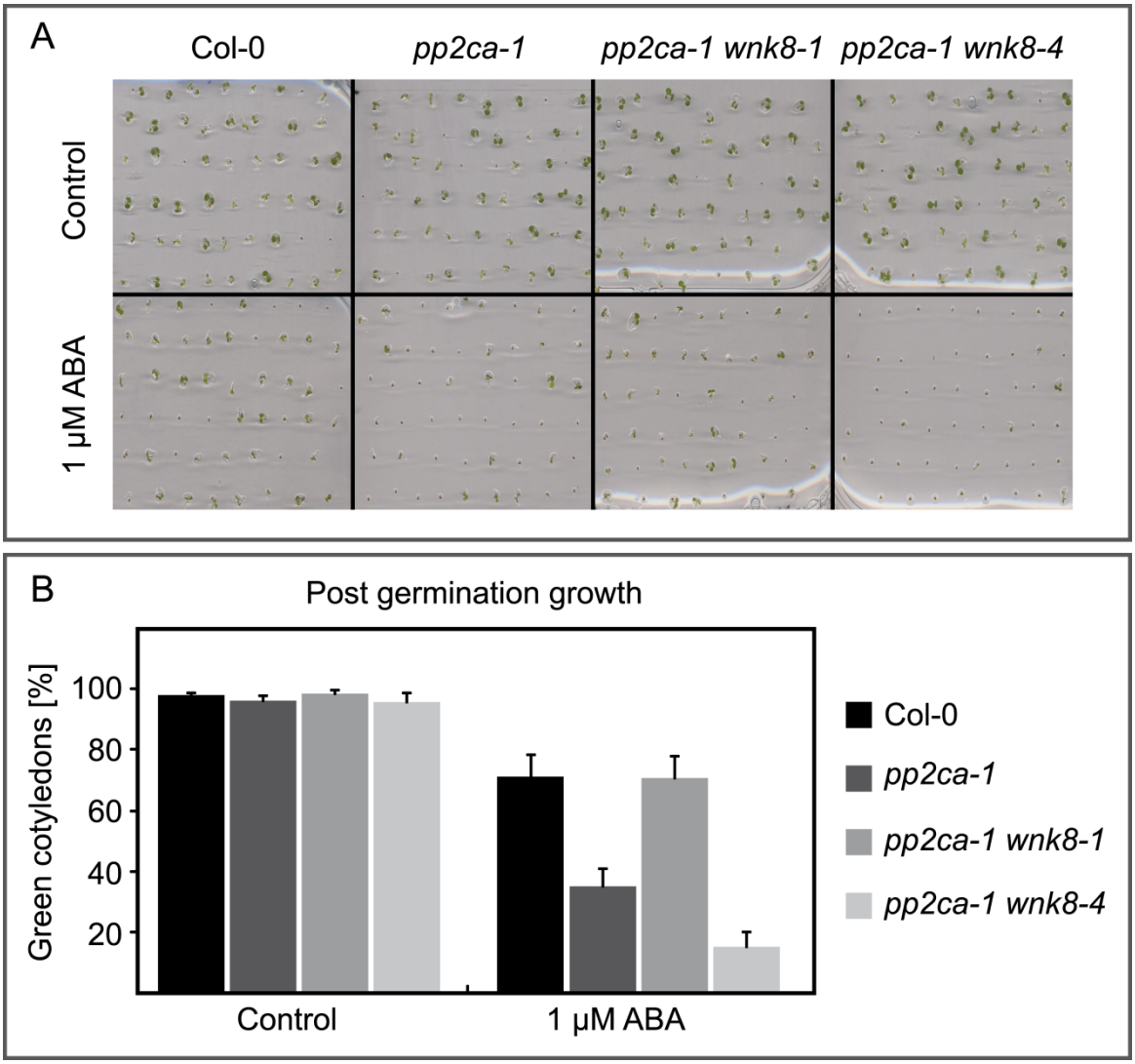

Figure 7. Modification of the *pp2ca-1* ABA-hypersensitive phenotype by *wnk8* alleles

(A) Postgermination growth of Col-0, *pp2ca-1*, *pp2ca-1 wnk8-1* and *pp2ca-1 wnk8-4* in the absence (control) or presence of 0.75 µM ABA. Plants were grown under long day conditions (16 h light, 8 h darkness). Pictures were taken 4 DAG.

(B) Postgermination efficiency. To investigate postgermination development in *pp2ca-1 wnk8* double mutants, formation of green cotyledons was monitored in 4 d old seedlings of Col-0, *pp2ca-1*, *pp2ca-1 wnk8-1* and *pp2ca-1 wnk8-4* in the presence of 0.75 µM ABA. No exogenous ABA was applied to plates under control conditions. Error bars represent the SE of 3 independent experiments with n = 45 – 50 seedlings.

Postgermination growth of *pp2ca-1 wnk8-1* and *pp2ca-1 wnk8-4* double mutants was analyzed in the presence of ABA (Fig. 7). All genotypes developed equally in the absence of exogenous ABA (control conditions). In the presence of ABA *pp2ca-1* mutant seedlings showed the expected ABA-hypersensitive phenotype which was modified by the *wnk8* alleles in the double mutants (Fig. 7A). Once more the formation of green cotyledons was used as a quantifiable parameter to measure postgermination growth (Fig. 7B). ABA-hypersensitivity of the *pp2ca-1* mutant was no longer detectable in the double mutant with *wnk8-1* and growth arrest was restored to wild type levels. In contrast, the ABA hypersensitive response in the *pp2ca-1 wnk8-4* double mutant was strongly increased. The observation of different phenotypes for the two *wnk8* mutants allowed us to identify *wnk8-4* as the recessive, loss-of-function allele opposite *wnk8-1*.

Physical interaction: AtWNK8 kinase domain and AtPP2CA interact in yeast

Genetic interaction could be observed between *AtWNK8* and *AtPP2CA* in *pp2ca-1 wnk8* double mutants as shown in the previous chapter. To investigate physical interaction between the kinase and the phosphatase first interaction studies were performed in yeast using the yeast-two-hybrid (Y2H) approach. The GAL4 DNA binding domain was fused to AtPP2CA to act as bait. AtWNK8, AtWNK8KD and AtWNK8KD$_{K41M}$, a kinase dead version of AtWNK8, were cloned into the activation domain vector and used as prey. Bait and prey constructs were transformed into the yeast strain pJ69-4A, auxotrophic for leucine and tryptophan allowing the selection of successfully transformed cells on SD medium –LW, lacking leucine and tryptophan. Upon interaction the reconstituted GAL4 transcription factor initiates transcription of the reporter genes histidine and adenine which is visualized by yeast growth on SD medium –LWAH, deficient in leucine, tryptophan, adenine and histidine.

Results of the Y2H assay showed that the AtWNK8 kinase domain (WNK8KD) is able to interact with AtPP2CA (Fig. 8). Transforming the AtPP2CA bait with the empty activation domain vector (pGADT) clearly showed that yeast growth was not simply due to auto-activation of the reporter through the DNA-binding domain. However, interaction in yeast depended on the activity of the kinase, because AtWNK8KD$_{K41M}$ (compare section 3.1) was not able to interact with the phosphatase. Also the full-length AtWNK8 did not interact with AtPP2CA in yeast. Specificity of the AtWNK8KD/AtPP2CA interaction was

verified using two other phosphatases of the same family, AtABI1 and AtABI2. AtABI1 and AtABI2 were cloned into the DNA-binding domain vector and used as bait together with the AtWNK8KD prey. Successfully transformed cells carrying both constructs were not able to grow on SD -LWAH medium indicating that there was no interaction between AtABI1 and AtABI2 with AtWNK8KD in yeast.

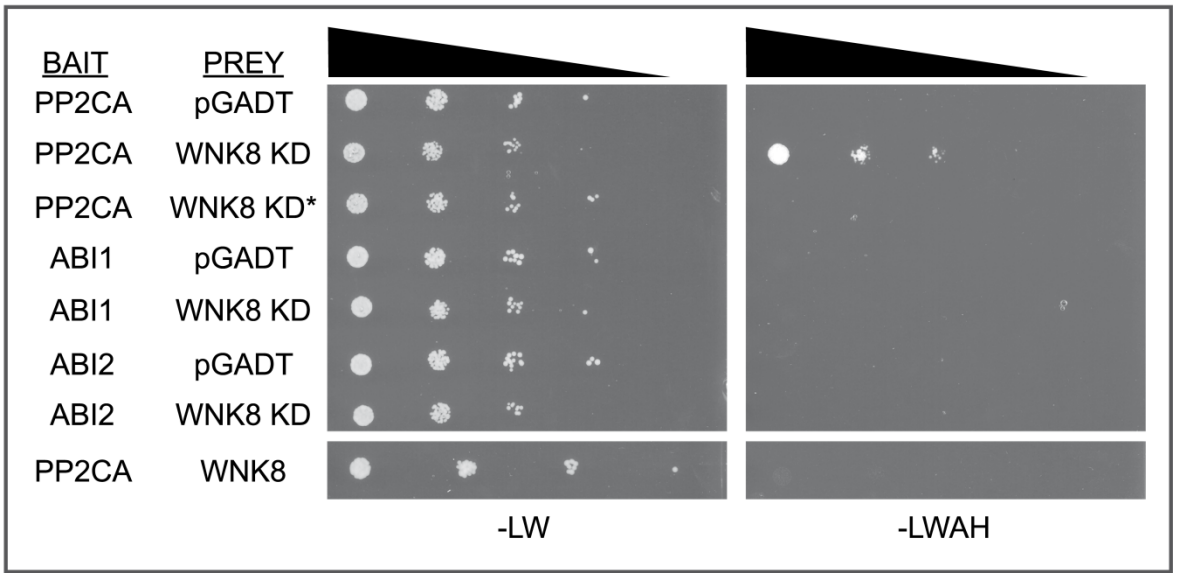

Figure 8. AtWNK8 kinase domain (WNK8 KD) and AtPP2CA interact in yeast
Yeast cells transformed with the indicated bait and prey constructs were grown on SD medium lacking leucine and tryptophan (-LW) to monitor successful uptake of both plasmids. Growth on SD medium (-LWAH) shows protein interaction. Co-transformation of the bait construct and the empty prey vector was used as a negative control. Cells were spotted in a serial of 1:10 dilutions as indicated by the black triangle. Plates were incubated for 2 d at 28°C.

Physical interaction: AtWNK8 and AtPP2CA interact in the nucleus of plant cells

Y2H analysis had shown that AtWNK8KD and AtPP2CA do physically interact in yeast. To learn more about the interaction in plants co-localization studies of the kinase and the phosphatase were performed. GFP and RFP translational fusions were generated with cDNA of *AtWNK8* and *AtPP2CA*, respectively, driven by the constitutive UBQ10 promoter. Both constructs were transiently co-expressed in *Nicotiana benthamiana* leaves. Two to four days after infiltration tobacco leaves were analyzed via confocal microscopy. As described previously, GFP-WNK8 expression was observed in the nucleus and the cytosol. In contrast, expression of RFP-PP2CA was restricted to the nucleus where it co-localized with the GFP-WNK8 signal (Fig. 9A).

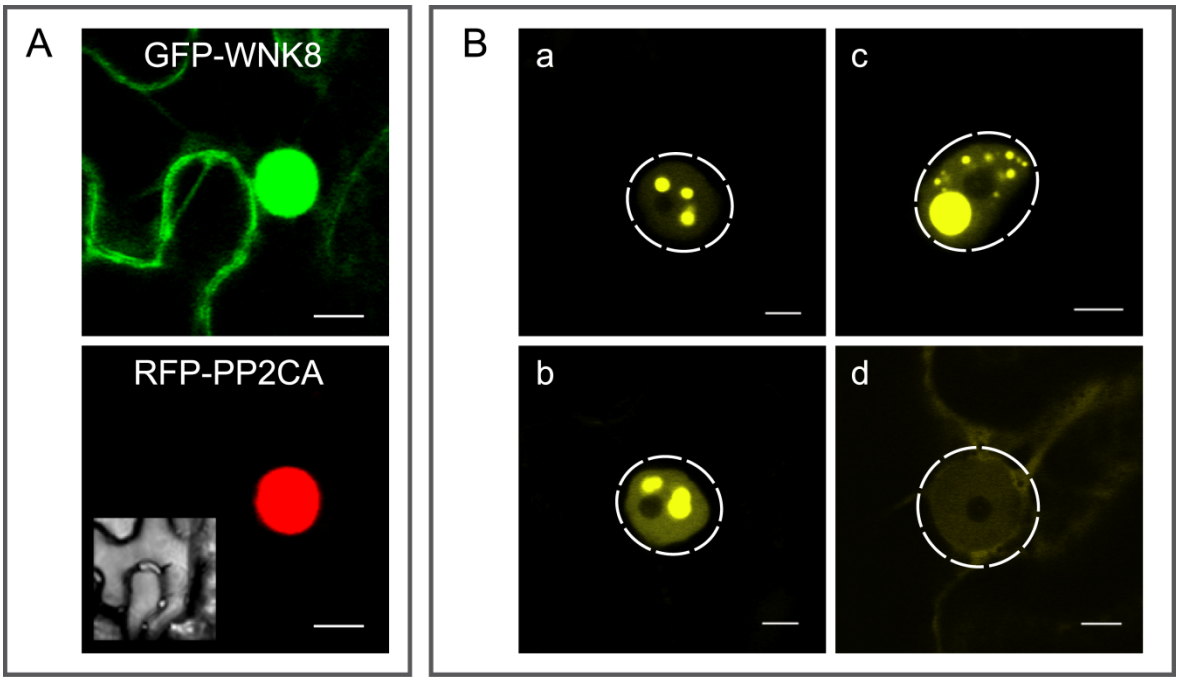

Figure 9. AtWNK8 and AtPP2CA interact inside the nucleus

(A) GFP-WNK8 and RFP-PP2CA were transiently co-expressed in *Nicotiana benthamiana* leaves via infiltration with *Agrobacterium tumefaciens*. Pictures showing a magnification of the nucleus were taken 2 d after infiltration. Scale bars = 10 µm

(B) Bimolecular fluorescent complementation assay showing the interaction of WNK8 and PP2CA in the nuclei of tobacco epidermal cells. Cells were infiltrated with a mixture of *Agrobacterium* suspension harboring the following constructs: (a) YFP^N-WNK8/PP2CA-YFP^C, (b) YFP^N-WNK8KD/PP2CA-YFP^C or (c) YFP^N-$WNK8KD_{K41M}$/PP2CA-YFP^C. YFP background fluorescence that could be detected when YFP^N-WNK8KD was combined with the non-interacting partner mCherry-YFP^C is depicted in (d). The dotted lines mark the nucleus. Pictures were taken 2 – 3 d after infiltration. Scale bars = 5 µm

Bimolecular fluorescence complementation (BIFC) was used to further study AtWNK8 and AtPP2CA interaction *in planta*. Co-expression of the C-terminal half of YFP fused to AtPP2CA (PP2CA-YFP^C) and the N-terminal half of YFP fused to AtWNK8, AtWNK8KD or $AtWNK8KD_{K41M}$ (YFP^N-WNK8, YFP^N-WNK8KD or YFP^N-$WNK8KD_{K41M}$) resulted in fluorescence exclusively in the nuclei of *N. benthamiana* leaves (Fig. 9B). This was in agreement with the subcellular localization of AtWNK8 and AtPP2CA described before. These results indicated that *in planta* interaction of the kinase and the phosphatase was independent of the length or the activity of the AtWNK8 protein. Furthermore, the results

showed that upon interaction the two proteins accumulate and form well-defined structures within the nucleus.

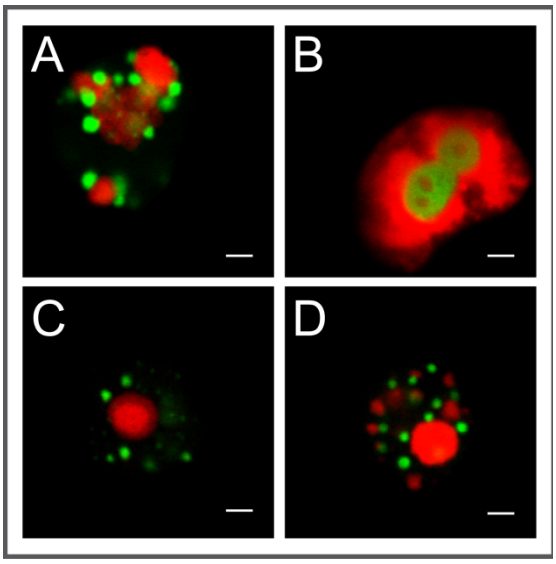

Figure 10. AtWNK8 / AtPP2CA BIFC complexes do not label specific nuclear compartments

The BIFC pair YFP^N-WNK8/PP2CA-YFP^C was transiently co-expressed with Coilin-RFP (A), Cyc64-RFP (B), FIB1-RFP (C) or RNPS1-RFP (D) in *Nicotiana benthamiana* leaves. To better visualize areas of co-localization the YFP signal is depicted in green colour. Pictures showing magnifications of the epidermal nuclei were taken 2 – 3 d after infiltration with *Agrobacterium tumefaciens* carrying the appropriate constructs. Scale bars = 2 µm

Compartmentalization of the nucleus is now accepted as an important feature for the organization of nuclear processes, for example the modification of RNAs (LORKOVICandBARTA 2004). To identify whether the structures, observed during BIFC experiments, label specific nuclear compartments, such as cajal bodies or speckles, different RFP-tagged markers were co-expressed with the BIFC pairs in *N. benthamiana* leaves. Plasmids of all marker proteins were kindly provided by the lab of Peter Shaw, John Innes Centre, Norwich, UK. RNPS1-RFP, also called SR45 (ARGININE/SERINE-RICH 45), and Cyc64-RFP (CypRS64) were used to identify splicing speckles. Coilin-RFP marks Cajal bodies and FIB1-RFP (FIBRILLARIN 1) labels the nucleolus (ALIandREDDY 2006, KOROLEVA *et al.* 2009). However, co-localization with any of the four markers with the YFP^N-WNK8/PP2CA-YFP^C complex was not observed (Fig.10).

<u>AtWNK8 is regulated by AtPP2CA and phosphorylates AtPYR1 *in vitro*</u>

Having shown that AtWNK8 and AtPP2CA physically interact and knowing that AtWNK8 is an active protein kinase (HONG-HERMESDORF *et al.* 2006) it remained to be determined if AtWNK8 is able to phosphorylate AtPP2CA or if interaction leads to dephosphorylation of the kinase. In order to investigate these two possibilities *in vitro* phosphorylation assays were performed. Recombinant full-length AtWNK8 and AtPP2CA were strepII-tag-purified

after *in vitro* translation and used in the assay. The hypothesis that AtWNK8 phosphorylates AtPP2CA was not confirmed. In turn, the results showed that AtPP2CA was able dephosphorylate AtWNK8, which strongly autophosphorylated in the absence of the phosphatase (Fig. 11A and B). AtWNK8 kinase activity could be restored if, in addition to AtPP2CA, an ABA-receptor protein (AtPYR1 or AtPYL8) and ABA were present (Fig. 11A). In the absence of the plant hormone AtWNK8 activity was not recovered efficiently. Furthermore, when in complex with the phosphatase and the receptor, AtWNK8 specifically phosphorylated the AtPYR1 but not the AtPYL8 receptor protein (Fig. 11A). Intensity of receptor phosphorylation correlated with AtWNK8 kinase activity. Controls confirmed that ABA alone was not sufficient to interfere with AtPP2CA activity or modify AtWNK8 autophosphorylation.

To investigate whether phosphorylation of AtPYR1 by AtWNK8 is also possible without the complex formation with AtPP2CA a second set of *in vitro* phosphorylation assays were performed. The results showed strong phosphorylation of the AtPYR1 protein by AtWNK8 in an ABA-independent manner (Fig. 11B). However, since this experiment allowed the direct comparison of the receptor phosphorylation status in the presence and the absence of AtPP2CA, there was evidence that the phosphatase was partially blocking modification of the receptor.

In order to identify phosphorylated residues in AtWNK8 cold *in vitro* phosphorylation assays were carried out using recombinant AtWNK8 and AtPP2CA proteins. Proteins were incubated with ATP prior to separation by SDS-PAGE. Following coomassie staining, bands corresponding to AtWNK8 in the absence and the presence of the phosphatase were cut from the gel. Tryptic digestion followed by mass spectrometry (MS) allowed the identification of 11 phosphorylated serine and threonine residues upon autophosphorylation of AtWNK8 (S3, S5, S77, T181, T187, S245, S251, S281, S336, S363 and S475). Furthermore, 6 putative, ambiguous residues were detected but could not be confirmed, yet (Fig. 12A). If AtPP2CA was present in the reaction the only phosphorylated residue left on AtWNK8 was S363 (Fig. 12B). Table S1 (supplementary material) contains detailed information on all the peptides identified during MS analysis.

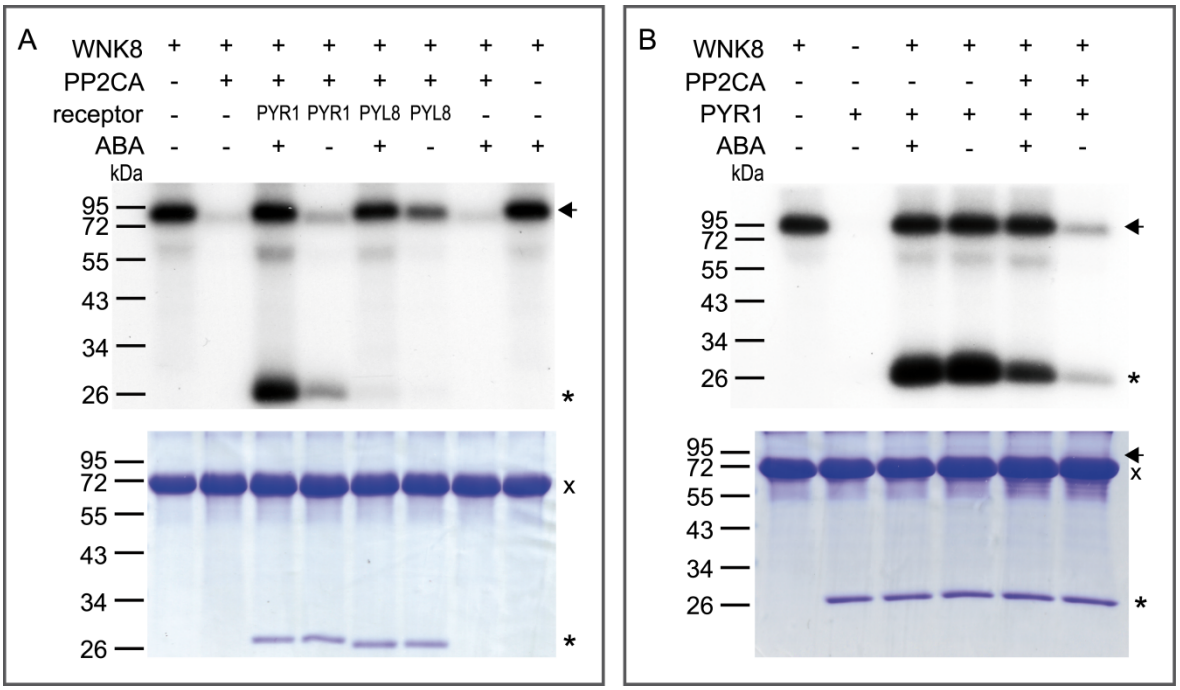

Figure 11. Control of AtWNK8 kinase activity by AtPP2CA and phosphorylation of AtPYR1

(A) Autoradiography (top) showing autophosphorylation status of AtWNK8 (arrowhead) dependent on AtPP2CA, the ABA receptor proteins AtPYR1 or AtPYL8 and ABA. Transphosphorylation of AtPYR1 is indicated by the asterix. Gel was exposed to x-ray film for 10 minutes. Coomassie staining (bottom) showing protein amounts loaded. The asterisk indicates PYR1 and PYL8 protein. Concentration of AtWNK8 and AtPP2CA protein was too low to be visible. BSA, added as a carrier protein, is detectable at approximately 72 kDa (x).

(B) Autoradiography (top) showing autophosphorylation of AtWNK8 (arrowhead) and transphosphorylation of the ABA receptor AtPYR1 (asterisk). Gel was exposed to x-ray film for 5 minutes. Coomassie staining (bottom) showing proteins amounts loaded. Arrowhead and asterix mark AtWNK8 and AtPYR1 protein, respectively. Concentration of AtPP2CA protein was too low to be visible. BSA, added as a carrier protein, is detectable at approximately 72 kDa.

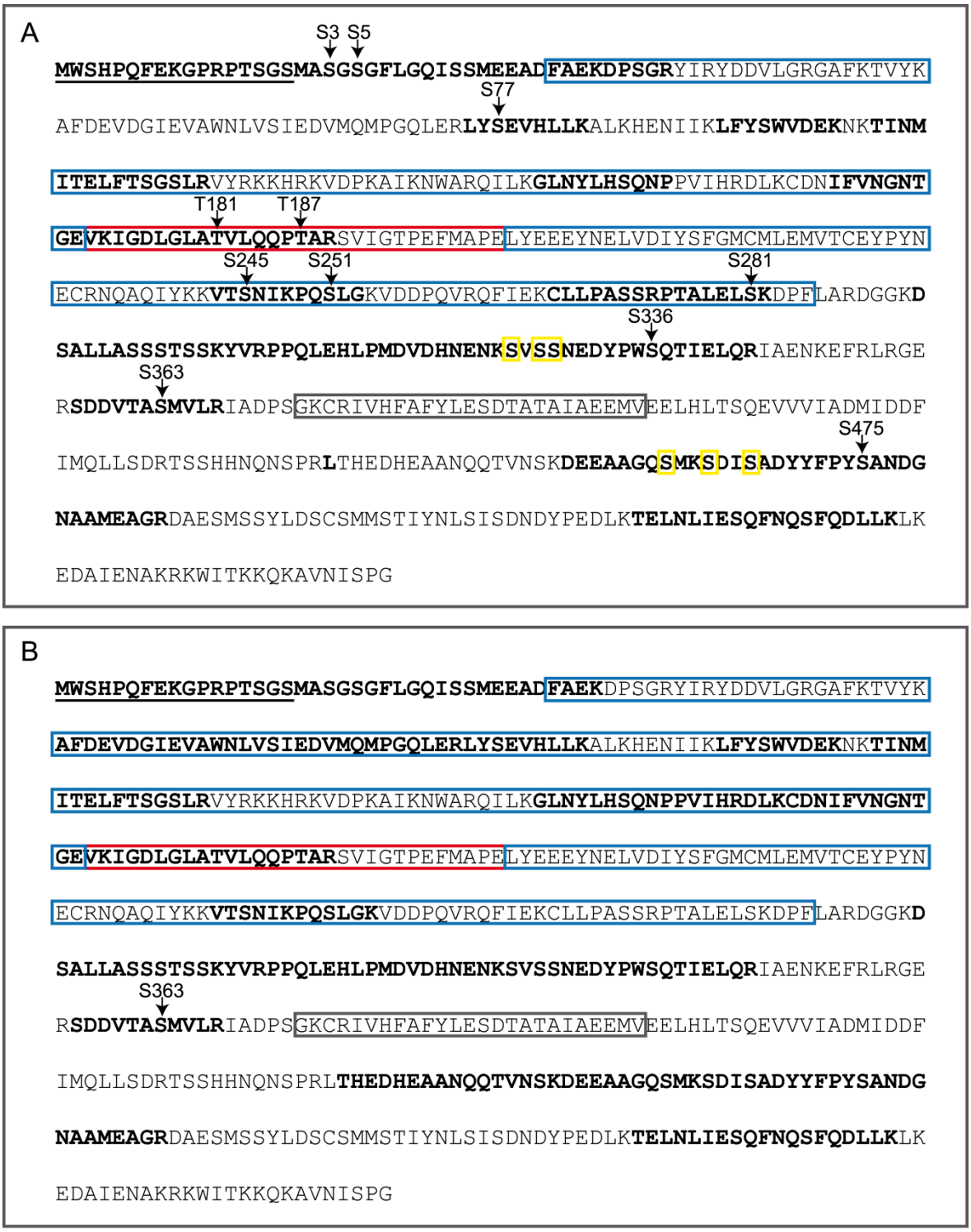

Figure 12. AtWNK8 phosphorylation sites

(A) Autophosphorylation of AtWNK8 and **(B) AtWNK8 dephosphorylated by AtPP2CA**

Phosphorylated residues were identified from recombinant AtWNK8 by MS analysis. Residues of the kinase domain are encircled in blue, within the kinase domain the activation loop is marked in red. The grey box labeles the autoinhibitory domain. The *strep*-tag is underlined. All detected peptides are printed in bold. Phosphorylated residues are marked with an arrow (confirmed) or with yellow boxes (putative and ambiguous).

We further aimed to identify phosphorylated residues on AtPYR1. Recombinant receptor protein was incubated with WNK8 protein in the presence of ATP. Proteins were separated by SDS-PAGE and AtPYR1 bands were cut from the gel after coomassie staining. Tryptic digestion followed by MS analysis revealed 7 phosphorylated residues (S16, S47, S85, T91, S92, T93 and S109) and three putative, ambiguous residues that still have to be confirmed (Fig. 13). Phosphorylation at position S85 is included in the so called Proline Cap motif ("Pro-Cap", Val^{83} – Asn^{90}) which together with the Leucine Lock motif ("Leu-Lock", Glu^{114} – Thr^{118}) folds over ABA to close the lid on the binding cavity of the receptor (NISHIMURA *et al.* 2009b). Pro-Cap and Leu-Lock are also referred to as gating loops (SANTIAGO *et al.* 2012).

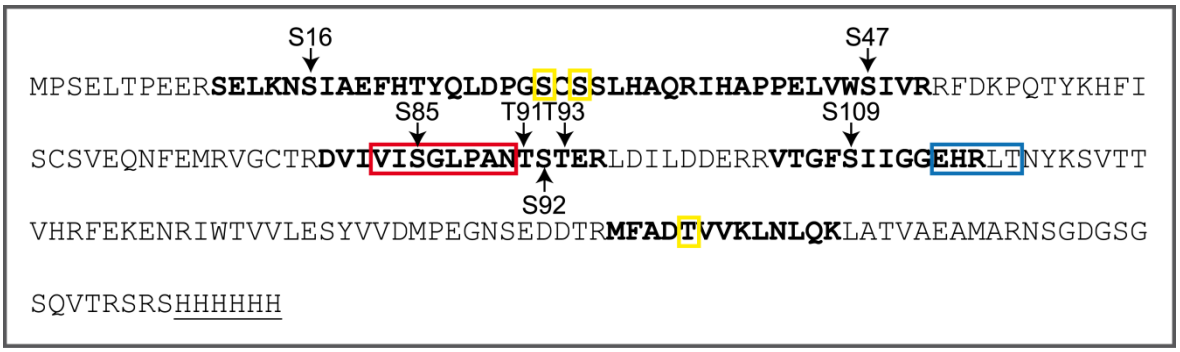

Figure 13. AtPYR1 phosphorylation sites

Phosphorylated residues were identified from recombinant AtPYR1 by MS analysis. Residues included in the Pro Cap motif are highlighted in red; the Leucine Lock region is marked in blue. The 6xHis-tag is underlined. All detected peptides are printed in bold. Phosphorylated residues are marked with an arrow (confirmed) or with yellow boxes (putative and ambiguous).

3.4 Putative AtWNK8 targets

AtWNK8 targets within the ABA signaling pathway

Following the identification of the direct interaction between the AtWNK8 kinase and the AtPP2CA phosphatase other members of the ABA signaling pathway were investigated as putative AtWNK8 targets. Since phosphorylation of the ABA receptor protein AtPYR1 could be observed *in vitro*, Y2H experiments were performed to confirm a direct interaction. AtPYR1 and AtPYL8 were cloned into the GAL4 DNA binding domain vector and used as baits. AtWNK8 full-length or AtWNK8 kinase domain (WNK8 KD) fused to the DNA activation domain of the GAL4 transcription factor served as preys. Bait and prey constructs were transformed into the yeast strain pJ69-4A. SD medium deficient in leucine and tryptophan (-LW) was used to identify the successful uptake of bait and prey plasmids. To identify interactions yeast cells were spotted onto SD medium lacking leucine, tryptophan, adenine and histidine (-LWAH). The interaction between AtPP2CA and AtWNK8 KD identified previously was used as a positive control (Fig. 14). Note that growth of cells transformed with the AtPP2CA bait and the empty vector (pGADT) was due to prolonged incubation time of 4 days (compare figure 8). In the yeast-two-hybrid system no physical interaction between AtWNK8 KD and AtPYR1 or AtPYL8 was observed (Fig. 14). Also the AtWNK8 full-length protein did not interact with any of the two receptors. The same experiment was repeated in the presence of (+) ABA (50 µM) which was incorporated in the SD medium. (+) ABA refers to the chiral configuration of the molecule due to an asymmetric C atom. Usually a mixture of (+/-) ABA was used. Still, yeast cells were not able to grow on –LWAH medium indicating that even in the presence of the ligand the ABA receptors do not interact with AtWNK8 in yeast.

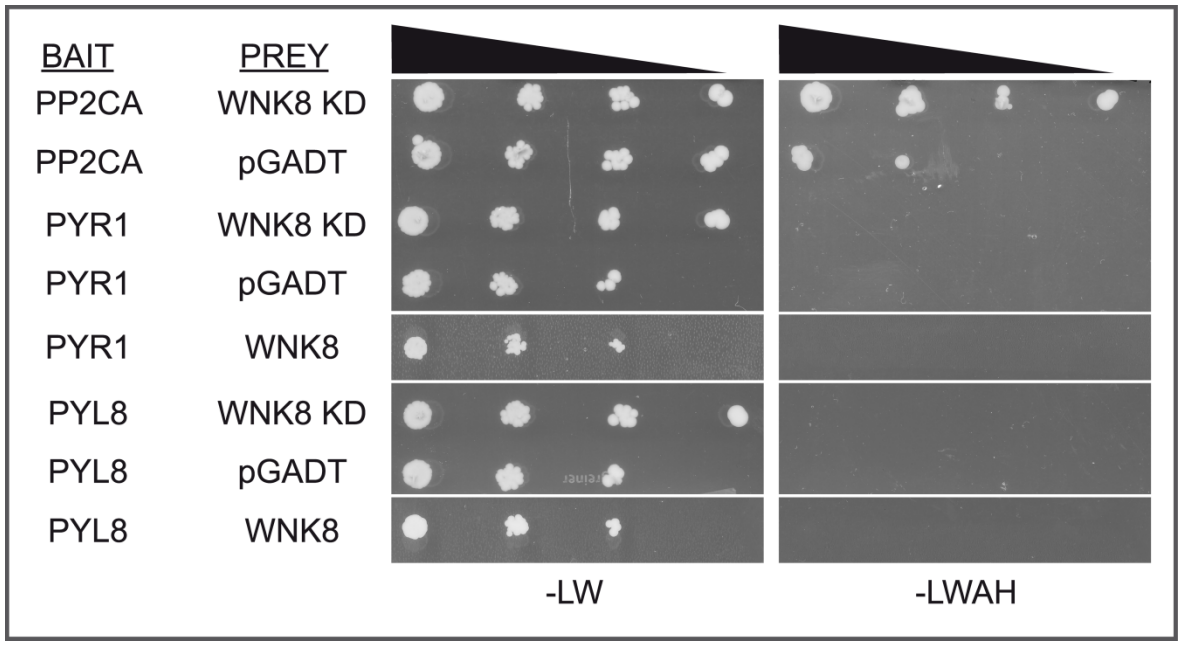

Figure 14. AtWNK8 and ABA receptor proteins AtPYR1 and AtPYL8 do not interact in yeast
Yeast cells transformed with the indicated bait and prey constructs were grown on SD medium lacking leucine and tryptophan (–LW) to monitor successful uptake of both plasmids. Growth on SD medium deficient in tryptophan, leucine, adenine and histidine (-LWAH) shows protein interaction. Interaction between AtPP2CA and AtWNK8 KD was used as a positive control. Cells were spotted in a serial of 1:10 dilutions as indicated by the black triangle. Plates were incubated for 4 d at 28°C.

Other putative AtWNK8 targets within the ABA signaling network are the main positive regulators, members of the *Arabidopsis* SNF1-RELATED PROTEIN KINASES FAMILY 2 (AtSnRK2). Since so far, no upstream kinases have been identified to regulate AtSnRK2 we used *wnk8-1* and *wnk8-4* mutants to determine whether AtSnRK2.2, 2.3 and 2.6 (AtOST1) could still be activated *in vivo*. It has been shown that the activation status of these kinases is highly regulated by ABA and can be detected *in vivo* by in-gel kinase assays using *Arabidopsis* crude extracts (FUJII *et al.* 2007, MUSTILLI *et al.* 2002). Total protein extracts were prepared from 5 week old *Arabidopsis* roots after exposure to 10 µM ABA for 30 minutes. Soluble protein extracts were run on an SDS-PAGE containing Histone III-S as a substrate for AtSnRK2s. Activity of all three kinases was induced in Col-0 wild type upon ABA treatment but AtSnRK2.2 and AtSnRK2.3 activity was reduced in the *snrk2.2 snrk2.3* double mutant (Fig. 15). No difference compared to wild type was observed in the *wnk8-1* or *wnk8-4* mutant background. Kinase activity was still induced by ABA indicated by phosphorylation of the substrate.

These results showed that neither the presence of a constitutively active AtWNK8 (*wnk8-1*) nor a missing AtWNK8 kinase (*wnk8-4*) influenced SnRK2 activity.

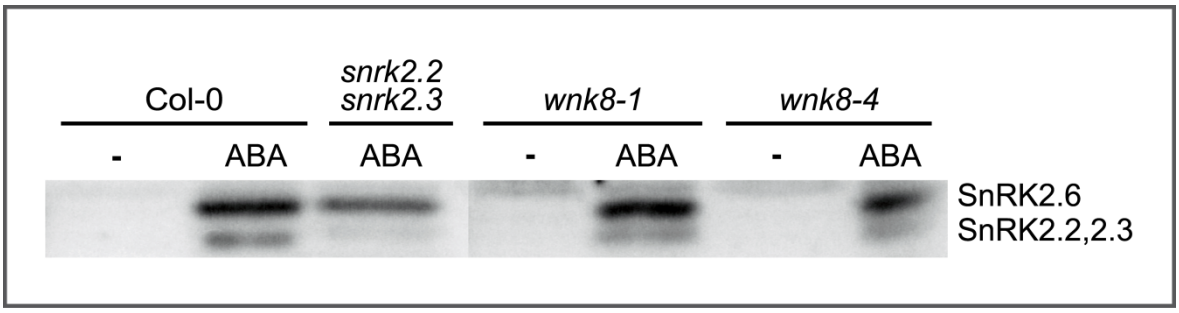

Figure 15. AtWNK8 does not influence ABA-induced AtSnRK2 activity
In-gel kinase assay with proteins extracted from Col-0, *snrk2.2 snrk2.3*, *wnk8-1* and *wnk8-4* roots under control conditions or 30 min after 10 µM ABA treatment. Histone III-S was used as a kinase substrate embedded in the gel. A 45 kDa and a 42 kDa band corresponding to SnRK2.6 (OST1) and SnRK2.2 and 2.3, respectively, were detectable after ABA treatment.

Besides AtSnRK2 kinases being involved in ABA signaling another family of *Arabidopsis* protein kinases has been shown to be implicated in regulating ABA responses. Together with individual members of the calcineurine-B like proteins (CBL) the CBL-interacting protein kinases (AtCIPKs) are linked to ABA and abiotic stress signaling in various publications. Whereas some were identified as negative regulators during seed germination and seedling development, others were proposed to negatively regulate ABA signaling in guard cells (CHEONG *et al.* 2007, D'ANGELO *et al.* 2006, KIM *et al.* 2003). Furthermore the direct regulation of several AtCIPKs by phosphatases of the PP2C family, such as AtPP2CA, was recently shown (LAN *et al.* 2011). Therefore AtCIPK protein kinases were investigated as putative AtWNK8 interacting candidates. In a Y2H screen all 26 members of the CIPK family were tested for their ability to interact with the AtWNK8 kinase. Bait constructs containing any AtCIPK fused to the DNA-binding domain and AtWNK8 prey construct with either AtWNK8 or the AtWNK8 kinase domain (WNK8 KD) fused to the activation domain were transformed into the yeast strain pJ69-4A. To identify interactions cells were spotted on SD medium deficient in leucine, tryptophan and histidine (-LWH). The results indicated that among the 26 candidates three were able to interact with AtWNK8 in yeast (Fig. 16). AtCIPK10 and AtCIPK14 interaction with AtWNK8 was slightly stronger than the interaction between AtCIPK26 and the kinase.

Moreover, interaction with AtWNK8 KD was strongly reduced indicating that the interaction might require the C-terminus of AtWNK8.

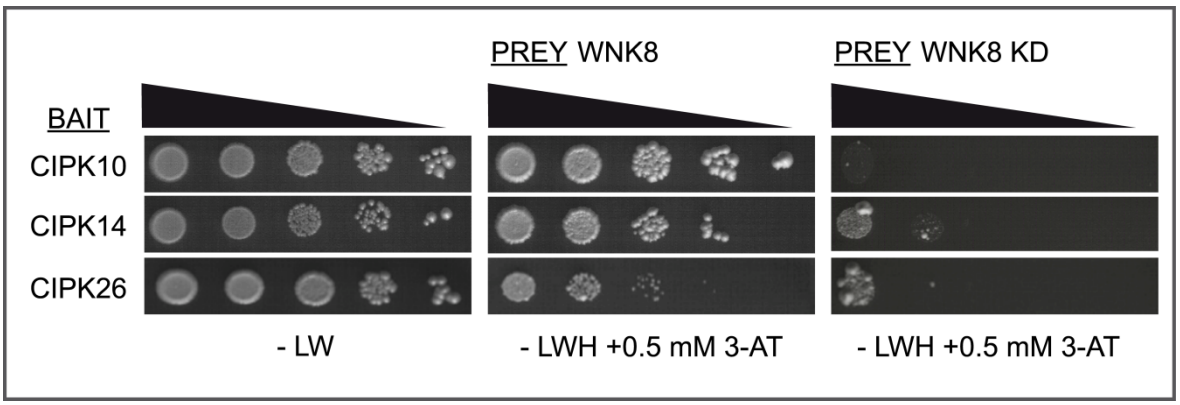

Figure 16. Several members of the AtCIPK family interact with AtWNK8 in yeast
Yeast (strain: pJ69-4A) transformed with the indicated bait and prey constructs were grown on SD medium lacking leucine and tryptophan (-LW) to monitor successful uptake of both plasmids. Protein interactions could be identified on medium lacking tryptophan, leucine and histidine (-LWH). Cells were spotted in a serial of 1:10 dilutions as indicated by the black triangle. Pictures were taken 15d after incubation at 30 °C.

Identification of putative AtWNK8 interaction partners via a large scale Y2H screen

To undertake a broader approach to identify AtWNK8 targets a large-scale Y2H approach was performed (Hybrigenics, Paris, France). Using the first 449 amino acids of the AtWNK8 protein as bait a cDNA library generated from one week old *Arabidopsis* seedlings was screened for interacting partners. Out of 64.2 million interactions 121 clones were processed and sequenced to identify the corresponding proteins (appendix, Table 2). All candidates were grouped according to an automatically generated score evaluating the confidence in the interaction (A = very high; B = high; C = good; D = moderate). Group D is the most difficult to interpret since it mixes two classes of interactions: It can either contain false positive interactions or interactions that are hardly detectable by the Y2H techniques due to low representation of mRNA in the library, prey folding or prey toxicity. For a few interactions a score cannot be attributed (N/A) because of fragments cloned out of frame, fragments that lie only in the 5'- or 3'-UTR or all fragments of the same reference are antisense.

From the potential interactions partners identified in the screen only some have been characterized in detail to date (supplementary material, Table S2). Among all candidate proteins AtEMF1 (EMBRYONIC FLOWER 1) is the only one which is has already been

published as an interacting partner for AtWNK8 (PARK *et al.* 2011). EMF proteins (AtEMF1 and AtEMF2) are known to be floral repressors because mutations in *EMF* genes cause early flowering (SUNG *et al.* 1992, YANG *et al.* 1995).

A number of candidates have been proposed to localize to compartments other than the cytosol or the nucleus (according to TAIR database) where interaction with AtWNK8 would most likely occur. RCA (RUBISCO ACTIVASE), ADT2 (AROGENATE DEHYDRATASE 2), PSBP-1 (PHOTOSYSTEM II SUBUNIT P1), DG1 (DELAYED GREENING 1) and CAF1 (CRS2-ASSOCIATED FACTOR 1) localize to plastids, LACS6 (LONG CHAIN ACETYL-COA SYNTHETASE 6) is described to be a peroxisomal acetyl-CoA synthetase. These proteins and all candidates that fall into the N/A group were not investigated any further. Several other candidates play a role in various cellular processes that we can currently not link to ABA signaling and the role of AtWNK8. Among these are two other proteins involved in acetyl-CoA biosynthesis, ACLB-1 (ATP-CITRATE LYASE B-1) and ACLB-2 (ATP-CITRATE LYASE B-2). RPL6A (60S RIBOSOMAL PROTEIN L6A) and RPL6B (60S RIBOSOMAL PROTEIN L6A) were shown to be involved in the structural constitution of ribosomes and translation, ASN1 (GLUTAMINE DEPENDENT ASPARAGINES SYNTHASE 1) encodes an enzyme needed for asparagines synthesis, GRV2 (GRAVITROPISM DEFECTIVE 2) and EYE (EMBRYO YELLOW) play a role in endosome formation and transport of Golgi-localized proteins, respectively.

Nevertheless, a number of identified interaction partners that can be linked to ABA signaling are known to localize to the same subcellular compartments as AtWNK8 or have been shown to interact with WNK proteins in other organisms. Therefore they will be more likely investigated in the future. From group A (Table 2) this includes one unknown protein, corresponding to *At5g62280*. Expression of this gene was identified to be weakly drought- and ABA-regulated (HUANG *et al.* 2008).

Three other candidate genes (*At5g14720*, *At4g24100* and *At4g10730*) are annotated as unknown proteins of a kinase family and fall into group B and D. These genes belong to the *Arabidopsis* family of mitogen-activated protein kinase kinase kinase kinase (MAP4K) of which are 10 in the *Arabidopsis* genome (CHAMPION *et al.* 2004b). The nomenclature suggests that MAP4Ks function in a classical MAP kinase pathway upstream MAP3Ks. However, MAP4Ks from yeast and mammals have been shown to be involved in other signaling pathways (DAN *et al.* 2001). The term MAP4K rather refers to phylogenetic

similarity and not necessarily to biochemical function. The mammalian MAP4 kinases OSR1 (OXIDATIVE STRESS-RESPONSIVE *KINASE*-1) and SPAK (STE20/SPS1-RELATED PROLINE/ALANINE-RICH K*INASE)* have been described to interact with HsWNKs (reviewed in DELPIREandGAGNON 2008).

Since interaction of AtWNK8 and AtPP2CA occurs in the nucleus proteins that have been described to localize to this subcellular compartment could be potential AtWNK8 targets. Among group B we identified the AtBIM3 (BES1-INTERACTING MYC-LIKE PROTEIN 3) basic helix-loop-helix transcription factor and the nuclear-localized AtUVR8 (UVB-RESISTANCE 8) protein. AtBIM3 has been shown to interact with AtBES1 (BRI1-EMS-SUPPRESSOR 1), a transcripton factor of the brassinosteriod signaling pathway (YIN *et al.* 2005, YIN *et al.* 2002). *Arabidopsis* perceives ultraviolet-B (UV-B) radiation with dimers of the AtUVR8 photoreceptor (RIZZINI *et al.* 2011). GFP-UVR8 localizes to the nucleus where it interacts with chromatin (BROWN *et al.* 2005). The only kinases described in the UVR8-stress activated signaling pathway so far are the mitogen-activated protein kinases AtMPK3 and AtMPK6 (GONZÁLEZ BESTEIRO *et al.* 2011).

Two other putative interaction partners in the nucleus are the B3 transcription factor AtHSL1 (HSI2-LIKE 1) which plays a role in regulating the transition from seed maturation to seedling growth, and a NAC domain-containing transcription factor AtNAM (NAC-REGULATED SEED MORPHOLOGY 2) which is active during embryogenesis and regulates seed development (KUNIEDA *et al.* 2008). Both AtHSL1 and AtNAM are classified in group D (appendix, Table 2).

The UBIQUITIN-ACTIVATING ENZYME 1 (AtUBA1) is the only candidate from group C which might be linked to AtWNK8 function. AtUBA1 has been described to bind ubiquitin and to transfer it to various ubiquitin-conjugating E2 enzymes thus marking proteins for degradation (HATFIELD *et al.* 1997). The results of the phenotypic analysis of *pp2ca-1 wnk8* mutants implied that WNK8 is a negative regulator of ABA signaling. In order to carry out this function an interaction with UBA1 seems plausible.

4 DISCUSSION

In the last years substantial progress has been made elucidating the key components of the ABA signaling pathway. Identification of the soluble ABA receptor proteins, the main phosphatase/kinase pairs and a number of downstream targets, including transcription factors and ion channels, has broadened our knowledge about this complex network. A lot of research has been focused on the composition of the so called core ABA signaling pathway (JOSHI-SAHA *et al.* 2011). This pathway includes only one type of protein kinases that function as positive regulators and one protein family of phosphatases that negatively regulate the ABA response. Knowledge about additional players in ABA signaling has been limited. Here we provide genetic and biochemical evidence that the plant protein kinase AtWNK8, previously described in our lab as an interaction partner of the C-subunit of the vacuolar V-ATPase (VHA-C), plays a role in negatively regulating ABA signaling (HONG-HERMESDORF *et al.* 2006).

4.1 AtWNK8 is a nuclear and cytosolic protein kinase

Initial information on the temporal and spatial activity of the *AtWNK8* promoter was obtained from the AtGenExpress Developmental data set (SCHMID *et al.* 2005). Promoter-GUS analysis confirmed that At*WNK8* is expressed throughout the plant. The expression pattern in 5 d old seedlings is supported by the observed *wnk8* mutant phenotype during postgermination development. The strong At*WNK8* expression in pollen, as indicated by the AtGenExpress data set, might suggest a role in male gametophyte development, but fertility in *wnk8* mutants is not affected. However, other members of the WNK family share this striking expression in pollen, such as the closest AtWNK8 homologs AtWNK6 (At3g18750), AtWNK7 (At1g49160) and AtWNK10 (At1g64630). If functionally redundant a mutant lacking the activity of more than one of these genes is needed to study the putative role of WNK kinases in pollen.

Using different transient expression systems we found that GFP-WNK8 localizes to the nucleus and the cytosol. This observation fits with role of AtWNK8 to phosphorylate the cytosolic tail of the C-subunit of the vacuolar H^+-ATPase (HONG-HERMESDORF *et al.* 2006) as well as with the interaction of the protein phosphatase PP2CA in the nucleus.

Though GFP-WNK8 could easily be expressed transiently, we failed to generate stable transgenic lines. Gene silencing is frequently observed in transgenic plants and prevents efficient expression of the transgene. Silencing occurs transcriptionally resulting from promoter inactivation or posttranscriptionally when mRNAs fail to accumulate (STAM *et al.* 1997). Here we might observe silencing of GFP-WNK8 but not of the kanamycin selection marker, since we were able to identify kanamycin resistant seedlings in T1. To exclude that kinase activity is preventing stable expression of GFP-WNK8 we transformed a kinase-dead version of AtWNK8 (GFP-WNK8$_{K41M}$). However, expression level of the transgene did not improve. Therefore, poor GFP-WNK8 expression could also be due to protein stability. Analysis of heat shock-inducible expression of GFP-WNK8 in stable transgenic plants supports the hypothesis that the fusion protein is highly unstable. Several hours after heat shock most of the GFP signal was no longer detectable. Results of preliminary experiments in which seedlings were treated with MG132 suggest that the 26S proteasome might be involved in this degradation process. Plants overexpressing the AtWNK8 protein without a tag have previously been reported and transcript levels have been shown to be significantly higher in these transgenic lines (PARK *et al.* 2011). However, an increase in protein amount was not confirmed.

4.2 AtWNK8 plays a role in ABA signaling: genetic evidence

In order to determine the *in vivo* function of the protein kinase AtWNK8 this work started with the characterization of two T-DNA insertion mutants, *wnk8-1* and *wnk8-4*. Phenotypic analysis provided evidence that AtWNK8 is mainly involved in regulating ABA-induced postgermination growth arrest. Most likely designed as a second checkpoint following germination this important adaptive mechanism allows seedlings to await unfavorable stress conditions (LOPEZ-MOLINA *et al.* 2001).

Careful analysis of the *wnk8-1* allele contradicts previous reports claiming that *wnk8-1* lacks total *AtWNK8* expression (PARK *et al.* 2011, WANG *et al.* 2008). Our results strongly suggest that *wnk8-1* is a dominant, gain-of-function allele resulting from a truncated transcript still present in the mutant. Dominance could be confirmed by examining the ABA-response of the F1 progeny of *wnk8-1*/+ seedlings. The intermediate phenotype observed here is likely to depend on a single copy of the *wnk8-1* allele which is still

sufficient to confer at least partial ABA-hyposensitivity as it can be observed for the homozygous mutant. Analysis of the recapitulation lines showed that the *wnk8-1* gain-of-function phenotype is caused by a constitutively active kinase. The truncated AtWNK8 protein lacks the C-terminally located autoinhibitory domain, thus, kinase activity is no longer regulated.

Unlike *wnk8-1*, *wnk8-4* seedlings did not show ABA-hyposensitivity, nor did they exhibit an ABA-hypersensitive phenotype. Even though the *wnk8-4* mutant seemed to contain a C-terminal transcript which originates from the T-DNA it can be considered a perfect knockout for kinase activity. Subsequent analysis of the *pp2ca-1 wnk8-4* double mutant supported the hypothesis that *wnk8-4* is the corresponding recessive, loss-of-function allele. Increased ABA-hypersensitivity of the *pp2ca-1 wnk8-4* double mutant further indicated genetic interaction.

Based on the ABA-hypersensitive phenotype identified for the *wnk8-4* mutant, we are now able to classify our dominant gain-of-function *wnk8-1* mutation according to one of three categories: antimorphic, hypermorphic and neomorphic (MULLER 1932). Antimorphic mutations act opposite to normal gene activity. This mutation might affect the function of a protein acting as a dimer. A dimer consisting of a normal and a mutated monomer is no longer functional and equals a loss-of-function mutation. However, since *wnk8-1* and the *wnk8-4* do not have the same phenotype classification of *wnk8-1* as an antimorphic mutation can be excluded. Neomorphic mutations are defined as mutations that cause a dominant gain of gene function different from normal gene activity. However, both *wnk8-1* and *wnk8-4* have an ABA-phenotype. Hypermorphic mutations, defined as an increase in normal gene function, often result from an increase in mRNA or protein expression or, as seen for *wnk8-1*, from a constitutively active protein.

These results help us to explain why the two mutants show opposite phenotypes. The ABA-dependent phenotype is linked to AtWNK8 kinase activity which is higher in *wnk8-1* but completely missing in *wnk8-4* seedlings. On the other hand both mutants are lacking the C-terminus of AtWNK8, including the putative coiled-coil domain. Therefore it is possible that *wnk8-1* and *wnk8-4* also show similar phenotypes if, for instance, contact with a respective interaction partner requires the C-terminus of AtWNK8. One example for interaction with AtWNK8 via the C-terminus is the C-subunit of the vacuolar H^+-ATPase (HONG-HERMESDORF *et al.* 2006). Strong binding of VHA-C to the most C-terminal 110 amino

acids was detected in Far-Western experiments indicating that the C-terminal coiled-coil domain is sufficient for the interaction. Currently phenotypic analyses of *det3 wnk8-1* and *wnk8-4 det3* double mutants are ongoing in our lab.

4.3 AtWNK8 directly interacts with the protein phosphatase AtPP2CA

Phenotypic analysis of the *pp2ca-1 wnk8* double mutants provided first indications that the protein phosphatase AtPP2CA and the protein kinase AtWNK8 are interacting partners. Yeast-two-hybrid analysis demonstrated that AtWNK8 directly interacts with AtPP2CA, being confirmed *in planta* using bimolecular fluorescence complementation (BIFC). Whereas interaction in yeast was restricted to the N-terminus of AtWNK8 harboring the kinase domain (WNK8KD), AtPP2CA and the full-length protein kinase did interact in plants. The autoinhibitory domain located at the C-terminus of AtWNK8 is an important regulator of enzyme activity. Through the interaction with the catalytic N-terminus the autoinhibitory domain maintains the inactive state of the kinase. Thus, steric effects might hinder AtPP2CA interaction with full-length AtWNK8 in yeast. On the other hand, yeast might lack potential AtWNK8 activators which are present in the plant. The hypothesis that interaction between AtPP2CA and AtWNK8 is dependent on kinase activity was further supported by the result that interaction with the inactive WNK8 kinase domain ($WNK8KD_{K41M}$) was not observed in yeast. Interaction of the same proteins in plants might be enabled by homodimerization of AtWNK8 (Diplomarbeit E. Görlich, unpublished) and subsequent transphosphorylation activity (HONG-HERMESDORF *et al.* 2006) to create an active AtWNK8 kinase.

BIFC experiments in plants showed that the interaction of AtPP2CA and AtWNK8 is restricted to the nucleus. Interestingly, the YFP signal detected in BIFC experiments was not homogeneously distributed but accumulated in distinct structures. What looks like separate compartments within the nucleus could be observed 24 – 36 hours after infiltrating *N. benthamiana* leaves. Today it is well known that the nuclear space is compartmentalized by nuclear bodies, which are dynamic but relatively stable structures composed of proteins and RNAs without delineating membranes (REDDY *et al.* 2012). The best investigated nuclear bodies in plants are the nucleolus, photobodies and nuclear speckles. Co-expression of known markers for splicing speckles, cajal bodies and the

nucleolus with the AtWNK8/AtPP2CA BIFC-complex revealed no co-localization indicating that the kinase/phosphatase pair is unlikely to participate in processing RNA in the nucleus. AtWNK8 Homodimerization likely contributes to accumulations observed in the nucleus. Once the YFP fluorophore is reconstituted after AtWNK8 interaction with AtPP2CA this complex is permanent. Simultaneous formation of AtWNK8 homodimers will lead to the formation of AtWNK8/AtPP2CA aggregates fused to functional YFP.

4.4 AtWNK8 kinase activity is inhibited by AtPP2CA

To further characterize the interaction between AtWNK8 and AtPP2CA in more detail we conducted *in vitro* phosphorylation assays testing for kinase and phosphatase function. Autophosphorylation of AtWNK8, as previously described in our lab, could be confirmed (HONG-HERMESDORF *et al.* 2006). We were able to identify numerous phosphorylated residues on AtWNK8. Within the kinase domain 6 residues were phosphorylated. In addition, two serine residues at the very N-terminus and three serine residues in the C-terminus were modified. However, more phosphorylated residues are likely to exist in AtWNK8. Even without radioactive labeling dephosphorylation of AtWNK8 by AtPP2CA was already observed in coomassie-stained SDS gels indicated by a significant band shift (supplementary material, Fig. S2). Since AtWNK8 provides only few trypsin cleavage sites, digestion results in long peptides which are not ideal to process and analyze in mass spectrometry. Thus, several phosphorylation sites could not be detected.

Members of the WNK kinase subfamily in mammals are well characterized concerning the importance of several residues for kinase activity and autoinhibition. Exchange of the conserved lysine (K41) or aspartate residue (D175) in the active center of RnWNK1 abolishes phosphorylation activity of the kinase (JOHNSON 2001, XU *et al.* 2000). The same was shown for AtWNK8 (HONG-HERMESDORF *et al.* 2006). It was also shown that phosphorylation of a conserved serine residue in the activation loop of the kinase domain, S382 in RnWNK1, is necessary to ensure kinase activity. The homologous residue in AtWNK8 is S190 which, unfortunately, was not yet detected to be phosphorylated in the recent analysis. Here we report the identification of two more phosphorylated residues within the activation loop, S181 and S187. If and how modification of these

residues might affect AtWNK8 activity or interaction with other proteins remains to be determined.

Phosphorylation sites at the C-terminus of AtWNK8 have been proposed in our lab previously. When truncated, inactive AtWNK8 substrates were used to test for intermolecular autophosphorylation they were still phosphorylated indicating that at least one phosphorylation site is located within the last 110 amino acids (Hong-Hermesdorf A., PhD thesis, 2005). Here we report phosphorylation of the serine residue S475. It will be interesting to investigate whether phosphorylation of S475 is required for the interaction of AtWNK8 and AtVHA-C which is primarily established via the C-terminus of the kinase.

On the contrary, the *in vitro* phosphorylation experiments did not verify the hypothesis that AtWNK8 targets the AtPP2CA. Instead, we found that WNK8 activity is negatively regulated by AtPP2CA. Upon contact with AtPP2CA, the kinase was very efficiently dephosphorylated except for a single serine residue, S363. Distinct domains within AtPP2CA which are required for interaction and dephosphorylation of AtWNK8 have not been described, yet. Some information might be transferred from the interaction between AtSnRK2 kinases and AtPP2C phosphatases. Recently the crystal structure of the *Arabidopsis* SnRK2.6-HAB1 complex has been analyzed and allowed the identification of the molecular mechanism by which the protein kinase AtSnRK2.6 (OST1) interacts with the protein phosphatase AtHAB1 (Soon *et al.* 2012). The overall structure revealed a monomeric kinase-phosphatase complex in which the active sites of the kinase and the active site of the phosphatase form the major binding interface. Thereby, the activation loop of AtSnRK2.6 is inserted into the catalytic cleft of AtHAB1 leading to the dephosphorylation of a key serine residue and thereby reducing AtSnRK2.6 activity to a basal level. Simultaneously, a conserved tryptophan residue of AtHAB1 (W385) locks into the active site of the AtSnRK2.6 to physically inhibit kinase function. Interestingly, ABA-bound receptors have adopted a similar surface feature. They interact with the active site of PP2C phosphatases and are linked via the conserved W385 (Fig. 2). Kinase inactivation by a phosphatase as observed in the AtSnRK2.6/ATHAB1 complex may also represent a mechanism for the AtWNK8/AtPP2CA interaction. BIFC experiments already confirmed that AtPP2CA interacts with the kinase domain of AtWNK8. Since AtWNK8 activity was restored once AtPP2CA was bound to PYR1, it is likely that the active site of the

phosphatase is simply changing its interaction partner. This idea can further be supported by the fact that interaction between AtPP2CA and some members of the *Arabidopsis* CIPK family of protein kinases has also been described to be kinase domain dependent (LAN *et al.* 2011). Future experiments should aim to answer the question whether interaction between the active sites of AtWNK8 and AtPP2CA alone is still possible. In addition, careful analysis of the conserved tryptophan residue, W280 in AtPP2CA, will help to determine if it plays a role in AtWNK8 inhibition.

4.5 AtWNK8 phosphorylates the ABA receptor AtPYR1

Few proteins have been identified to interact with AtWNK8 so far (HONG-HERMESDORF *et al.* 2006, PARK *et al.* 2011, TSUCHIYAandEULGEM 2010). Except for the C-subunit of the vacuolar V-ATPase, phosphorylation of these proteins by AtWNK8 has not been shown, yet. Here we report *in vitro* data on the phosphorylation of the ABA receptor protein AtPYR1 by AtWNK8. All phosphorylated residue of AtPYR1 that have been detected are shown as blue ball-and-stick models in Figure 1. The crystal structure and the mechanism of ABA binding to AtPYR1 have been very well described (NISHIMURA *et al.* 2009a). *In vivo* AtPYR1 assembles as a homodimer, but only one of the two monomers binds ABA (NISHIMURA *et al.* 2009a). The ABA molecule sits in a deep cavity forming interactions with polar site chains of the AtPYR1 protein via hydrogen bonds. ABA-induced conformational changes of the AtPYR1 monomer include the gating loops (Val^{83} – Asn^{90} and Glu^{114} – Thr^{118}) which fold over ABA to complete ABA enclosure (Fig. 1). Furthermore, these conformational changes are crucial to generate a favorable interaction surface for the binding of the PP2Cs (MELCHER *et al.* 2009, MIYAZONO *et al.* 2009, NISHIMURA *et al.* 2009a, SANTIAGO *et al.* 2009a). Single residues contribute to ABA binding or to the interaction with the PP2C in particular and could be identified as being phosphorylated by AtWNK8. Among these the serine residue S92 is one of 17 residues which form the large internal binding cavity (NISHIMURA *et al.* 2009b). Phosphorylation at this position is likely to interfere with ABA binding to the receptor (Fig. 1).

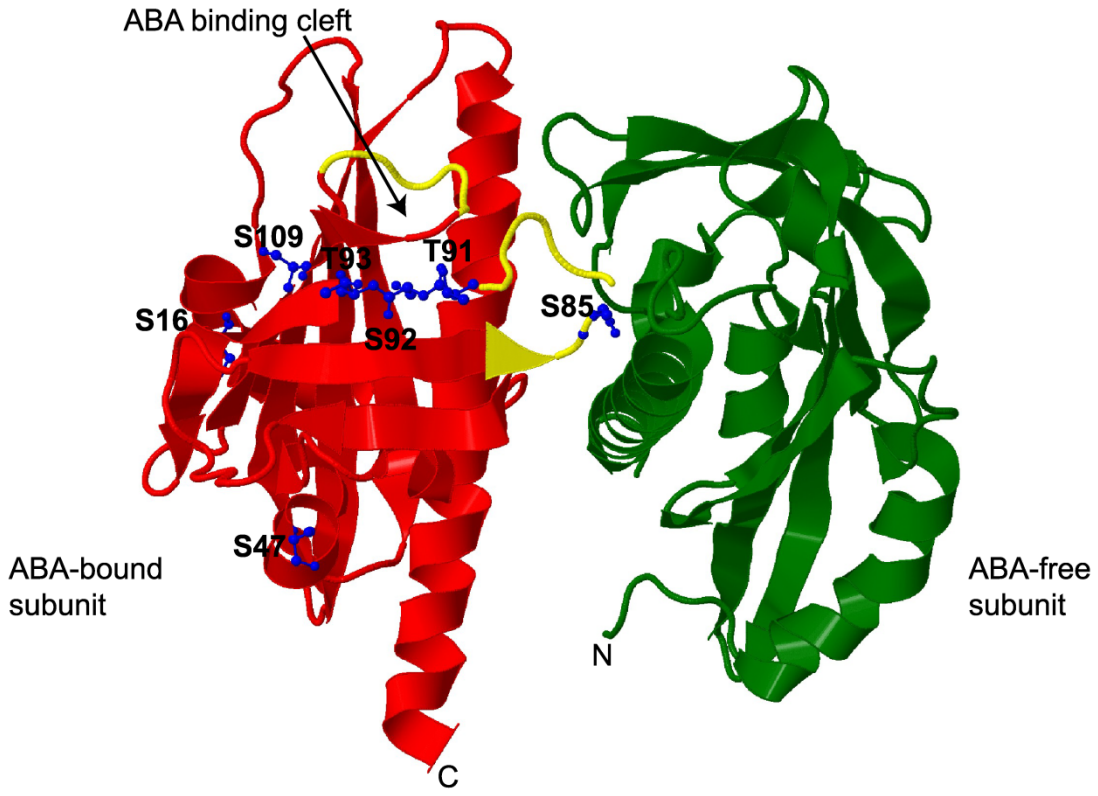

Figure 1. Crystal dimeric structure of the ABA receptor AtPYR1
Asymmetric dimer (ABA-bound and ABA-free subunit) is shown as ribbons (ß-strands and helices) and strands (loops) with ABA-binding cleft indicated by arrow. The two gating loops are labeled in yellow. Individual residues are highlighted in blue ball-and-stick models. The figure was designed using Geneious v5.5, Drummond et al. 2011.

The neighboring residues T91 and T93 are not described to contribute to the ABA binding site though they are close by. Phosphorylation at these two threonines is unlikely to interfere with ABA-binding since the phosphorylated side chain is facing away from the binding cleft. On the contrary, phosphorylation at position S85 will probably influence the interaction with AtPP2CA. Figure 2 shows the structure of the AtPYR1/AtHAB1 complex and highlights critical regions in the two molecules (DUPEUX *et al.* 2011). The interaction between AtPYR1 and AtPP2CA is likely to form a similar structure since AtHAB1 and AtPP2CA are from the same PP2C family of protein phosphatases and share identical active sites and conserved residues. Serine S85 has been described to be crucial for the interaction with the active site of the PP2Cs (reviewed by SANTIAGO *et al.* 2012). This residue is exposed on the surface of AtPYR1 upon binding of ABA (Fig. 2). It has been shown that a mutation of the equivalent residue in AtPYL2 (S89) leads to loss of interaction with AtABI1 (MELCHER *et al.* 2009, YIN *et al.* 2009). Future experiments will aim

to address the function of these phosphorylations in order to understand how the receptor itself and the receptor interaction with a PP2C phosphatase might be regulated by AtWNK8. Mutations mimicking constitutively phosphorylated or dephosphorylated AtPYR1 proteins will be used for *in vitro* phosphorylation assays to examine AtWNK8 activity in the presence of AtPYR1 and AtPP2CA. Direct interaction of AtWNK8 and AtPYR1 remains to be determined.

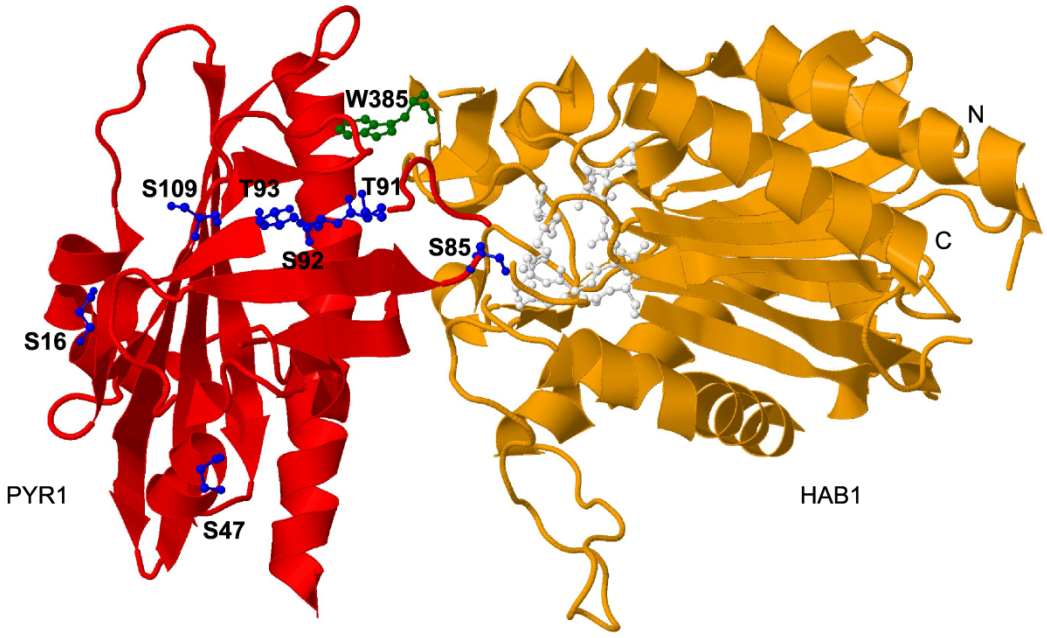

Figure 2. Crystal structure of the AtPYR1-HAB1 complex (adapted from DUPEUX *et al.* 2011)
The ABA-bound AtPYR1 receptor is colored in red, the catalytic core of AtHAB1 (residues 172-511) in white. The blue ball-stick model marks the threonine residue which gets phosphorylated by WNK8. The two gating-loops enclosing the ABA in the binding cavity are labeled in yellow. The green ball-stick model highlights the conserved tryptophan residue locking into the active site of the ABA-receptor. Orange residues mark critical active-site residues of AtHAB1. (The figure was designed using Geneious v5.5, Drummond et al. 2011)

Previous experiments to confirm AtPYR1/AtWNK8 interaction in yeast failed (compare section 3.5). BIFC or FRET (Fluorescence Resonance Energy Transfer) analysis will be used in the future to investigate receptor kinase interaction *in planta*. However, interactions between kinases and their respective targets are likely to be very short events and do not require strong protein-protein binding.

4.6 Different kinases are putative AtWNK8 targets

To better understand the role of AtWNK8 in ABA signaling direct interactions with other members of the core pathway and related pathways have been tested. Members of the AtSnRK2 family of protein kinases are well known as positive regulators of ABA signaling. Their ABA-dependent autophosphorylation and activation has been well described on a molecular and structural level (NG *et al.* 2011, SOON *et al.* 2012, YUNTA *et al.* 2011). However, the identification of molecular components responsible for ABA-independent AtSnRK2 regulation remained unknown. Our results demonstrated that AtWNK8 does not regulate the activity of SnRK2.2, 2.3 or 2.6. Even in a *wnk8-1* or *wnk8-4* background the SnRK2s were still able to phosphorylate Histone III-S. AtWNK8 and AtSnRK2 kinases are more likely to act in parallel pathways controlling ABA signaling, this way AtWNK8 could act as a negative regulator opposite AtSnRK2s.

AtCIPKs are found to be differentially regulated by a variety of stress conditions, including drought, salt and ABA (D'ANGELO *et al.* 2006, KIM *et al.* 2003). In addition, some AtCIPKs (AtCIPK1 and AtCIPK6) directly interact with the protein phosphatase AtPP2CA (LAN *et al.* 2011). We were interested to know whether any of these kinases might interact with AtWNK8. Our results provide three promising AtCIPK interaction partners for AtWNK8: AtCIPK10, 14 and 26. Since interaction with the full-length AtWNK8 protein was much stronger than interaction with the kinase domain alone, there is evidence that the C-terminus of AtWNK8 is required for the interaction. The function of AtCIPK10, 14 and 26 is still poorly understood. While there is no information on AtCIPK10 and AtCIPK26, AtCIPK14 was described to be involved in the regulation of salt and ABA responses and in phytochrome A-mediated far-red light inhibition of greening in *Arabidopsis* seedlings (QIN *et al.* 2010, QIN *et al.* 2008). In addition, the crystal structure of the regulatory domain of CIPK14 and its interacting calcium binding protein, CBL2, has been solved (AKABOSHI *et al.*

2008). In the future we will further investigate the functional relationship of AtWNK8 and AtCIPK10, 14 and 26.

The results of the Y2H screen provided us with additional, promising AtWNK8 interacting candidates. However, due to the strong autoactivation signals of the AtWNK8 C-terminus only the first 449 aa were used as bait. Interactions that require the C-terminal end of the kinase, such as the C-subunit of the vacuolar H^+-ATPase, were therefore not detected in this screen (HONG-HERMESDORF *et al.* 2006). However, out of 121 clones we identified three of the 10 *Arabidopsis* MAP4 kinases (AT5G14720, AT4G24100 and AT4G10730) as putative interacting partners for AtWNK8. According to the automatically generated score the interactions with AT5G14720 and AT4G24100 have a high level of confidence and are likely to be true interactions. AT4G10730 falls into group C, which indicates only moderate confidence in the interaction. In addition, group C contains those candidates represented by low mRNA levels. This classification fits with the AtGenExpress Developmental set which shows ubiquitous but very low expression of *AT4G10730* (SCHMID *et al.* 2005). Interestingly, *AT5G14720* shows the same strong expression in pollen as observed for AtWNK8.

MAP4 kinases were first described in yeast when the yeast Ste20p kinase was shown to phosphorylated MAP kinase kinase kinase (MAP3K) targets (WU *et al.* 1995). All Ste20-related kinases fall into the so called Ste20 superfamily, which include the mammalian OSR1 and SPAK1 as well as plant MAP4Ks (CHAMPION *et al.* 2004b). Compared to MAP kinase sequences of other plants, mammals, insects, *Caenorhabditis elegans*, *Dictyostelium* and fungi the plant MAP4Ks cluster within the STE family, in the group of MAP4Ks (Fig. 3). However, the possibility that all kinases, that cluster within the group of MAP4Ks, act upstream of a MAP3K can no longer be supported. Though some target MAP3Ks, others phosphorylate MAP kinase kinases (MAP2Ks) or have been shown to be involved in other signaling pathways (CHAMPION *et al.* 2004b). The term MAP4K rather refers to phylogenetic similarity and not necessarily to biochemical function. Evidence that the AtMAP4Ks identified in the screen are promising interaction partners for AtWNK8 comes from data about mammalian WNKs. Several studies have identified two members of the WNK family (HsWNK1 and HsWNK4) as upstream kinases for HsSPAK and HsOSR1 (MORIGUCHI *et al.* 2005, VITARI *et al.* 2005). Following HsSPAK and HsOSR1

activation through phosphorylation, they in turn phosphorylate key ion transporters, thus contribute to fluid and ion homeostasis. Furthermore, there is recent data describing a role of HsSPAK and HsOSR1 in regulating the activity of their upstream activators via a feedback loop (THASTRUP *et al.* 2012).

Preliminary data on the three AtMAP4Ks indicated that these kinases localize exclusively to the cytosol, supporting a role for AtWNK8 outside the nucleus.

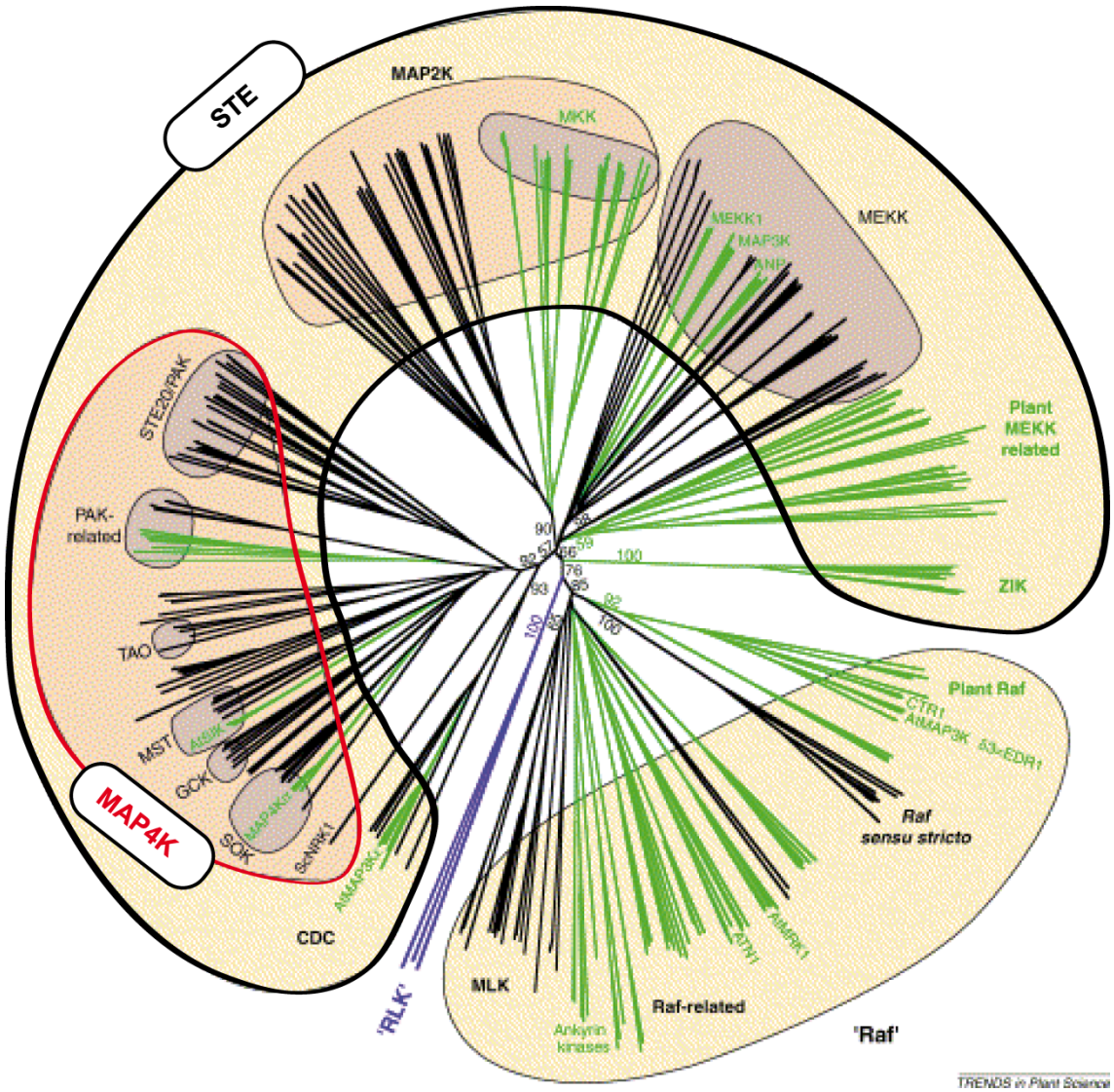

Figure 3. A general view of the STE family and the RAF superfamily of kinases (modified from CHAMPION *et al.* 2004b)

The figure shows a relationship tree (phenogram) based on an extensive survey of mammals, plants, insects, *Caenorhabditis elegans*, *Dictyostelium* and fungi STEs and Rafs. The plant lineage is shown in green. The group of MAP4Ks, red, belongs to the STE family (black ring).

4.7 Conclusion

In conclusion we describe here the function of the protein kinase AtWNK8 in the ABA signaling pathway. Genetic as well as biochemical data provide evidence that AtWNK8 acts as a negative regulator. Figure 4 illustrates AtWNK8 function in ABA signaling and points out putative targets. The earliest events of the ABA signaling pathway occur through a central signaling module which is present in the cytosol as well as in the nucleus (HUBBARD *et al.* 2010). It is made up of three protein classes: protein phosphatase 2Cs, protein kinase SnRK2s and PYR/PYL ABA receptors. AtWNK8 has been included opposite the SnRK2 kinases. AtWNK8 was shown to interact with the protein phosphatase AtPP2CA which in the absence of ABA inhibits kinase activity by dephosphorylating AtWNK8 and AtSnRK2s. Once ABA is present it binds to the receptor AtPYR1 which in turn inhibits AtPP2CA. Subsequently, both AtWNK8 and AtSnRK2s undergo autophosphorylation and become active.

In order to function as a negative regulator of ABA signaling AtWNK8 might interfere with the ABA response in different ways:

Except for protein phosphatases regulators of the AtSnRK2s have not been described, yet. Though, physical interaction of AtSnRK2s and AtWNK8 has not been tested, it would provide the most direct way to negatively regulate ABA signaling.

A second way to interfere with the ABA signal transduction could be the interaction with transcription factors which usually promote the ABA response. It is well known that protein stability can be regulated via phosphorylation and subsequent degradation via the 26S proteasome (AL-SADY *et al.* 2006, DUEK *et al.* 2004). Destabilization of ABA-responsive transcription factors by phosphorylation could therefore allow AtWNK8 to negatively regulate the ABA response. The key transcription factor regulating ABA-induced postgermination growth arrest in young seedlings is AtABI5. Phenotypic analysis of the *wnk8* mutants strongly suggests a role for AtWNK8 during this particular stage of development. Phosphorylation of AtABI5 might provide a role for AtWNK8 to fine tune the expression of AtABI5 target genes.

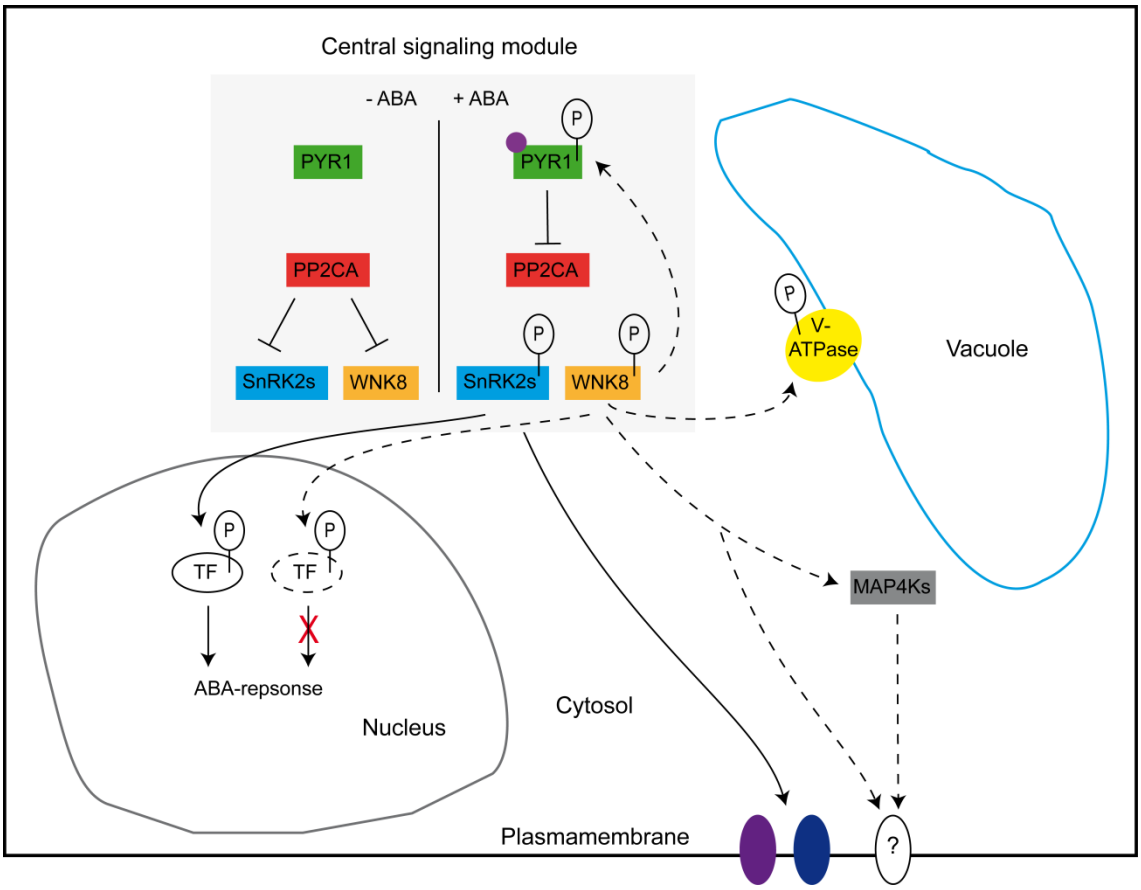

Figure 4. Proposed model for AtWNK8 action in ABA signaling and other pathways
A schematic drawing of a cell including nucleus, cytosol, plasma membrane and vacuole is shown to illustrate different compartments where AtWNK8 is active. The central signaling module of ABA signaling is present in the nucleus as well as in the cytosol (modified according to ANTONI *et al.* 2011, CUTLER *et al.* 2010, HUBBARD *et al.* 2010). Depending on the subcellular compartment SnRK2s and WNK8 act on different targets. Continuous lines indicate confirmed interactions; dotted lines represent relations that have not been confirmed, yet. An ABA-molecule is depicted as a purple ball attached to the ABA receptor PYR1. Phosphorylation is symbolized by the letter P.

Another way to control ABA signaling could be via phosphorylation of the ABA receptor protein. We showed that AtWNK8 phosphorylates AtPYR1. Particularly phosphorylation of serine S85 might be a promising way to interfere with the stability of the receptor/phosphatase complex. A reduction in phosphatase inhibition would lead to decreased activity of SnRK2s and ultimately attenuate the ABA response.

The model is consistent with the phenotypes observed for the *wnk8* mutants. The constitutively active kinase in the *wnk8-1* mutant functions as an enhanced negative regulator, thus causing ABA hyposensitivity. The *wnk8-4* allele increases ABA hypersensitivity in the *pp2ca-1 wnk8-4* double mutant. The lack of negative regulator in

the *wnk8-4* single mutant is not sufficient to cause a visible phenotype. Though few transcription factors might be stabilized if AtWNK8 is missing, this is not likely to alter ABA responses significantly. If in addition AtPP2CA is missing in the double mutant, the effect of two negative regulators is additive and an increase in ABA hypersensitivity can be observed.

How exactly AtWNK8 decreases the ABA response has to be determined. A close examination of the receptor/kinase interaction and further analysis of downstream targets are necessary to characterize the function of AtWNK8 as a negative regulator in ABA signaling in more detail. Furthermore, AtWNK8 is likely to be involved in other pathways that are not directly linked to ABA signaling (Fig. 4). Interaction with the cytosolic MAP4 kinases and the C-subunit of the vacuolar H^+-ATPase indicate that AtWNK8 functions also outside the nucleus. Since the mammalian MAP4 kinases OSR1 and SPAK control the activity of ion channels together with WNKs it remains to be determined whether AtWNK8 has a similar role in plants.

5 SUPPLEMENTARY MATERIAL

Primer for construct design

Primer	Sequence (5' – 3')
HSP18.2FWD	ATA GAG CTC CGA CAA GTA GGT TTG TTC
HSP18.2REV	ATA GGT ACC TGT TCG TTG CTT TTC GGG AG
RFP_BamHI_F	GGA TCC ATG GCC TCC TCC GAG GAC GT
RFP_BamHI_R	GGA TCC GGC GCC GGT GGA GTG GC
GFP5_BglII_F	ATA AGA TCT AAG GAG ATA TAA TCA TGA G
GFP5_BamHI_R	ATA GGA TCC TTT GTA TAG TTC ATC CAT GCC
MCS_for	GAT CCG ACG TCT CTA GAT TAA TTA ACC ATG GAC TAG TG
MCS_rev	TCG ACA CTA GTC CAT GGT TAA TTA ATC TAG AGA CGT CG
UBIQ10_BamHI_for	CTA GGA TCC CGA CGA GTC AGT AAT AAA CG
UBIQ10_BamHI_rev	CAT GGA TCC GCT GTT AAT CAG AAA AAC TC
YN173_BglII_F	ACT AGA TCT ATG GTG AGC AAG GGC GAG GAG
YN173_BamHI_R	GAA GGA TCC AAG ATC CTC CTC AGA AAT C
cYN_SpeI_for	CCA ACT AGT ATG GAG CAA AAG TTG ATT TC
cYN_XbaI_rev	CAT TCT AGA CTA CTC GAT GTT GTG GCG GAT C
nYC_AatII_For	CAT GAC GTC ATG GAC AAG CAG AAG AAC
nYC_AatII_Rev	CCT GAC GTC AGC GTA ATC TGG AAC ATC G
cYC_SalI_For	CAT GTC GAC ATG TAC CCA TAC GAT GTT C
cYC_SalI_Rev	GAT GTC GAC TTA CTT GTA CAG CTC GTC
WNK8PromFW2	ATA TCT AGA GAA GAA GAT CGG AGA TGA TG
WNK8PromRV2	ATA GGA TCCAAGA AGC CAT ATA TGT ATG CAC
WNK8SacII_fwd	ATA CCG CGG ATA TGG CTT CTG GTT CTG GAT
WNK8SalI_rv	TCT GTC GAC TCA AGA GAT GTT AAC TGC
mutK41Mfwd	GGA GAG GAG CTT TCA TGA CTG TAT ATA AGG CGT
mutK41Mrev	ACG CCT TAT ATA CAG TCA TGA AAG CTC CTC TCC
WNK8_BamHI.for	ATA GGA TCC ATG GCT TCT GGT TCT GGA
WNK8_XmaI.rev	CGA CCC GGG TCA AGA GAT GTT AAC TGC
WNK8NTnoStop_BamHI.rev	GGA TCC GCA AGG AAT GGG TCC TTC
WNK8-BamHI.Fwd	ATA GGA TCC ATA TGG CTT CTG GTT CTG GA
WNK8.NT-XhoI.Rev	ATA CTC GAG TCA GCA AGG AAT GGG TCC TTC
WNK8NdeFW	ATA CAT ATG GCT TCT GGT TCT GGA
WNK8NT-BamHI-REV	GGA TCC TCA GGC AAG GAA TGG GTC CTT C
PP2CA-NdeI For	GTT AGA CAT ATG ATG GCT GGG ATT TGT TGC G
PP2CA-SalI REV	CAC GTC GAC TTA AGA CGA CGC TTG ATT ATT C
PP2CA_PacI.for	GAC TTA ATT AAC ATG GCT GGG ATT TGT TGC G
PP2CAnoStop_SpeI.rev	GCA ACT AGT AGA CGA CGC TTG ATT ATT CCT CC
mCherry-AatII.for	ATA GAC GTC ATG TTG AGC AAG G
mCherry-SpeI.rev	TAT ACT AGT CTT GTA CAG CTC GTC CAT G
PP2CA-XmaI.FOR	CCC GGG ATG GCT GGG ATT TGT TGC GGT G
PP2CA-cmyc-SpeI.REV	ACT AGT TTA CAA ATC CTC CTC AGA GAT AAG CTT CTG CTC AGA CGA CGC TTG ATT ATT C
WNK8Bam.fw	ATA GGA TCC AAT GGC TTC TGG TTC TGG A
wnk8-1.rev2	GGA TCC TTA AAA ACG TCC GCA ATG GTT ATG ATC CAC ATC CAT G

Primer for RT-PCR and qRT-PCR

Primer	Sequence (5' – 3')
AtWNK8.fwd5	ATG GCT TCT GGT TCT GGA T
WNK8RT.rev2	CTT ACG ATA TAC CCT GAG ACT AC
vcip1160.fwd	ACA CAG CAA CAG CAA TCG C
WNK8SalI_rv	TCT GTC GAC TCA AGA GAT GTT AAC TGC
qRT1021_For	CTC TTT TAC TCT TGG GTT GAC G
qRT311.1_For	CCA GTC CTT CCA AGA TCT GCT G
TL2	CAA TCA GCT GTT GCC CGT CTC AC
pp2ca-1.fwd	GCA GTC TCC TCA GTG TGA CGC CG
pp2ca-1.rev	TCA CCA ATT GCT CTA GAC ATG GC
wnk8-4.fw	CAA CTT GAA AGG CTA TAT TCT G
wnk8-4.rv	AAC TGT GAG AGG AAT GAA TTG GAT AG
wnk8-1.fw	TGA GCG TAA TCA GGT ACT CC

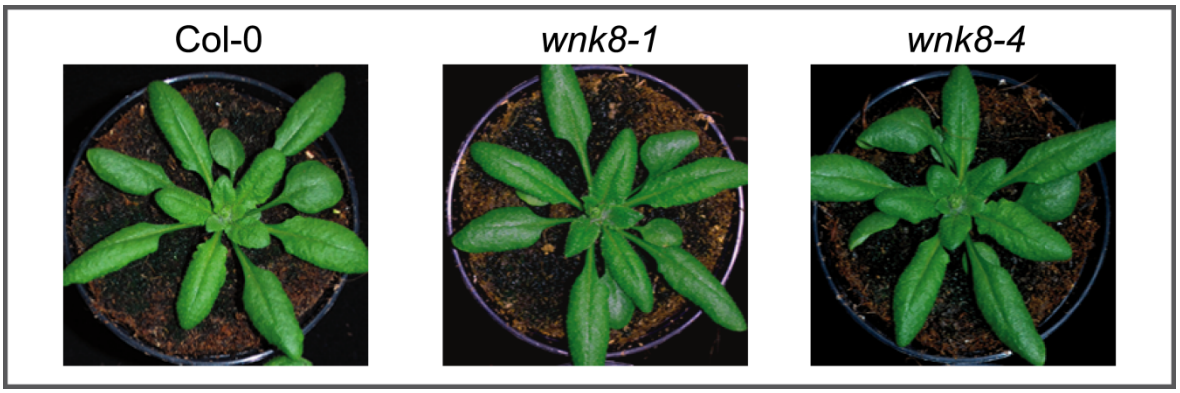

Figure S1. Soil-grown *wnk8* mutants are indistinguishable from Co-0 wild type
Col-0 wild type, *wnk8-1* and *wnk8-4* mutant plants were grown under long day conditions (16 h light, 8 h darkness). Pictures were taken 28 DAG.

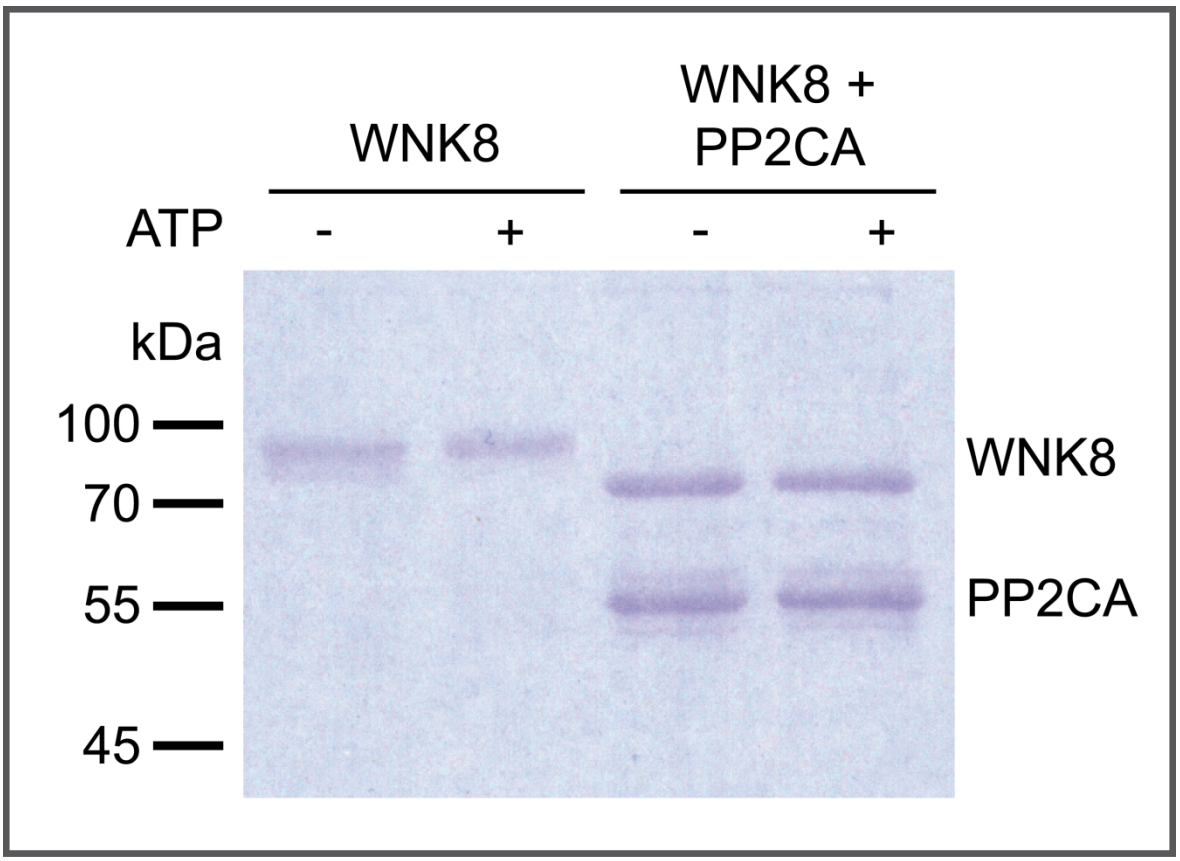

Figure S2. AtPP2CA dephosphorylates AtWNK8
AtWNK8 and AtPP2CA recombinant proteins were incubated in the absence and presence of 10 µM ATP, for 30 minutes at 30 °C. Proteins were separated on a 12 % SDS-PAGE and stained with coomassie for 1 hour, following destaining overnight. AtWNK8 fully autophosphorylated after ATP treatment. AtWNK8 mobility shift indicates desphosphorylation through AtPP2CA.

Table S1. ESI-MSA analysis of phosphorylated and non phosphorylated peptides derived from WNK8 incubated with ATP

Phosphorylation sites are printed in bold.

Peptide	Precursor ion mass (calculated)	Precursor ion mass (observed)	Comments
MW**S**HPQFEK	635.2597 (MH^{+2})	635.2618 (MH^{+2})	Part of N-terminal *strep*-tag
GPRP**T**SG**S**MA**S**GSGFLGQISSMEEADFAEK/ GPRPT**S**G**S**MA**S**GSGFLGQISSMEEADFAEK/ GPRP**TS**GSMA**S**GSGFLGQISSMEEADFAEK/	1091.0969 (MH^{+3})	1091.1018 (MH^{+3})	Phosphorylation sites not unambiguously assignable. N-terminus includes part of *strep*-tag
GPRP**T**SG**S**MASGSGFLGQISSMEEADFAEK/ GPRPT**S**G**S**MASGSGFLGQISSMEEADFAEK/ GPRP**TS**GSMASGSGFLGQISSMEEADFAEK/	1064.4415 (MH^{+3})	1064.4458 (MH^{+3})	Phosphorylation sites not unambiguously assignable. N-terminus includes part of *strep*-tag
GPRPTSGSMA**S**G**S**GFLGQISSMEEADFAEK	1037.7860 (MH^{+3})	1037.7897 (MH^{+3})	
GPRPTSGSMA**S**G**S**GFLGQISSMEEADFAEKDPSGR	1235.1901 (MH^{+3})	1235.1862 (MH^{+3})	
LY**S**EVHLLK	591.3019 (MH^{+2})	591.3040 (MH^{+2})	
IGDLGLA**T**VLQQPTAR	866.9533 (MH^{+2})	866.9557 (MH^{+2})	
IGDLGLATVLQQP**T**AR	866.9533 (MH^{+2})	866.9563 (MH^{+2})	
VT**S**NIKPQ**S**LGK	716.3359 (MH^{+2})	716.3387 (MH^{+2})	
VT**S**NIKPQSLGK	676.3527 (MH^{+2})	676.3551 (MH^{+2})	
PTALEL**S**KDPFLAR	819.4186 (MH^{+2})	819.4219 (MH^{+2})	
SVSSNEDYPW**S**QTIELQR	1109.9863 (MH^{+2})	1109.9898 (MH^{+2})	
SV**S**SNEDYPWSQTIELQR / **S**VS**S**NEDYPWSQTIELQR / SV**SS**NEDYPWSQTIELQR	1149.9694 (MH^{+2})	1149.9742 (MH^{+2})	Phosphorylation sites not unambiguously assignable
SVSSNEDYPWSQTIELQR / SV**S**SNEDYPWSQTIELQR / SVS**S**NEDYPWSQTIELQR	1109.9863 (MH^{+2})	1109.9917 (MH^{+2})	Phosphorylation sites not unambiguously assignable
SDDVTA**S**MVLR	637.2783 (MH^{+2})	637.2813 (MH^{+2})	
DEEAAGQ**S**MK**S**DI**S**ADYYFPYSANDGNAAMEAGR	1291.1453 (MH^{+3})	1291.1488 (MH^{+3})	Peptide not unambiguously identified
SDISADYYFPY**S**ANDGNAAMEAGR	1333.0311 (MH^{+2})	1333.0352 (MH^{+2})	

SDISADYYFPY**S**ANDGNAAMEAGR / SDI**S**ADYYFPY**S**ANDGNAAMEAGR	1373.0142 (MH^{+2})	1373. 0195 (MH^{+2})	First phosphorylation site not unambiguously assignable
LFYSWVDEK	1186.5779 (MH^{+})	1186.5812 (MH^{+})	**Non-phosphorylated peptides**
TINMITELFTSGSLR	841.9427 (MH^{+2})	841.9455 (MH^{+2})	
GLNYLHSQNPPVIHR	872.9657 (MH^{+2})	872.9691 (MH^{+2})	
CDNIFVNGNTGEVK	755.3537 (MH^{+2})	755.3561 (MH^{+2})	
CLLPASSRPTALELSK	843.4664 (MH^{+2})	843.4688 (MH^{+2})	
DLKCDNIFVNGNTGEVK	933.4567 (MH^{+2})	933.4605 (MH^{+2})	
CDNIFVNGNTGEVK	755.3537 (MH^{+2})	755.3561 (MH^{+2})	
DSALLASSSTSSK	627.3146 (MH^{+2})	627.3168 (MH^{+2})	
YVRPPQLEHLPMDVDHNENK	811.0656 (MH^{+3})	811.0607 (MH^{+3})	
TELNLIESQFNQSFQDLLK	1134.0813 (MH^{+2})	1134.0852 (MH^{+2})	

Table S2. Putative AtWNK8 interaction partners identified via a yeast-two hybrid screen

The first 449 amino acids of the AtWNK8 protein were used as bait in a Y2H screen to find interacting partners. Out of 64.2 million interactions 121 clones were processed and sequenced to identify the corresponding proteins. Letters A – D refer to an automatically generated score classifying the confidence in the interaction (A = very high; B = high; C = good; D = moderate; N/A = an automatic score could not be attributed). Descriptions for each gene were obtained from the Arabidopsis Information Resource (TAIR) database.

	AtG-number	Name	Description (according to the Arabidopsis Information Resource (TAIR) database	Reference
A	AT5G62280	Unknown protein	Gene expression was identified to be weakly drought- and ABA-regulated	(HUANG *et al.* 2008)
A	AT2G39730	RCA (Rubisco Activase)	Nuclear-encoded chloroplast protein required for the light activation of rubisco and involved in jasmonate-induced leaf senescence	(ZHANGandPORTIS 1999) (SHAN *et al.* 2011)
A	AT4G04570	CRK40 (Cysteine-rich RLK 40)		
B	AT3G06650	ACLB-1 (ATP-Citrate Lyase B-1)	One of two genes encoding subunit B of the trimeric enzyme ATP citrate lyase; involved in acetyl-CoA biosynthesis	(FATLAND *et al.* 2005)
B	AT3G07630	ADT2 (Arogenate Dehydratase 2)	Encodes a plastid-localized arogenate dehydratase involved in phenylalanine biosynthesis	(CHO *et al.* 2007)
B	AT5G14720 AT4G24100	Unknown protein of kinase family	Belongs to the family of MAP4 kinases	(CHAMPION *et al.* 2004b)
B	AT5G38860	BIM3 (Bes1-Interacting Myc-Like Protein 3)	Functions in DNA binding and regulation of transcription	(YIN *et al.* 2005)
B	At5G11530	EMF1 (Embryonic Flower 1)	*EMF* Genes Maintain Vegetative Development by Repressing the Flower Program in Arabidopsis; are involved in histone methylation	(CALONJE *et al.* 2008, MOON *et al.* 2003, PARK *et al.* 2011)
B	AT1G18540	RPL6A (60S ribosomal protein L6)	Ribosomal protein L6 family protein; functions in structural constituent of ribosome and is involved in translation	
B	At1G06680	PSBP-1 (Photosystem Ii Subunit P-1)	Encodes a 23 kD extrinsic protein that is part of photosystem II and participates in the regulation of oxygen evolution	(KOCHHAR *et al.* 1996)
B	AT5G63860	UVR8 (UVB-Resistance 8)	UV-B-specific signaling component, located in the nucleus and the cytosol. Associates with chromatin via histones. UV-B light promotes URV8 protein accumulation in the nucleus	(RIZZINI *et al.* 2011)
B	AT2G42490		Putative copper amine oxidase family protein	
C	AT2G30110	UBA1 (Ubiquitin-Activating Enzyme 1)	Encodes a ubiquitin-activating enzyme (E1), involved in the first step in conjugating multiple ubiquitins to proteins targeted for degradation	(GORITSCHNIG *et al.* 2007, HATFIELD *et al.* 1997)

C	AT5G16320	FRL1 (Frigida Like 1)	Member of FRI-related genes that is required for the winter-annual habit	(SCHMITZ *et al.* 2005)
D	AT1G74060	RPL6B (60S ribosomal protein L6)	Ribosomal protein L6 family protein functions in structural constituent of ribosome and is involved in translation	
D	AT5G49460	ACLB-2 (ATP Citrate Lyase Subunit B 2)	One of the two genes encoding subunit B of the trimeric enzyme ATP citrate lyase; involved in acetyl-CoA biosynthesis	(FATLAND *et al.* 2005)
D	AT3G47340	ASN1 (Glutamine-Dependent Asparagine Synthase 1) or DIN6 (Dark-inducible 6)	Encodes a glutamine-dependent asparagine synthetase and is expressed predominantly in shoot tissues, where light has a negative effect on its mRNA accumulation	(LAM *et al.* 2003)
D	AT5G67570	DG1 (Delayed Greening 1)	Encodes a chloroplast protein; mutants have pale young leave and reduced accumulation of plastid encoded transcripts suggesting a role for DG1 in regulation of plastid gene expression	(CHI *et al.* 2010)
D	AT2G25560		DNAJ heat shock N-terminal domain-containing protein	
D	AT2G29970		Double Clp-N motif-containing P-loop nucleoside triphosphate hydrolases superfamily protein	
D	AT2G26890	GRV2 (Gravitropism Defective 2)	*grv2* mutants result in a reduction in gravitropic response in hypocotyls and shoots but do not affect root gravitropism; GRV2 is required for proper formation of the endosomes involving protein trafficking to the vacuoles	(SILADY *et al.* 2008, TAMURA *et al.* 2007)
D	AT1G12320	Unknown protein		
D	AT4G32010	HSL1 (HSI2-like 1)	B3 transcription factor; HSL1 repress the sugar-inducible expression of the seed maturation program in seedlings and plays an essential role in regulating the transition from seed maturation to seedling growth	(TSUKAGOSHI *et al.* 2007)
D	AT3G05970	LACS6 (Long Chain Acetyl-CoA Synthetase 6)	Encodes a peroxisomal long-chain acyl-CoA synthetase (LACS) isozyme	
D	AT1G52880	NAM (Nac-Regulated Seed Morphology 2 or No Apical Meristem)	Transcription factor with a NAC domain, expressed in the embryo	(KUNIEDA *et al.* 2008)
D	AT4G16250	PHY D (Phytochrome D)	Encodes a phytochrome photoreceptor with a function similar to that of phyB that absorbs the red/far-red part of the light spectrum and is involved in light responses	(THOMAS 2006)
D	AT4G10730	Unknown protein of kinase family	Belongs to the family of MAP4 kinases	(CHAMPION *et al.* 2004b)
D	AT5G51430	EYE (Embryo Yellow)	Encodes a protein that is homologous to Cog7, a subunit of the conserved oligomeric Golgi (COG) complex, which is required for the normal morphology and function of the Golgi apparatus. Likely to be involved in transport or retention of Golgi-localized proteins and in maintenance of Golgi morphology	(ISHIKAWA *et al.* 2008)
D	AT2G20020	CAF1 (CRS2-associated factors 1)	Promotes the splicing of chloroplast group II introns. Splices clpP-1 and ropC1 introns	(OSTHEIMER *et al.* 2006)

N/A	AT5G46110	APE2 (Acclimation Of Photosynthesis To Environment 2)	Encodes a chloroplast triose phosphate translocator that transports triose phosphates derived from the Calvin cycle in the stroma to the cytosol for use in sucrose synthesis and other biosynthetic processes	
N/A	AT4G38210	EXP20 (Expansin 20)	Expansin -like protein, involved in the formation of nematode-induced syncytia in roots of *Arabidopsis thaliana*	
N/A	AT4G30610	BRS1 (BRI1 Suppressor 1)	Encodes a secreted glycosylated serine carboxypeptidase with broad substrate preference involved in brassinosteroid signaling via BRI1. It is proteolytically processed *in vivo* by a separate as yet unidentified protease	(ZHOUandLI 2005)
N/A	AT4G24570	DIC2 (Dicarboxylate Carrier 2)	Encodes one of the mitochondrial dicarboxylate carriers	
N/A	AT3G06455		Ubiquitin-like superfamily protein	
N/A	AT3G01490		Protein kinase superfamily protein: Putative MAPKKK protein	(GROUP *et al.* 2002)
N/A	AT1G02920	GSTF7 (Glutathione S-Transferase 7)	Encodes a glutathione transferase belonging to the phi class of GSTs	
N/A	AT2G07690	MCM5 (Minichromosome Maintenance 5)	Member of the minichromosome maintenance complex, involved in DNA replication initiation; binds chromatin	
N/A	AT1G21640	NADK2 (NAD Kinase 2)	Encodes a protein with NAD kinase activity. The protein was also shown to bind calmodulin.	(TURNER *et al.* 2004)
N/A	AT1G12440		A20/AN1-like zinc finger family protein	
N/A	AT3G46440	UXS5 (UDP-Xyl Synthase 5)	Encodes a protein similar to UDP-glucuronic acid decarboxylase.	
N/A	AT3G01930		Nodulin family protein	

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