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Exploring the Role of ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERASE 4 (AHP4) in Cytokinin Signalling

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Exploring the role of AHP4 in cytokinin signalling

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ABSTRACT

Plant hormones are key players in providing the communication signals necessary for plants to coordinate growth, development and respond to environmental stimuli. Among them cytokinins are now widely studied and understood. Cytokinins are being perceived by cells through a two-component His-Asp phosphorelay system where histidine kinases receive the phosphoryl group, histidine phosphotranfer proteins (HPts) convey the signal to response regulators which in turn, activate or inhibit cytokinin inducible genes. In *Arabidopsis*, four of the HPts (AHP1, 2, 3, 5) are well characterised for their redundant role in cytokinin signalling but the function of AHP4 remains a mystery.

In this work, I investigate the role of AHP4 in *Arabidopsis* using a combination of molecular, biochemical, and physiological approaches. Phylogenetic analysis reveals that AHP4 is conserved across angiosperms, and it forms a distinct clade very closely related to known monocot cytokinin signalling inhibitors. Functional modelling analysis of all AHPs show that AHP4 might not be able to function as an HPt due to positional effect of its conserved His region. Consistent with this, *ahp4* plants exhibit subtle phenotypes attributed to cytokinin responses suggesting a minor role in cytokinin signalling. However, the sensitivity of *ahp4* plants observed under osmotic stress, and the upregulation of *AHP4* in water limiting conditions, suggest a novel function of AHP4 in root drought tolerance. Here I show that AHP4 is essential to preserve the primary root meristem in drought stress conditions through the maintenance of phloem transport.

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Abbreviations

ARR	Arabidopsis Response Regulator
CK	Cytokinin
SAM	Shoot apical meristem
RAM	Root apical meristem
BA	Benzyladenine
tRNA	transfer RNA
IPTs	Isopentenyltransferases
cZ	cis-zeatin
tZ	trans-zeatin
LOG	LONELY GUY
CKXs	cytokinin oxidases/dehydrogenases
PIN	PIN-FORMED
AHP	Authentic Histidine Phosphotransferase
PHP	Pseudo Histidine Phosphotransferase
HPt	Histidine Phosphotransferase
PUPs	Purine permeases
TCS	Two components signalling
ENTs	Equilibrative Nucleoside Transporters
ABCG	ATP-BINDING CASSETTE TRANSPORTER G
(A)HKs	(Arabidopsis) Histidine Kinase
ER	Endoplasmic Reticulum
GUS	β-glucuronidase
GFP	GREEN FLUORESENCE PROTEIN
CZ	Central Zone
WUS	WUSCHEL
STM	SHOOTSTEMLESS
KNOX	KNOTTED-like Homeobox
GA	Gibberellic Acid
CVL3	CLAVATA3
AM	Axillary Meristems
TIR1/AFB	TRANSPORT RESISTANCE PROTEIN 1–AUXIN SIGNALLING F-BOX
MAX3	MORE AXILLARY GROWTH 3
TZ	transition zone
EZ	elongation zone (
MZ	meristematic zone
SHY2	SHORT HYPOCOTYL 2.
QC	Quiescent Centre
WOX5	WUSCHEL RELATED HOMEOBOX5

LR	Lateral Root
WT	Wild type
Col	Columbia ecotype
CDS	Coding Sequence
cDNA	complementary DNA
IPTG	β-D-1-thiogalactopyranoside
crRNA	CRISPR RNA
tracrRNA	small transactivating crRNA (
DSB	double strand brake
TagRFP	Red fluorescent tagged
NOS	nopaline synthase
RPS5A	ribosomal protein S5
gRNA	gRNA
UTR	Untranslated Region
PAM	Protospacer Adjacent Motif
PTC	Premature Termination Codons
NMD	Nonsense- Mediated mRNA decay
CaMV	Cauliflour Mosaic Virus
HDGS	Homology- dependent gene silencing
Chl	Chlorophyll
PCA	Principal Component Analysis
Fpkm	fragments per kb mapped reads
LCM	laser confocal microscopy
scRNAseq	Single cell sequencing
SE	sieve elements
CC	companion cells
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
CFDA	carboxylflorescein diacetate
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
CRISPR	Clustered Regularly Interspaced Short Palidromic Repeats
ABA	Abscisic acid

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Chapter 1: Introduction

Ever since plants colonised the land, about 450 million years ago (Plackett and Coates, 2016), they evolved to be an extremely diverse group of organisms morphologically and geographically spread to every corner of the world. Unlike animals, plants are sessile organisms and thus are largely dependent on fast adaptations to the changing conditions throughout their development. Such adaptations involve root and shoot architectural changes when plants are under abiotic stress, responses against pathogenic organisms and many more. For example, plants have evolved to sense changes in light intensity caused by neighbouring vegetation. Reduction in photosynthetically active radiation causes large morphological changes to plants including hypocotyl elongation, reduced shoot branching and early flowering (Reviewed by Casal, 2012; Zhao et al., 2017). On the other hand, below the ground, roots sense a myriad of signals regarding nutrient and water availability resulting in changes in their architecture for optimal resource acquisition. When phosphorous availability is low the primary root growth is inhibited, and the root network is expanded with an increase in the lateral root (LR) branching and enhanced root hair production. This way, the plant occupies more ground space to increase the uptake of phosphate (P) (Abel, 2011; Shahzad and Amtmann, 2017). Under water deficit conditions, primary roots grow longer and the LR emergence angle is decreased to create a steep root system, which is able to reach the deeper layers of the soil (xerotropism) (Lynch, 2013; Feng *et al.*, 2016). These are some of the many examples of adaptations that demonstrate the developmental plasticity of plant organs in response to external stimuli which ensures their survival in an ever-changing environment.

On the cellular level, these adaptations are achieved by coordinating the regulation of various cellular responses mainly through hormonal signalling. In plants, there are several phytohormones, namely: auxins, cytokinins, brassinosteroids, gibberellins, jasmonic acid, ethylene, strigolactones, and abscisic acid. An example of how hormones can regulate the cellular responses is the increase in root hair length observed in the plants grown under low phosphate

mentioned above. This adaptation in the root architecture due to low phosphate, is tightly regulated by the induction of *ETHYLENE INSENSITIVE 3* (EIN3), an ethylene signalling component, which directly targets the transcription factor *ROOT HAIR DEFECTIVE 4* (RSL4) inducing root hair elongation (Song *et al.*, 2014; Shahzad and Amtmann, 2017). However, at the organismal level, these phytohormones do not act independently to regulate these responses, there is a degree of cross talk between them which enables them to act either synergistically or antagonistically to regulate cellular processes. For example, in the shoots, auxin suppresses *ARR7* and *ARR15* expression, these are proteins that negatively affecting the cytokinin (CK) signalling. Through this suppression, CK response is enhanced and therefore auxin and CK work cooperatively to control stem cell identity in the shoot apical meristem (SAM) (Zhao *et al.*, 2010). However, auxin and cytokinin have opposing roles in the suppression of these CK signalling regulators in the specification of the stem cell niche at the root apical meristem (RAM) (Müller and Sheen, 2008).

Historically, the notion of a mobile signal in plants which could act as a phytohormone was hypothesised and identified in studies investigating phototropism and geotropism in the late 19th century. It wasn't until the beginning of the 20th century that this signal was termed as auxin (Reviewed by Enders & Strader, 2015) and since then the auxin was found to be involved in many developmental processes. In the second half of the 20th century another phytohormone started appearing the scientific literature as a compound that promoted cell division in tissue cultures. The compound found was first isolated form herring sperm DNA and was named kinetin (Miller *et al.*, 1955) but later another compound called zeatin was isolated from maize endosperm (Letham, 1973) and coconut milk. These compounds together with other forms with similar properties and chemical structures were then grouped together to form the cytokinins. In terms of their chemical composition naturally-occurring CKs are purine derivatives that closely resemble the adenine nucleotide. The N^6 position of the adenine ring is substituted with an isoprenoid or aromatic side chain to form the different types of CKs (Fig 1.1). Additional variation in the chemical structure is achieved by selective hydroxylation of the CKs and by their conjugation with various

sugars, sugar phosphates and amino acids. The most abundant natural CKs are those belonging to the isoprenoid group while the aromatic CKs were considered to only exists synthetically. However, derivatives of benzyladenine (BA) were identified in many plant species (Tarkowská *et al.*, 2003; Fré bort *et al.*, 2011; Kieber and Schaller, 2014). Since their discovery, many biological and developmental processes have been assigned to be regulated by CKs throughout the plant development (Hwang, Sheen and Uller, 2012). This is partly ensured by the large variation in the chemical structures of the CKs which determines their activity (Frébortová, Frébort and Nascimento, 2021).

The focus of this thesis lies in elucidating aspects in cytokinin signalling and their effect in plant physiological process. Therefore, the following sections of the introduction will present several known aspects of cytokinin metabolism, transport, signalling and its important role in plant development. The plant species chosen for this work is the model plant *Arabidopsis thaliana.*

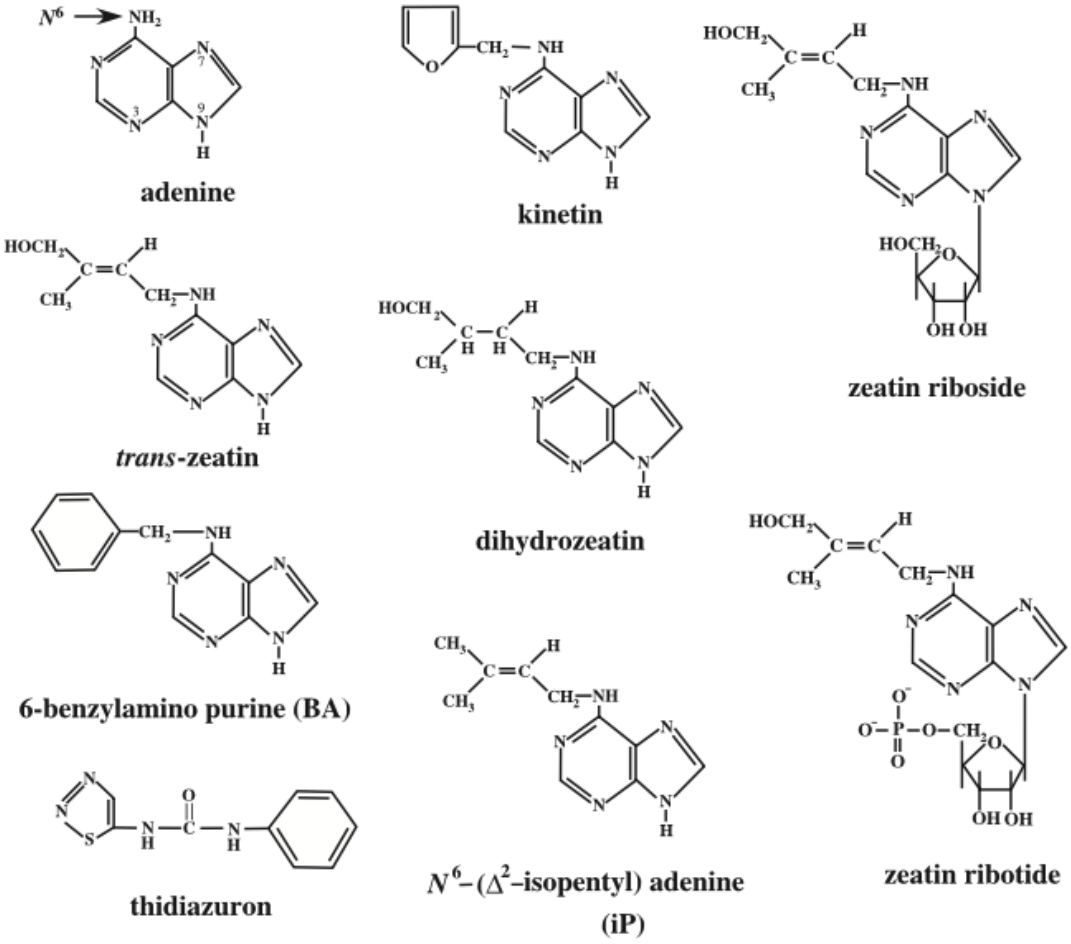


Fig 1.1 Chemical structure of the different cytokinins (CK). Adenine structure is shown on the top left. All CKs are adenine derivatives with the substitution of an isoprenoid or aromatic side chain at the N6 position. This figure was taken from the Arabidopsis book, Cytokinins chapter (Kieber and Schaller, 2014).

Metabolism of cytokinin

Despite the existence of aromatic forms of CK in higher plants, our understanding about CK biosynthesis is concentrated on the biosynthesis of isoprenoid CKs due to their abundance. Biosynthesis of isoprenoid CKs starts with the addition of the isopentyl side chain, a specific carbon chain, to the N^6 position of the adenoside 5'-phosphate (AMP, ADP, or ATP) molecule, a process called N-prenylation (Fig 1.2). These carbon chains derive from the dimethylallyl diphosphate (DMAPP), or the 1-hydroxy-2-methyl-2-E-butenyl 4-diphosphate

(HMBDP) donor molecule and the reaction is catalysed by a group of enzymes known as the adenosine phosphate- isopentenyltransferases (IPTs, Fig 1.2) (Kamada-Nobusada and Sakakibara, 2009; Frébortová, Frébort and Nascimento, 2021). The resulting molecules: isopentyl adenine (iP) riboside 5'-triphosphate (iPRTP), iP riboside 5'-diphosphate (iPRDP) or iP riboside 5'-monophosphate (iPRMP) are the inactive forms of iP type CKs. These can either be converted into active forms (iP) in reactions catalysed by LOG enzymes (explained below), or they are hydroxylated by cytochrome P450 monooxygenase (CYP735A) to form the zeatins (Letham, 1963; Fré bort *et al.*, 2011).

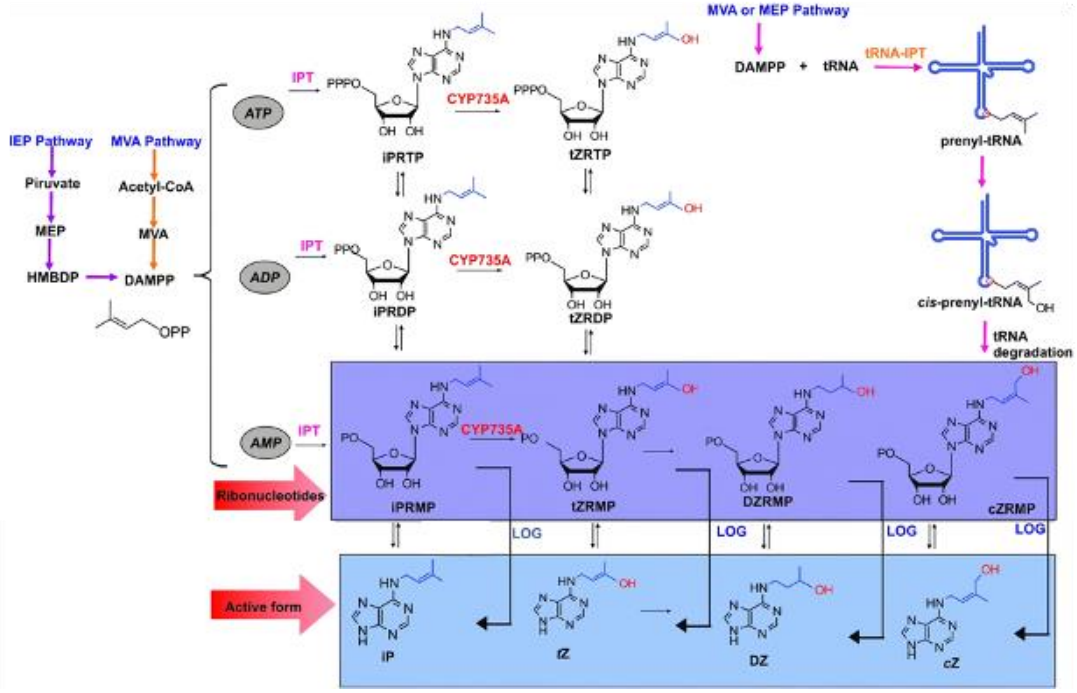


Fig 1.2 The cytokinin biosynthesis pathway in plants. The first step in *de novo* CK biosynthesis is the prenylation of AMP, ADP or ATP from DMAPP and HMBDP which are produced by the MEP or MVA pathway. This reaction is catalysed by IPT enzymes (pink). The resulting molecules become active forms of CK (iP) immediately with the help of LOG enzymes (blue) or they are hydroxylated by CYP735A (red) for the formation of zeatin type CKs, specifically the trans-zeatin (tZ). On the right the CK biosynthesis though the degradation of tRNA with the help of tRAN-IPT enzymes. These biochemical reactions produce the cis-Zeatin (cZ) CK active form. This figure is a simplified version taken by the following publication (Márquez-López, Quintana-Escobar and Loyola-Vargas, 2019).

Besides the *de novo* biosynthesis, CK is synthesised through the degradation of tRNA too. tRNAs can undergo prenylation with an addition of a cis-hydroxyl group by tRNA-IPTs. Therefore, subsequent degradation of the prenyl-tRNA results in the production of cis-zeatin (one of the zeatin types CK, Fig 1.2) (Kamada-Nobusada and Sakakibara, 2009; Márquez-López, Quintana-Escobar and Loyola-Vargas, 2019).

The regulation of CK biosynthesis relies not only on the availability of precursor molecules like DMAPP and HMBDP but also on several other factors. These factors include the expression of genes encoding the enzymes required for catalysing the biosynthesis process, as well as the activity of the enzymes involved in the pathway.

One of these essential group of enzymes in CK biosynthesis are the IPTs. In *Arabidopsis* there are nine *AtIPT* genes and based on phylogenetic analysis form two distinct clades: one with seven genes and the other with the remaining two *AtIPTs*. Disruption of *AtIPT2* and *AtIPT9* led to a decrease in cis- zeatin CK (cZ) which is produced by the degradation of tRNA pathway of CK biosynthesis. Conversely, defects in the remaining *IPTs* resulted in decrease in the production of trans-zeatin (tZ) which is produced primarily by the hydroxylation of iPRTP, iPRDP, iPRMP. These findings support their phylogenetic relationship (Takei, Sakakibara and Sugiyama, 2001). Transcriptional reporters indicated that *IPT3* and *IPT5* are expressed in phloem companion cells (CC) and later root primordia (LRP) whereas *IPT4* and *IPT8* are expressed in seeds (Miyawaki, Matsumoto-Kitano and Kakimoto, 2004; Takei *et al.*, 2004a). At the cellular level *IPT1, 3, 5, 8* are expressed in plastids but *IPT4* and *IPT7* are found in the cytosol and mitochondria (Kasahara *et al.*, 2004). Furthermore, not all *IPTs* are able to catalyse all possible reactions with the same strength. For example, *IPT4* uses ATP and ADP as a substrate preferentially over AMP (Kakimoto, 2001). The presence of multiple *IPT* genes, which are expressed in tissue and organelle specific manner and exhibiting different substrate affinities biochemically, all contribute as factors to the diversity of the biological processes CK regulates.

A second group of enzymes that are important to the CK biosynthesis are encoded by the *LONELY GUY (LOG)* genes. In *Arabidopsis* there seven genes (Kuroha *et al.*, 2009) encoding for functional proteins despite the fact that nine genes were initially predicted. The name derived after their very distinct mutant phenotype in rice which has flowers with one stamen and no pistil (Kurakawa *et al.*, 2007). Their enzymatic activity is essential to catalyse the reaction converting the some of the ribonucleotides (especially those resulting from AMP prenylation) into active CKs. Between them there is a degree of redundancy but many of those appear to have distinct roles too. Mutations in all *LOGs* (*log1-5,7,8*) resulted in plants with severe impairment of the shoot and root growth which appeared to be milder in lower order mutants (Tokunaga *et al.*, 2012). Moreover, disruption of *LOG7* caused severe retardation in the SAM development and together with *LOG3* and *LOG4* are important for primary root growth (Tokunaga *et al.*, 2012).

The levels of CK in the cells are not only determined by the anabolic processes of its biosynthesis, but by its catabolism as well. To achieve the homeostasis in the cells, various enzymes contribute to the degradation of the CKs. Such enzymes are the cytokinin glucosyl transferases (*UGT76Cs*) and the cytokinin oxidases/dehydrogenases (*CKXs*). The former enzymes catalyse glucose conjugation reactions on the active CKs leading to either permanently inactive CKs (through *N*-glycosylation) or CKs that can be stored and reactivated when needed (*O*-glycosylation) (Wang *et al.*, 2011; Jin *et al.*, 2013). Mutations of *UGT76Cs* are responsible for the permanent *N*-glycosylation produce hypersensitive to CK plants (Wang *et al.*, 2011) whereas disruptions or overexpression of the *O*-glycosylation responsible genes have no effect on plant development, indicating that the latter process can be compensated by other components of the CK biosynthesis or signalling to ensure the appropriate CK levels (Jin *et al.*, 2013).

The second group of genes encoding enzymes involved in CK catabolism are the *CKXs*. The CKX enzymes cleave the N^6 chains of the CKs to adenine, an alteration which permanently renders the free bases inactive. Because CKX cleavage

inactivates CK in a single enzymatic step, they are considered important for controlling local CK levels in cells participating in the regulation of CK-depended processes (Kieber and Schaller, 2014) . There are seven genes in the *Arabidopsis* genome (*CKX1-7*), and their respective proteins are located either in the vacuole, in the cytoplasm or the extracellular space based on translational reporter studies (Werner *et al.*, 2003; Fré bort *et al.*, 2011). Besides subcellular localisation, individual proteins differ in their affinity for different CK (Schmülling *et al.*, 2003; Fré bort *et al.*, 2011), their expression regulation and their expression patterns in the different organs of the plant (Werner *et al.*, 2003; Fré bort *et al.*, 2011). Overexpression of CKX causes defects associated with CK deficiency such as smaller shoot and SAM, and enhanced primary root growth (Werner *et al.*, 2003). Mutants of CKXs expressed specifically in the shoot (*ckx3/ckx5*) developmentally exhibit phenotypes associated with high CK levels in the shoot such as larger flowers, more siliques, and larger seed size (Bartrina *et al.*, 2011a).

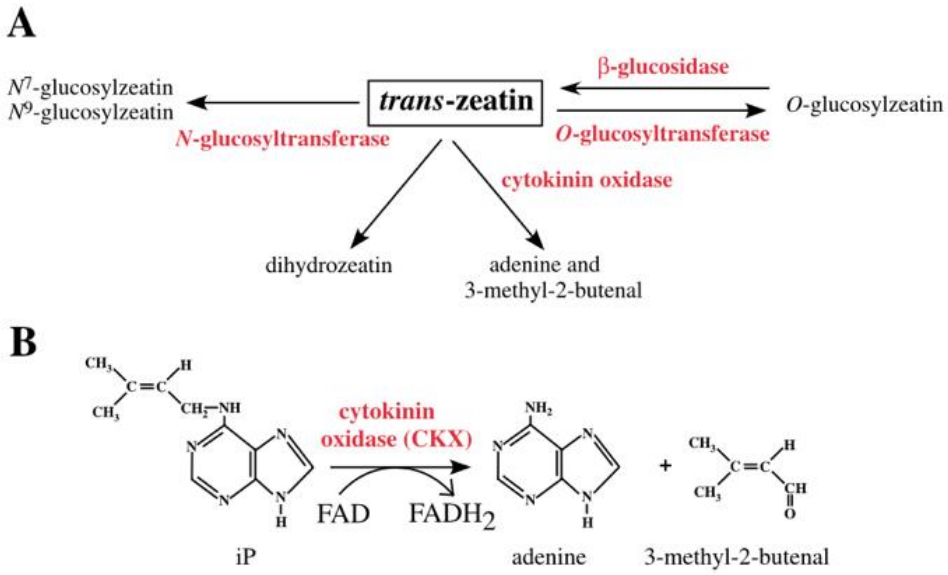


Fig 1.3 Catabolic processes of CKs. A) The glucosylation of trans-zeating by the indicated enzymes (red) can be permanent (N-glycosylation) of reversable (O-glycosylation). B) Cleavage of the N-side chain permanently degrade the CKs to adenine and the side chain derivatives. This diagram was taken from the Arabidopsis Book (Kieber and Schaller, 2014).

Transport of cytokinin

Cytokinins, like all phytohormones are chemical signals that need to be transported to different sites of the plant to enable cell and organ communication. Therefore, the signal needs to be able to travel both short distances to enable neighbouring cell to cell communication and long distances across different organs in response to environmental and internal stimuli. CK synthesis occurs in various plant organs including the roots, shoots, developing seeds and in general areas where rapid cell proliferation occurs, evident from the different expression patterns of *IPT* biosynthesis genes (Miyawaki, Matsumoto-Kitano and Kakimoto, 2004).

Even though CK biosynthesis happens throughout the plant, there is spatial specificity in the synthesis of each of the active CK forms, the directionality (root to shoot or vice versa) and the way of transport (symplastically or apoplastically) (Hirose *et al.*, 2008; Liu, Zhao and Zhang, 2019; Márquez-López, Quintana-Escobar and Loyola-Vargas, 2019). For example, the gene for the biosynthetic enzyme CYP735A which catalyses the reaction for tZ synthesis (Fig 1. 2) is expressed predominantly in the roots (Takei, Yamaya and Sakakibara, 2004). In addition, tZ is the main CK found in the xylem sap (Takei *et al.*, 2001), suggesting that tZ is synthesised primarily in the root and travels to the shoot to promote growth of the aerial parts through the transpiration stream in the xylem. Further evidence for the long-distance apoplastic transport of tZ from root to shoot come from grafting experiments in which *ipt1,3,5,7* quadruple mutant shoots were grafted onto WT roots. These mutant plants were defective in the ability to produce both tZ and iP cytokinins but grafting recovered the levels of only tZ in the shoot (Matsumoto-Kitano *et al.*, 2008; Kieber and Schaller, 2014).

In contrast to tZ transport, iP mainly follow the opposite route. They are synthesised in the vegetative parts and travel to the root via the phloem (Corbesier *et al.*, 2003; Hirose *et al.*, 2008; Liu, Zhao and Zhang, 2019). In fact, in the grafting experiment descripted above, when WT shoots were grafted onto *ipt1,3,5,7* roots, the iP levels in the root were recovered but not the tZ levels (Matsumoto-Kitano *et*

al., 2008; Kieber and Schaller, 2014). The symplastic rootward direction of the iP is important for maintaining the vascular pattern close to the root meristem by regulating *PIN-FORMED (PIN)* activity and auxin concentrations in specific cell types. Specifically, iP type CK induce *PIN* expression in the cells near the xylem pole. PINs are then directing auxin to enter these cells. Increased local concentration of auxin induces *AHP6* expression in those cells, which is one of the CK signalling inhibitors (Bishopp, Lehesranta, *et al.*, 2011). This cross talk between two major growth regulators ensures the specification and formation of protoxylem in the root. Additionally, shoot derived iP is responsible for the regulation of nodulation in response to nitrogen levels in the leaves. Nodulations are important for improving the symbiotic relationship between roots and nitrogen-fixing bacteria however, over-nodulation burdens the fitness of the plant and therefore when N is at satisfying concentrations in the shoots, CK acts as a signal to control the number of nodules (Sasaki *et al.*, 2014; Liu, Zhao and Zhang, 2019).

Perception of CK happens within the intracellular space to enable the induction of CK inducible genes leading to CK responses. Therefore, aside from long distance transport, CK signal must be perceived intracellularly via the interaction of CK molecules with the CK receptors. The position of the receptors is not clear yet. There are indications that they might be situated in the plasma membrane where they directly perceive the signal from the apoplast. Conversely, the second opinion states that the CK receptors are located on the endoplasmic reticulum (ER) membrane. This would require the CK molecules to be transported across the plasma membrane to enable interaction with the receptors (See Cytokinin perception and signalling section). In the latter case, the CK perception is only possible in the presence of specialised CK transporters. There are three types of transmembrane transporters which were implicated in this process (Kieber and Schaller, 2014; Liu, Zhao and Zhang, 2019; Márquez-López, Quintana-Escobar and Loyola-Vargas, 2019). The first family are members of purine permeases (PUPs). In Arabidopsis, there are 23 members if the PUP family but only, PUP1, PUP2 and PUP14 were able to show demonstratable CK uptake (Bürkle *et al.*, 2003; Zürcher *et al.*, 2016) even though their activity was not specific to CKs. Furthermore, the

expression patterns of those three genes differs, with *PUP1* and *PUP2* expressed in restricted areas in the leaves whereas *PUP14* having a wider expression pattern (Bürkle *et al.*, 2003; Zürcher *et al.*, 2016). Their subcellular localisation, however, remains unclear as there are contradicting opinions whether PUP14 is a transmembrane protein of the plasma membrane or on the ER membrane (Zürcher *et al.*, 2016; Romanov, Lomin and Schmülling, 2018). The PUP14 is expressed in developing cotyledons, areas where CK signalling was not observed , based on signalling reporters (TCS::GFP). This led to the theory that PUP14, by importing CK into the cytoplasm depletes the local apoplastic CK levels, and therefore prevents the CK receptors histidine kinases (HKs) from binding and in turn blocks downstream signal transduction (Zürcher *et al.*, 2016). However, the majority of the HK receptors are thought to be localised on the ER membrane (Reviewed in Romanov et al., 2018). Therefore, PUP14 expression in the developing cotyledons could result in CK signalling inhibition by depletion of the CK molecules as they would have been in the cytoplasm readily available to interact with the HKs on the ER resulting in the TCS signal. Furthermore, the PUP14 transporter on the plasma membrane should be able to interact with greater affinity with the CK substrates than the HKs do on the plasma membrane to be able to compete for the apoplastic CKs; something that was not demonstrated (Romanov, Lomin and Schmülling, 2018). Generally, there is some uncertainty about the localisation of PUPs and their precise function as CK transporter which remains to be elucidated.

The second family of transporters which act as influx CK carriers are the Equilibrative Nucleoside Transporters (ENTs). *Arabidopsis* genome has eight *ENT* genes, and ENT1, 3, 6 and 7 demonstrated ability to transport nucleosides and ribosides (tZR and iPZ) across the plasma membrane (Wormit *et al.*, 2004) and vacuole (Bernard *et al.*, 2011). Transiently expressed ENT6:GFP in tobacco leaf protoplasts was localised on the plasma membrane and transcriptional *GUS* reporter of *AtENT6* was expressed in the vasculature of the root, leaf and flowers and stomata (Hirose *et al.*, 2008). Furthermore, yeast uptake studies demonstrated that AtENT6 has higher affinity for iPR ribosides than iPZ whereas AtENT3 and 7 had very weak interactions with any of the cytokinin type ribosides (Hirose *et al.*, 2005).

Even though ENT function is also not completely clear, collectively the above results suggest that ENTs (especially ENT6) are possibly involved in the long distance transport of CK. Additionally, the difference in the substrate affinity between the ENT6 and the two ribosides it is thought to be important for the spatial regulation of cytokinin signal.

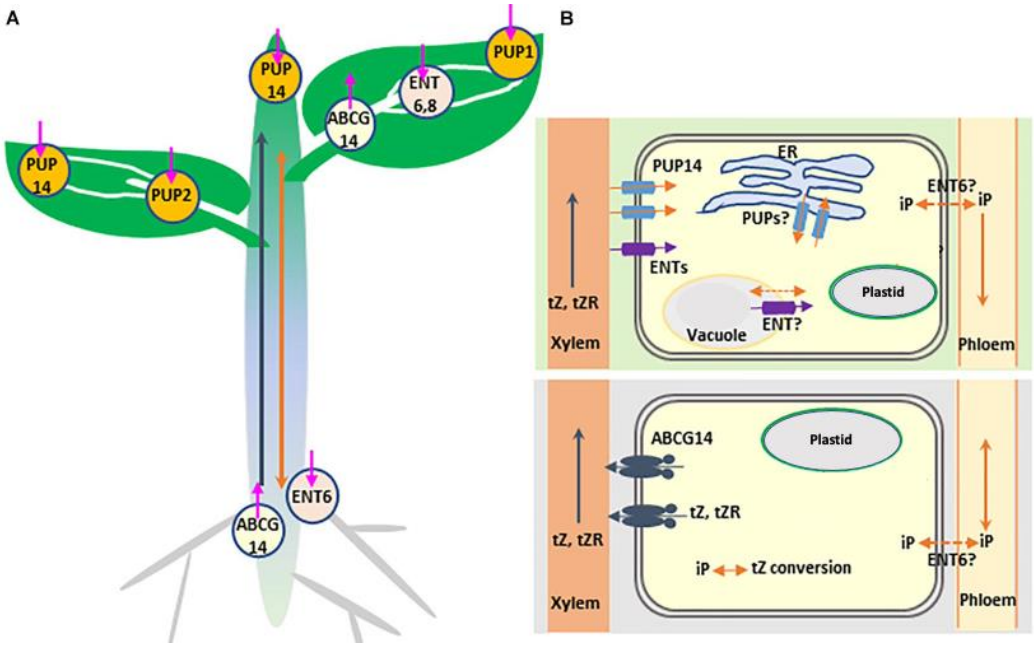


Fig 1.4 Cytokinin transport route and key players. A) CK is transported from areas where biosynthesis occurs to areas of action via the transporter proteins indicated in within the coloured circles. tZ active form of CK travel through the transpiration stream in the xylem tube from roots to shoots (black arrow). iP-type CKs have a bidirectional movement through the phloem tube (red arrows). The pink arrows on the transport proteins indicate whether they function as influx carriers (arrow pointing down) or efflux carriers (arrow pointing up). B) A zoomed in diagram showing the type of CKs are being transferred through the xylem and phloem tubes on either side of the image. In addition, subcellular localisation of the influx PUPs and ENTs, and the efflux ABCG14 carrier is indicated. This diagram is a slight modification of a diagram published in the following review article (Liu, Zhao and Zhang, 2019).

Finally, a third CK transporter is a protein belonging to the ATP-binding cassette transporter G (ABCG) subfamily, which unlike the PUP and ENT, acts as efflux pump. Disruption of ABCG14 resulted in developmental defects associated with decreased CK levels or inhibition of CK perception both in above and below the

ground organs. Furthermore, hormonal profiling of the roots and shoots demonstrated that *abcg14* plants accumulated more tZ CKs in the roots and at the same time shoot tissues were having reduced tZ levels, suggesting that ABCG14 is specifically involved in the translocation of shoot bound tZ synthesised in the root, thus acting as ATP-dependent efflux carrier. Consistent with its function, ABCG14 was localised on the plasma membrane in the root vasculature (Ko *et al.*, 2014; Zhang *et al.*, 2014; Liu, Zhao and Zhang, 2019).

Cytokinin perception and signalling

Cytokinin signals through the multi-step two-component signalling (TCS) mechanism. Based on phylogenetic analyses, this mechanism of signalling originated in bacteria, and it is employed as their main signalling pathway to respond to external cues and regulate their physiological processes such as cell division and sporulation (Reviewed in Schaller et al., 2011). The importance of this signalling type in prokaryotes is demonstrated by the amount of different TCS systems are found in their genomes. While numbers differ depending on the complexity of the cells, the average number of TCS pathways in bacterial species is around 30 (Schaller, Shiu and Armitage, 2011; Kieber and Schaller, 2014). TCS involves a transmembrane sensor protein with a conserved histidine kinase domain which acts as a transmitter of the signal, and a variable input domain (Fig 1.5). Upon signal perception of the input domain, ATP donates a phosphoryl residue to phosphorylate the HK domain of the sensor kinase. Following this autophosphorylation, the phosphoryl group is taken up by the aspartate (D) of a response regulator (RR) at the receiver domain of the protein to activate an output domain associated with the RR (Fig 1.5 A). Some RRs have a DNA binding domain and therefore act as transcriptional activators whereas those lacking this domain have other functions including inhibitions of the above signal by preventing transcriptional induction (Schaller, Shiu and Armitage, 2011; Kieber and Schaller, 2014).

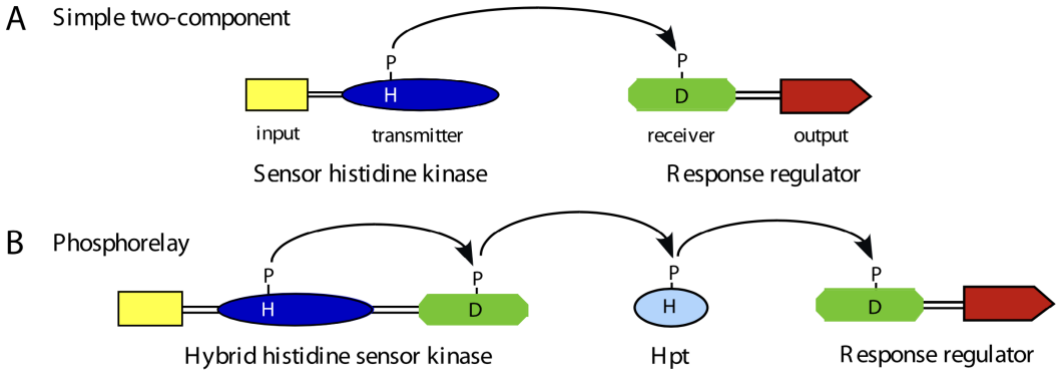


Fig 1.5 Schematic of the two-component phosphotransfer signalling systems (TCS). A) Simple TCS found in bacteria with a sensor transmembrane protein containing a variable input sequence where the signal is recognised attached to the highly conserved His containing sequence which is phosphorylated by ATP, and the response regulator with the Asp containing sequence as the receiver domain. B) the more complex multi-step TCS which utilises the hybrid sensor protein (containing an input, His and Asp domains), the Authentic phosphotranferases and the response regulators for the phosphorelay from the membranes, across the cytoplasm and into the nucleus. Image was taken from "The Arabidopsis Book" (Kieber and Schaller, 2014).

Advances in genome sequencing allowed identification of several components of the TCS to be identified in plants and fungi, which most likely were acquired through lateral gene transfer during endosymbiotic events that led to mitochondrial and plastids development (Anantharaman, Iyer and Aravind, 2007; Schaller, Shiu and Armitage, 2011). The TCS mechanisms in these two kingdoms is an expanded version of that found in bacteria. One of the differences is the structure of the transmembrane sensor kinases which in plants and fungi is termed a hybrid HK sensor. This is because, aside from the input and transmitter domains with the H residue, it also contains a receiver domain with a D residue (Fig 1.5 B). The phosphorylation happens in sequential phosphorelay steps between H and D residues of the HK. The phosphoryl group then reaches the D on the receiver domain of the RR with the help of the Authentic histidine phosphotransferases (AHP of HPt) which can move between the nucleus and the cytoplasm (Fig 1.5 B) (Punwani and Kieber, 2010). The multi-step TCS with the hybrid sensor kinases and the AHPs is thought to have evolved to accommodate the increased complexity of

plant and fungal cells since signal transduction needs to occur across greater distances and pass a nuclear membrane, with phosphorylated histidine forming a more stable linkage than that of phosphor-aspartate (Schaller, Shiu and Armitage, 2011). Interestingly, the complexity of the plant and fungal systems is also addressed via the expansion of genes encoding for proteins involved in the TCS. For example, in plants, there are multiple HKs, AHPs and RRs with the latter being a significantly larger protein family (Schaller, Shiu and Armitage, 2011; Kieber and Schaller, 2014). Similarly, fungi genomes encode for multiple proteins in their corresponding TCS, with the difference that HKs are found in greater numbers than AHPs or RRs (Schaller, Shiu and Armitage, 2011).

In plants ethylene and cytokinin are the only two hormones that signal via the TCS but for the purpose of this project only cytokinin signalling components and signal transduction will be discussed. In *Arabidopsis*, there are six histidine kinases but not all of them are very well understood. Then function of AHK2, AHK3 and AHK4/CRE1/WOL1 and their involvement in CK signalling has been well demonstrated (Kieber and Schaller, 2014). Phylogenetic studies indicate that these three kinases are closely related. Conversely, the remaining three histidine kinases, AHK1, AHK5/CKI2 and CKI1, display a more distant phylogenetic relationship (Kieber and Schaller, 2014) and less information exists in the literature about their function in the CK signalling pathway. There are some reports about CKI1 inducing CK signalling when overexpressed (Deng, Dong, Mu, Ren, Zheng, Ji, W.-C. Yang, *et al.*, 2010; Nongpiur *et al.*, 2012) and AHK5 being a cytoplasmic only sensor is involved in H_2O_2 homeostasis (Desikan *et al.*, 2008; Nongpiur *et al.*, 2012) but whether they function in the CK signalling remains to be proven. The HKs interact with themselves or each other to form homo-or hetero-dimers (Caesar et al., 2011) and despite the initial thought that they were plasma membrane proteins, most of them were found to be predominantly localised on the ER membrane by various reports (Caesar *et al.*, 2011; Wulfetange *et al.*, 2011; Romanov, Lomin and Schmülling, 2018).The input sequence on the outer side of the cell or the inside of the ER lumen is a CHASE (cyclase/histidine kinases associated sensor extracellular) domain which acts as the recognition site of CKs (Anantharaman and Aravind,

2001). Through utilisation of yeast and E. coli TCS systems it was possible to determine that AHK proteins perceive active forms of CK only and that there is a degree of specificity to that interaction (Kieber and Schaller, 2014). All AHKs have affinity for all bioactive forms of CK but there is a subtle difference in relative affinities between the AHKs and the CK type. For example, AHK3 has higher affinity for tZ than iP whereas AHK2 and AHK4 have high affinity for iP (Takei *et al.*, 2004b). Inside the cytoplasm, signal transduction is initiated by the activation of HK resulting in the autophosphorylation of the His residue, the phosphate is then transferred to the Asp of the receptors receiver domain of the protein containing the H and D residues respectively (Inoue *et al.*, 2001; Schaller, Shiu and Armitage, 2011; Kieber and Schaller, 2014).

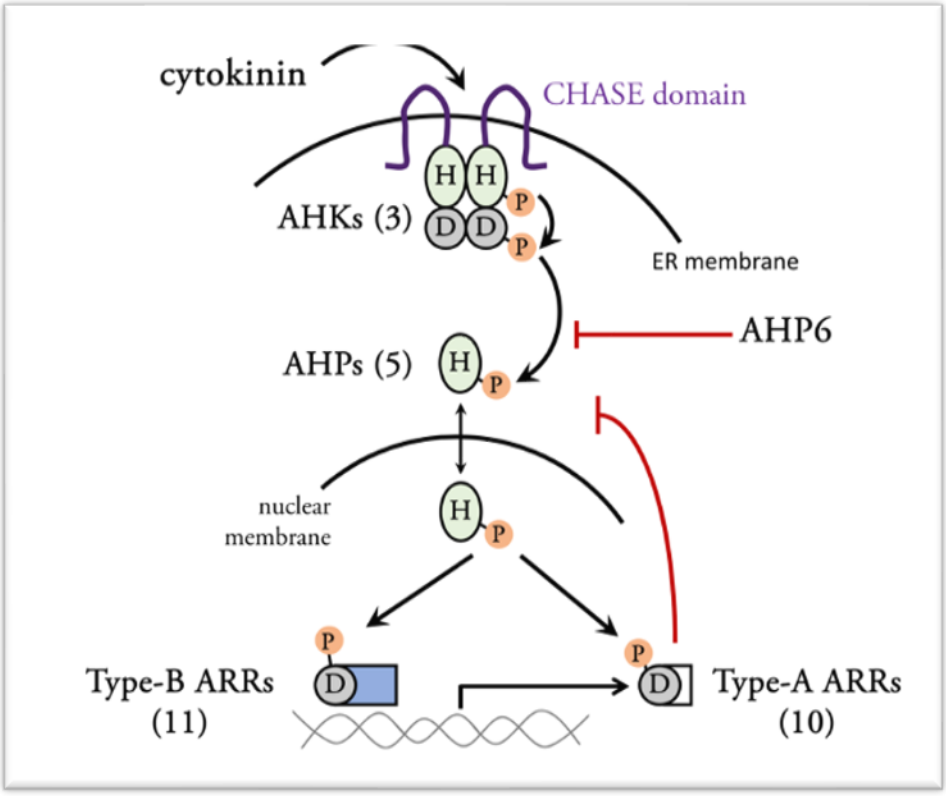


Fig 1.6 The CK signalling model. The perception of bioactive forms of CK happens on the extra-cytoplasmic CHASE domain of the AHKs which causes the autophosphorylation of the kinase domain on the His (H) residue followed by the phosphorylation of the receiver domain of the AHK on the Asp (D) residue. The phosphorelay continues with the AHPs which become phosphorylated as a result of their interaction with the AHKs and transfer the signal to the nucleus to phosphorylate and therefore active type-A and type-B ARRs. These inhibit or induce respectively the induction of CK inducible genes. AHP6/PHP1 is a protein with very high sequence homology with AHPs which has an Asn residue in the place of the H-accepting residue in the conserved region of the protein. This modification in the structure is enough to cause the protein to be unable to participate in the phosphorelay in a positive manner. Instead, it competitively interacts with AHKs to prevent them from interacting with AHPs and inhibits the phosphorylation between AHPs and ARRs. This image was taken from the Arabidopsis book (Kieber and Schaller, 2014)

The phosphorylated receptor receiver domain then passes the phosphate to the phosphotransferases. These are cytoplasmic small proteins (about 20kDa) which can move between the cytoplasm and the nucleus transporting the signal from the membrane bound AHKs to the RRs in the nucleus (Hwang and Sheen, 2001; Hutchison and Kieber, 2002; Punwani and Kieber, 2010). There are four AHP proteins in *Arabidopsis* (AHP1-3 and 5) and they all contain a highly conserved sequence in the domain where the His accepting residue is located. Quantitative analysis of translational fusion with GFP indicated that AHPs move between the nucleus and cytoplasm independently from the level of CK or their phosphorylation status (Punwani and Kieber, 2010; Punwani *et al.*, 2010). An explanation to this phenomenon might be to ensure a quicker response to the fluctuating levels of CK in the extra-cytosolic environment. Having a pool of phosphorylated AHPs moving freely from nucleus to cytoplasm is beneficial in the cases where CK levels drop rapidly. This can cause the interacting phosphatases to dephosphorylate AHPs. Such interaction is observed both in *in-vitro* and *in-planta* experiments with the AHK4, where it acts as kinase under high CK levels but when CK levels decrease it switches to a phosphatase state to dephosphorylate the AHPs (Mähönen *et al.*, 2006). Biochemical and physiological assays show that there is a level of redundancy between the AHPs. For example, when chemical associations of AHPs were tested with AHK5 the majority of AHPs showed associations albeit with different affinities (Bauer *et al.*, 2013). Additionally, loss-of-function experiments of only higher order *AHP* mutants demonstrated changes in CK sensitivity on various CK associated phenotypes (Hutchison *et al.*, 2006; Jeon and Kim, 2013; Nishiyama *et al.*, 2013). AHP4 is a protein believed to belong to the family of the phosphotransferases because of its great homology with the AHPs and its canonical structure. Interestingly, despite its canonical structure with a His in the phospho-accepting conserved domain, AHP4, was found not to function in the same manner as other members of its family raising the question whether it functions as a positive regulator of the CK signalling. In the examples mentioned above, AHP4 failed to show any interaction with AHK5 while all other AHPs were able to form an interaction with this histidine kinase (Bauer *et al.*, 2013). Furthermore, when *ahp4*

was included in the loss-of-function analyses of the higher order mutants, it appeared to have a negative action on the CK responses (Hutchison *et al.*, 2006).

A sixth member of the phosphotranferases family exists in *Arabidopsis*, AHP6. This is a protein similar to the other AHPs with the difference that lacks the His residue in the conserved domain of the protein. The His residue is substituted with an asparagine (Asn) and this change has rendered the protein unable to become phosphorylated (Mähönen *et al.*, 2006). Because of its inability to accept the phosphoryl group, the AHP6 is also named pseudo-phosphotransferase 1 PHP1 and it is a negative regulator of the TCS system. This was demonstrated with *in-vitro* phosphotransfer assays which demonstrated that phosphorylation is inhibited between AHP1 and ARR1 in the presence of AHP6 (Mähönen *et al.*, 2006). Additionally, biochemical assays revealed that AHP6 has the capacity to interact with AHK5 albeit with lower affinity compared with the AHPs (Bauer *et al.*, 2013). Even though interactions with other AHKs have not been shown, this might be another mechanism of AHP6 inhibition by competing for AHK interaction with AHPs, thus reducing CK signal transduction.

Negative regulation of the CK signalling was demonstrated in crop plants too. Specifically, in rice this is achieved through the action of the OsPHPs. Rice genome encodes for 3 OsPHPs (PHP1-3) as oppose *Arabidopsis* which has one PHP (AHP6). Mechanistically, rice PHPs are functioning similar to the Arabidopsis PHP. This is because they have Gln at the crucial domain of the protein where His is normally situated for the protein to function as a HPt. Consequently, this changes renders the OsPHPs unable to accept and subsequently pass on the phosphate (Vaughan-Hirsch *et al.*, 2021).

The final step for the TCS is the phosphorylation of Arabidopsis Response Regulators (ARRs) by the AHPs. These are transcription factors which activate or inhibit the transcription of CK inducible genes. There are two types of ARRs: type-A and type-B ARRs. Phylogenetic analysis of the receiver domain of ARRs clearly

separates the two types suggesting that they have clear and diverse roles in regulating CK responses (Fig 1.7 A).

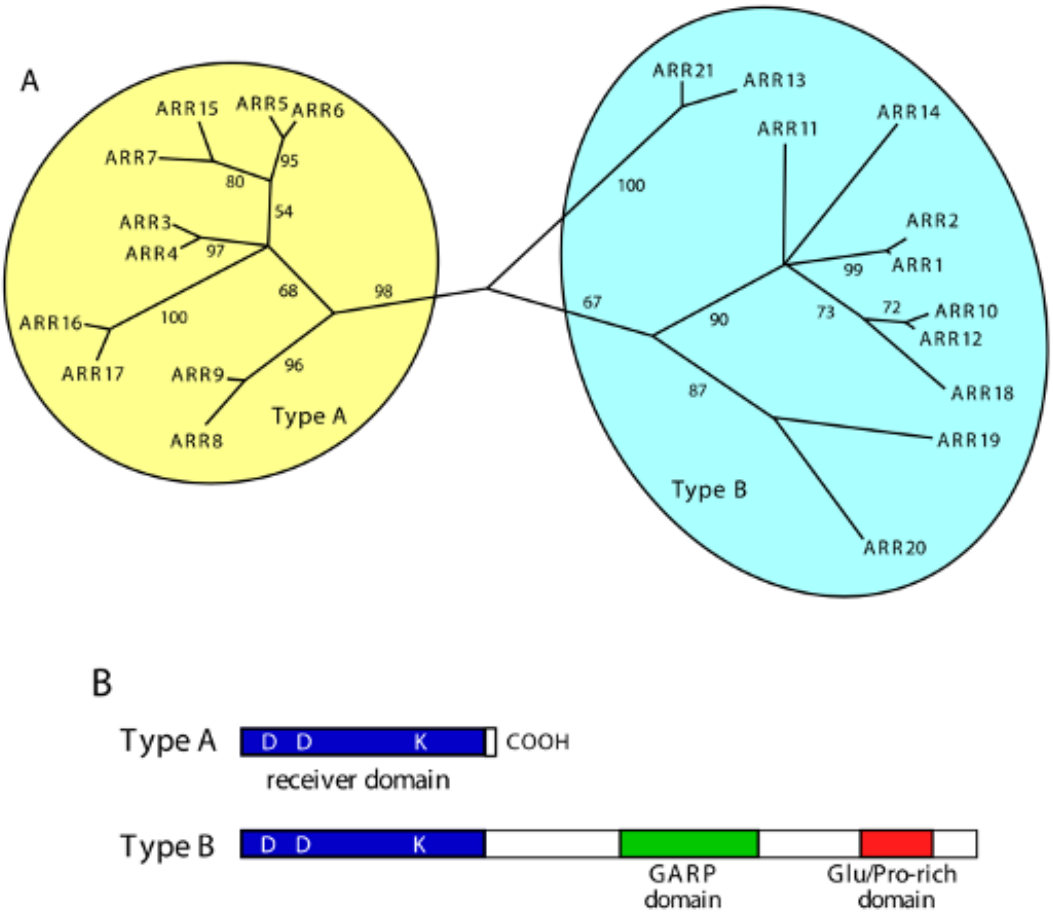


Fig 1.7 The structure of the two types of the Arabidopsis Response Regulators. A) Phylogenetic tree showing the relationship of the ARR receiver domains. The proteins form two distant clusters indicative of their diverse function. The numbers on the branches represent the bootstrap values. B) The structure of the two ARR types. Proteins belonging to the type-A ARRs have the receiver domain only but those in type-B family they contain an additional C-terminal domain with GARP DNA binding sites and Glu/Pro rich site necessary for nuclear localisation signal. The figure was taken from the Arabidopsis book, Chapter: Cytokinin (Kieber & Schaller, 2014).

The *Arabidopsis* genome encodes for eleven type-B ARRs, and they are responsible for regulating the CK transcriptional response. In *type-B ARR* mutant backgrounds (especially of higher order), expression of genes previously enriched by CK treatment is reduced and fails to increase when CK is exogenously applied (Argyros *et al.*, 2008). Their structure consists of a receiver domain and a C terminal output domain where DNA binding sites are located (Fig 1.7 B). These DNA binding sites are the GARP domain (named after the proteins it is found in, the GOLDEN2 in maize, the ARRs and Psr1 in Chlamydomonas) and the glutamine/proline (Glu/Pro) rich domains which act as nuclear binding and localisation signal sequences respectively (Lohrmann *et al.*, 2001; Hosoda *et al.*, 2015) . In fact, a concatemer subset of the GARP domain fused with GFP is widely used to detect the CK signal by tracking the activity of type-B ARRs, termed the *Two component signalling sensor* (TCS:GFP) (Zürcher *et al.*, 2013). On the N-terminal of the protein, the receiver domain is the part of the protein that interacts with AHPs, and it contains the phospho-accepting Asp (Sakai *et al.*, 1998; Lohrmann *et al.*, 2001; Veerabagu *et al.*, 2012). *In-vitro* assays demonstrated that when Asp was replaced with Glu in ARR2 the phosphorylation was inhibited (Hass *et al.*, 2004). Similarly, ARR18 failed to activate transcriptional responses when the crucial Asp was changed to either Glu or Asn *in-planta* (Veerabagu *et al.*, 2012). Another function of the receiver domain of the type-B ARRs was found in domain mapping experiments of ARR18 where deletion of the receiver domain resulted in pleiotropic growth defects directly associated with increased CK transcriptional activation (Liang *et al.*, 2012). Therefore, the default state of type-B ARRs is transcriptionally inactive because of the inhibition applied by their receiver domain by reducing the DNA binding capacity. Only upon phosphorylation of that domain the repression is lifted and transcription of CK inducible genes is activated (Liang *et al.*, 2012; Kieber and Schaller, 2014).

Like all CK signalling components, the type-B ARRs exhibit functional overlap between them, and phenotypes associated with CK responses (similar to those observed in multiple *ahk* or *ahp* knockout plants) are only observable when

multiple genes of this family are disrupted (Kieber and Schaller, 2014). Furthermore, while some members of the family are expressed broadly in plant tissues, others exhibit specificity in their expressions pattern. For example, *ARR2* transcripts were detected in many areal plant tissues but it is unclear whether there is any expression in the root. Some reports indicate high expression in root tissue (Sakai *et al.*, 1998) and others find no transcripts in the same tissues (Lohrmann *et al.*, 2001). Additionally, when looking at GUS transcriptional reporter, ARR2 expression is found only in the mature pollen unlike the broader transcript detection reported earlier (Lohrmann *et al.*, 2001). The above studies, with the often contradicting results indicate that expression pattern regulation of the *type-B ARRs* might be influenced greatly by the growth conditions and together they contribute to the regulation of the vast CK related responses (Hutchison and Kieber, 2002; Kieber and Schaller, 2014).

The type-A ARRs are significantly smaller proteins than type-B ARRs as they only consist of a receiver domain which is between two short amino acid sequence at the N- and C-terminal of the protein (D'agostino, Deruè and Kieber, 2000). There are ten type-A ARRs in Arabidopsis and unlike B-ARRs whose expression is not influenced from the CK levels (Kiba *et al.*, 1999), A-ARRs are upregulated at elevated CK levels (D'agostino, Deruè and Kieber, 2000). This is likely due to the fact that their promoter regions have many B-ARR binding sites and upon phosphorylation the activated B-ARRs induce the expression of *A-ARRs* (Kieber and Schaller, 2014). Phosphorylation of type-A ARRs is essential for the function of the proteins and just like in B-ARRs happens via the interaction with AHPs to transfer the phosphoryl group from their His to Asp residue on the receiver domain of A-ARRs, resulting in a stable form of a protein able to suppress CK responses (To *et al.*, 2004, 2007). Evidence of the interaction of A-ARRs with AHPs comes from molecular studies, yeast-two-hybrid studies, and in-vitro phosphotransfer assays (Imamura *et al.*, 1999; To *et al.*, 2007). The suppression of CK responses is likely to happen through competition of A-ARRs and B-ARRs for the interaction with AHPs. This would mean that in increased CK concentrations, the population of phosphorylated AHPs within the cell is increased which subsequently increases the

B-ARRs's phosphorylated status. These then activate expression CK inducible genes, including A-ARRs which compete with B-ARRs for the interaction with AHPs, therefore inhibiting B-ARRs action.

Expression of *A-ARRs* were found in all tissues of the plant but, like in all other signalling components, there is some specificity for the individual genes. For example, the GUS transcriptional reporter of *ARR5* indicated that its expression was mainly localised in the apical meristems of shoot and root under basal CK levels (D'agostino, Deruè and Kieber, 2000). In contrast, similar transcriptional reporters for *ARR15* and *ARR16* show that their expression was limited to the vasculature of the root and the endodermis (Kiba, Yamada and Mizuno, 2002). Additionally, *arr3,4,5,6* plants were more sensitive to elevated levels of CK in many physiological responses whereas single mutants do not have any responses (To *et al.*, 2007). This suggests that many of *A-ARRs* have redundant functions in negatively regulating the CK responses.

Role of cytokinin in plant development

CK is implicated in modulating many aspects of plant growth and development. Tight regulation of such diverse and important processes is achievable firstly through the involvement of many signalling, biosynthesis, and transport components (evident by the number of different components each gene family has) and secondly through the interaction of those components with other plant hormones in a complex network, knows as hormonal crosstalk.

Cytokinin in embryogenesis

During embryogenesis, auxin and cytokinin work antagonistically to coordinate the establishment of the root and shoot apical meristems (RAM and SAM respectively). In the early globular stages of embryogenesis, PIN7 redistributes auxin to the lower embryonic cell where hypophysis is formed. Asymmetrical division of the hypophysis results in one upper cell in which CK signalling components are active and one larger daughter-basal cell where auxin concentration induces the expression of *ARR7* and *ARR15* which in turn suppress CK signalling (Müller and Sheen, 2008). The CK signalling suppression by auxin in this cell ensures the preservation of the root-stem cell niche (Su, Liu and Zhang, 2011).

Cytokinin in regulating Shoot Apical Meristem

Coordination of cytokinin signalling with other phytohormones is necessary for the maintenance of SAM post-embryonically. The SAM consists of three zones. In the middle, the central zone (CZ), provides cells to neighbouring zones. In the periphery of CZ there are zones which give rise to lateral tissues and leaf primordia. These are named the peripheral zones (PZ). The rib zone is below the CZ and generates the stem tissues (Fig 1.8) (Su, Liu and Zhang, 2011). In the CZ a group of cells express *WUSCHEL* (*WUS*), a homeodomain transcription factor, and *SHOOTSTEMLESS* (*STM*) which is a member of the KNOTTED-like homeobox (KNOX) proteins. Together these proteins regulate cell proliferation, ensuring that the cells

in CZ will retain their stem cell identity (Su, Liu and Zhang, 2011; Kieber and Schaller, 2014). In *Arabidopsis* SAMs, increased CK activates *STM* which ensures the CK levels will remain high by activating the induction of *IPT7.* Increase in CK concentration in the area induces the expression of other *KNOX* members which facilitate the repression of the biosynthesis of a phytohormone promoting cell elongation known as gibberellic acid (GA) and enhancing genes related to GA catabolism (Su, Liu and Zhang, 2011; Kieber and Schaller, 2014). Furthermore, CK induction leads to the expression of *WUS* (which induces cell proliferation). *WUS* expression is controlled by *CLAVATA3* (CVL3) via a negative feedback loop since one downregulates the other (Fig 1.8 A). A second negative feedback loop regulates CK levels in the CZ of the SAM involves *ARR7 and ARR15* expression. These genes are induced by CK but they themselves inhibit CK transcriptional action. To overcome this CK inhibition, auxin signalling steps in via ARF5/MONOPTEROS to inhibit *ARR7 and ARR15* expression providing an example of how auxin and cytokinin interact synergistically to regulate the stem cell identity in the SAM, unlike their interaction in the RAM (Su, Liu and Zhang, 2011; Kieber and Schaller, 2014). However, at the PZ, auxin accumulation is essential for organ initiation and in these cells cytokinin biosynthesis is inhibited via the inhibition of *STM* (Reinhardt *et al.*, 2003; Su, Liu and Zhang, 2011).

Increases in the levels of CK, achieved by disruptions of single or multiple *CKXs*, prolong the undifferentiated stem cell identity of cells in the SAM, resulting in larger meristems. The increased size of the meristem enables for more and larger flowers which directly impact positively the yield in *Arabidopsis* as well as for economically important plants such as rice and maize (Schmülling *et al.*, 2003; Ashikari *et al.*, 2005a; Bartrina *et al.*, 2011a).

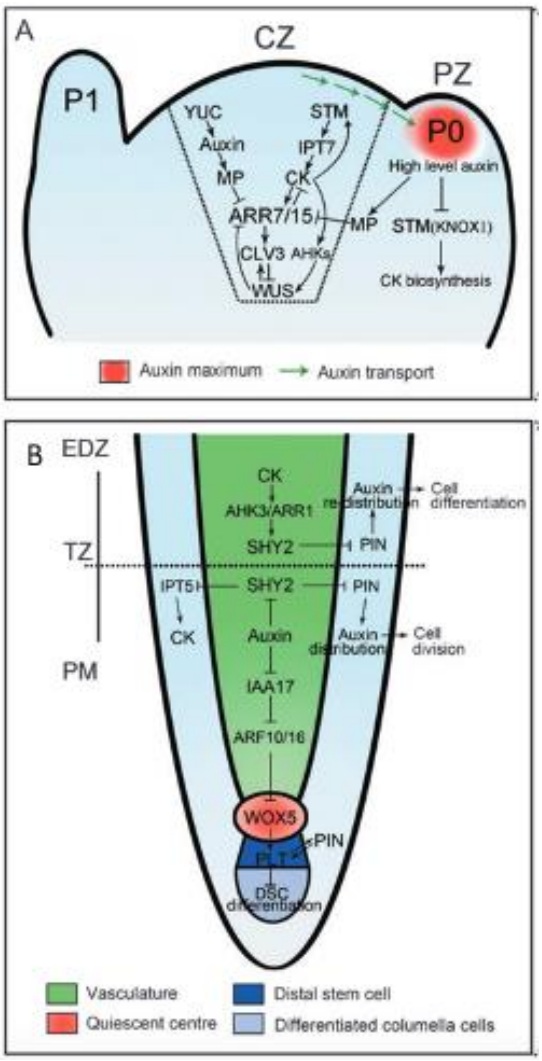


Figure 1.8 Interaction between CK and auxin in modulating the function of the two apical meristem. A) At the shoot apical meristem (SAM), the central zone (CZ) contains the stem cells and feeds the surrounding zone undifferentiated cells therefore they are areas of high cell proliferation rates. There CK signalling include WUS expression which is important for rapid cell division and WUS inhibits *ARR7/15* expression to block CK inhibition. Auxin signalling mechanism contributes to *ARR7/15* repression via ARF5/MP action and together WUS and MP ensure the CK levels in the CZ at SAM is high. Around the CZ there are the periphery zones (PZ) from which differentiation of new tissues occurs. In those areas of the SAM CK and auxin work antagonistically. B) At the root apical meristems, the meristematic zone (MZ) or proximal meristem (PM) cells originating from stem cells around the quiescent centre (QC) proliferate with auxin driving this process through repression of CK. Past the transition zone (TZ) is the elongation zone (EZ) where auxin distribution is inhibited by CK. In this area cells elongate and differentiate into their respective cell files. This image is a simplified diagram from the following review article (Su, Liu and Zhang, 2011).

Regulation of shoot branching

Production of larger flowers by maintaining SAM activation is one way to increase yield, however increasing the number of stems able to carry flowers, known as branching, results in even larger yield increases. Branching comes from the activation of axillary meristems (AM) situated at the base of each leaf and divide rapidly to give rise to leaves, stems, and flowers. AM activation is largely influenced by the surrounding environment of the plant but is tightly controlled through the interaction of various hormones (Domagalska and Leyser, 2011; Muller and Leyser, 2011; Wheeldon and Bennett, 2021). CK accumulation at AM leads to their activation. Auxin originating from the SAM is transported basally and inhibits CK biosynthesis through the negative interaction of the AXR1/AFB (AUXIN RESISTANCE PROTEIN 1–AUXIN SIGNALLING F-BOX PROTEIN) with *IPTs* (Muller and Leyser, 2011). At the same time, auxin causes the upregulation of strigolactone biosynthetic genes *MORE AXILLARY GROWTH 3* (*MAX3*) and MAX4 to inhibit axillary bud outgrowth and preserve the apical dominance (Domagalska and Leyser, 2011).

Development of SAM and AM is also controlled by root derived tZ CKs which travel via xylem to the shoot tissues (Hirose *et al.*, 2008). Reduction of the level of tZ in the shoot by disruption of CK transporter genes like *ABCG14* (See "Transport of cytokinin") resulted in several shoot developmental defects such as, smaller SAM, shorter inflorescence stem, smaller rosettes, and reduction in flower numbers (Reviewed in Wheeldon & Bennett, 2021). These are the same developmental defects observed in plants with disruptions in CK biosynthesis genes (for example *IPT* and *LOG;* See "Metabolism of cytokinin") or genes involved in the signalling pathway (for example *AHKs, AHPs* and B-*ARRs;* See "Cytokinin perception and signalling"). The root derived CK is essential in shaping shoot architecture and has evolved as a communication signal between the two organs to enhance adaptation to the soil heterogeneity (Wheeldon and Bennett, 2021). Specifically, changes in nutrient availability are conveyed through CK levels. For example, nitrate, phosphate, sulphate, and iron stimulate the expression of CK biosynthetic enzymes IPT3 and CYP735A2 in the roots which result in higher levels of CK in shoot (Takei *et*

al., 2001, 2004b; Hirose *et al.*, 2008). Such communication allows the modification of shoot growth based on nutrient availability.

Additionally, in the aerial parts of the plant, CK determines the arrangement of organs spatiotemporally (phyllotaxy) by working antagonistically with auxin (Besnard *et al.*, 2014). And at the growing leaves, CK promotes cell division and inhibits leaf senescence (Kieber and Schaller, 2014). Collectively, as indicated above, CKs in the shoot promote growth and development of those tissues.

Maintenance of Root Apical Meristem by cytokinin

CK plays a central role in shaping the root growth and regulating its development. However, unlike in the above the ground tissues where it promotes growth, in the roots cytokinin plays an inhibitory role.

Post-embryonically, the size of the RAM is determined by the antagonistic interaction of auxin and CK between the elongation zone (EZ) where cells differentiate and the meristematic zone where cell division is happening (MZ) (Fig 1.8 B). In the EZ, shoot derived CK in the form of iP activates the transcriptional abilities of B-ARRs and these induce the auxin signalling inhibitor *SHORT HYPOCOTYL 2 (SHY2)*. SHY2 influences PIN expression and by doing so, auxin transport and redistribution is blocked beyond the MZ (Dello Ioio *et al.*, 2007; Sabatini, Bianco and Giustini, 2013; Kieber and Schaller, 2014). Mutations in either of these genes as well as *ahk3* or *arr1* result in enlarged meristem suggesting that CK levels in the TZ are essential for the maintenance of cell differentiation (Sabatini, Bianco and Giustini, 2013; Kieber and Schaller, 2014).

On the other hand, at the MZ, auxin maxima causes degradation of the *SHY2* and repression of *PINs* is lifted. This way auxin is distributed throughout the MZ ensuring cell division activity. Conversely, cytokinin levels determine the activity of the quiescent centre (QC) (Zhang *et al.*, 2013). The QC consists of eight mitotically inactive cells and their function is to maintain stem cell identity of the cells around

them. Inactivity of those cells comes in the form of inhibition of their ability to divide and this is achieved by auxin dependent restriction of *WUSCHEL RELATED HOMEOBOX5 (WOX5)* expression in the QC. Disruption of this hormonal equilibrium in the area results in the destruction of the QC by activation of mitotic divisions indicating that QC cell identity is ensured in low CK and at the same time high auxin environment (Su, Liu and Zhang, 2011; Zhang *et al.*, 2011, 2013; Kieber and Schaller, 2014).

Cytokinin in root vascular development

The *Arabidopsis* root vasculature is a cylindrical structure running along the centre of the root. At the centre of this cylindrical structure is the xylem which is composed of protoxylem cells at the periphery and the metaxylem in the centre. The phloem poles are located at 90° of each of the protoxylem cells. Between the phloem and xylem poles there are the procambial cells (Sabatini, Bianco and Giustini, 2013).

Cytokinin signalling, and its interplay with auxin, plays a significant role in shaping this pattern of vascular development. CK signalling as determined by *TCS* and *ARR5* transcriptional reporters is active in the procambial and phloem cells but not in protoxylem and metaxylem cells. This expression pattern is reversed for auxin signalling reporters indicating that these two hormones have distinct functions in the vascular patterning (Bishopp, Help, *et al.*, 2011). With the exception of protoxylem, CK is essential for the specification of other vascular cell identities. Disruption of the *AHK4* results in defective root vascular development without proper differentiation of the phloem (Mähönen *et al.*, 2000). Similar defects of the root vasculature are observed in other mutants of CK signalling components (Argyros *et al.*, 2008) and biosynthesis genes (Matsumoto-Kitano *et al.*, 2008). In the procambial cells adjacent to protoxylem poles CK induces the expression of *PIN7* which drives auxin accumulation in the xylem axis. In turn, auxin induces *AHP6* expression which inhibits CK signalling and enables protoxylem differentiation by

restricting CK response out of the protoxylem cell (Mähönen *et al.*, 2006; Bishopp, Lehesranta, *et al.*, 2011).

Cytokinin regulation of Lateral Root development

Lateral root (LR) formation is important for increasing the below ground mass of the plant providing better access to nutrient and water absorption while providing anchorage to the plant. LR initiate at the pericycle cells near the protoxylem pole, where a series of anticlinal and periclinal divisions occur to give rise to a LR primordium (LRP). The initiation takes place early in the EZ where cytokinin signalling dominates (Bielach *et al.*, 2012), However, in the pericycle cell adjacent to protoxylem, *PIN1* expression is increased, driving auxin to concentrate in those cells known as LR initials (Dello Ioio *et al.*, 2007; Bielach *et al.*, 2012). The localisation of the PIN1 is connected with *AHP6* expression throughout the LR development process. This is especially important for the establishment and orientation of LRP (Moreira *et al.*, 2013). Together this evidence indicates that CK inhibition is important for controlling LR initiation. In fact, disruption of several *A-ARRs* results in reduction of LR numbers (To *et al.*, 2004) and mutations of *AHK* or *B-ARR* genes lead to opposite effects of LR numbers (Werner *et al.*, 2003; Riefler *et al.*, 2006).

Essentially CK inhibits LR development and increased CK response in the pericycle cells between LR primordia is essential to prevent the initiation of new LR so close to existing LRs as mechanism of optimizing the shape of the plant's root architecture (Bielach *et al.*, 2012; Sabatini, Bianco and Giustini, 2013).

Project Aims and Rationale

Currently our understanding of the cytokinin signalling is quite strong. However, like in all areas of biology, there are several knowledge gaps. One central question relates to how cytokinin can regulate so many different developmental processes within the plant.

As all CK signalling components are represented by large gene families, specificity could occur via subfunctionalisation of components. This has been shown for the receptors. Although all three receptors need to be knocked out to see strong CK related phenotypes (Higuchi *et al.*, 2004; Riefler *et al.*, 2006), the individual receptors display specificity in expression patterns (Mähönen *et al.*, 2000; Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006) and each has distinct response. For example, AHK4 is predominately expressed in the root (Mähönen *et al.*, 2000) and controls primary root growth, cell proliferation and elongation (Nishimura *et al.*, 2004). AHK3, on the other hand, is responsible for regulating photomorphogenesis and chlorophyl synthesis. AHK2, together with AHK3 have roles in root branching (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004). This level of understanding is not currently present in the downstream components. For example, there is limited knowledge for the specific functions associated with any of the AHPs.

The main aim of this project is to determine the role of AUTHENTIC HISTIDINE PHOSPHOTRANSFERASE 4 (AHP4) in *Arabidopsis thaliana*. AHP4 is one of the five Histidine Phosphotransferases (HPts) involved in the cytokinin two-component signalling (TCS) system, that act alongside a single pseudo phosphotransfer protein, AHP6. Currently, there is a lack of clarity about how AHP4 fits into this wider picture. Many review articles (Hutchison & Kieber, 2002; Kieber & Schaller, 2014; Schaller et al., 2015) list AHP4 as one of 5 positive regulators of cytokinin signalling. However, the evidence for this is mostly based on its sequence similarity to the other AHPs. Whilst there is strong biochemical evidence that AHP1, 2, 3 and 5 act during phosphorelay (Mähönen *et al.*, 2006; Bauer *et al.*, 2013); this is

missing or weak for AHP4. Additionally, some genetic experiments (Hutchison *et al.*, 2006) suggest that it may act as a negative regulator of cytokinin signalling. This question is particularly timely as recent work has explored the role of the PHPs in rice (Vaughan-Hirsch *et al.*, 2021). These have been shown to be negative regulators of cytokinin signalling. Surprisingly, when the authors constructed phylogenetic trees of the AHP and PHP families, they observed that AHP4 clustered away from the other AHPs and in a clade with the monocot PHPs (Vaughan-Hirsch *et al.*, 2021). This cast doubt on the status of AHP4 as a positive regulator of cytokinin.

In this thesis, I investigate the role of AHP4 in the CK signalling pathway starting from the hypothesis that it might have a negative role in CK signal transduction. I then use a reverse genetics approach to investigate phenotypes specific to AHP4. To address these questions, I have devised the following aims.

Aim 1: Determine whether AHP4 is a positive or negative regulator of cytokinin signalling

To do this, I will firstly explore the phylogenomic relationship between AHPs and PHPs across a variety of flowering plants. Previous analysis shows that AHP4 clades with the group of monocot PHPs (negative regulators) and away from the other AHPs that have been confirmed as positive regulators (Vaughan-Hirsch *et al.*, 2021). However, these phylogenies do not contain many dicot species. This means that it is not possible to know if this is a specific feature of the Arabidopsis AHP4 gene in general, or whether it is representative of a wider group of AHP4 genes in dicots. I will address this by creating new phylogenies with a greater number of species.

Secondly, it has been assumed that AHP4 is a positive regulator due to the presence of a histidine at the conserved phospho-accepting location. Whilst this would be a pre-requisite for AHP4 to be a positive regulator, it could only accept the phospho-message if the histidine was accessible to AHKs and ARRs. I will

address this by working with structural biologists to predict the protein structure of AHP4 to see if the His residue is exposed in a similar way to AHP1,2,3 and 5.

Additionally, there is strong *in-vitro* and *in planta* evidence that other AHPs can be phosphorylated by histidine kinases, but this data is missing for AHP4. To address this, I will express all the *Arabidopsis* AHPs and AHP6 in *E. coli*, purify these and use them in *in-vitro* kinase assays. This will tell whether, like AHP1, 2, 3 and 5, AHP4 has the potential to act as a positive regulator of cytokinin signalling. To test whether AHP4 is a negative regulator, I will test whether it can supplement for AHP6 activity when expressed under the AHP6 promoter. If AHP4 can trans-complement an *ahp6* mutant, this will provide strong evidence, that like AHP6, AHP4 is a negative regulator of cytokinin signalling.

Finally, because CK is associated with several developmental processes, such as regulating root development and architecture, modulating shoot development, flower and seed development, mutations of CK signalling components were used in the past to determine their position in the signalling cascade. For example, disruptions of *AHP1,2,3 and 5* result in reduced sensitivity of CK in the aforementioned CK responses (Hutchison *et al.*, 2006; Nishiyama *et al.*, 2013). Such experiments often included *ahp4* mutants but these alleles were unlikely to be null. Therefore, to investigate the effect of AHP4 in CK responses I will generate *ahp4* knockout mutants using the CRISPR-Cas system. Using the improved alleles, I will investigate how the plants respond to various levels of exogenously applied CK through monitoring several physiological processes (i.e., primary root length, chlorophyl retention, shoot branching and seed size). This will enable to understand whether AHP4 affects the CK signalling pathway positively or negatively. Furthermore, comparison of the transcriptome of *ahp4* plants in response to exogenous cytokinin will firstly enhance our understanding about the AHP4 regulation of CK signalling (i.e., whether CK responsive genes are increased or decreased in respect to WT) and secondly it will tell the possible targets of AHP4 in this process.

Aim 2: Investigation of the role of AHP4 in plant physiology

CK signalling is implicated in abiotic stress tolerance with several components being either upregulated or downregulated during exposure to abiotic stresses (Kieber and Schaller, 2014). For example, three of the HPts (*AHP2,3 and 5*) negatively influence drought tolerance in a redundant manner (Nishiyama *et al.*, 2013). Previous analyses considering the response of CK signalling components to stresses showed that AHP4 was the only HPt to be upregulated by a variety of abiotic stresses. These let to the question whether AHP4 had a specific function in regulating abiotic stress responses in plant development.

To investigate this, I will use the new *ahp4* alleles, generated for aim 1, in an effort to understand the abiotic stress (drought and osmotic stress) effect on the root and shoot development. If the effects observed are similar to what is known for HPts then AHP4 is likely to act in a similar way to modulating drought responses. The working hypothesis for this experiment is that AHP4 has a different function than the HPts.

Furthermore, I will develop transcriptional reporters to localise the expression of AHP4 in *Arabidopsis* seedlings. This will indicate the exact tissues in which *AHP4* expression is concentrated. By localising the expression, it will be able to see how drought stress affects those tissues and whether disruption of AHP4 has any specific effect on the expression area.

Chapter 2: AHP4 proteins in dicot species differ from the HPts and PHPs

2.1 Introduction

CK signalling and regulation in plant tissues is crucial for plant development and adaptation to their environment. This is why many of the CK signalling components have been well characterised and their function determined. However, like in all major processes in plant biology, there are players which are less characterised and AHP4 is amongst those. As mentioned earlier, AHP4 belongs to the family of phosphotransfer proteins. These are small proteins able to travel between the cytoplasm and the nucleus to transfer the phosphoryl group received from the HKs to the RR (Punwani and Kieber, 2010; Punwani *et al.*, 2010). To be able to participate in the phosphorelay, proteins must be able to interact with both the HKs and the RRs. As a second important requirement these proteins must contain the phosphate receiving histidine (H/His) residue in a specific region of the protein which is highly conserved. The His residue is responsible for the phosphorylation of the protein.

The *Arabidopsis* HPt family consists of six proteins, one of which, the AHP6 has an asparagine (N) in the position where the His residue is situated. This amino acid change means that the protein can no longer be phosphorylated at this position, and this is sufficient to change make AHP6 an inhibitor of the CK signalling (Mähönen *et al.*, 2006). The inhibition of the signal it is thought to occur via competition of the AHP6 with the HPts for the interaction with the HKs (Bauer *et al.*, 2013).

In angiosperms, the CK signalling pathway is conserved across the species with similar complexity in terms of the number of proteins involved (Table 2.1). However, the specific role of each of the components in CK signalling has diversified between monocots and dicots, especially in the inhibition of the signalling cascade. Specifically, previous studies in rice, demonstrated that OsPHPs do not function in

the same biological processes as AHP6 to modulate vascular patterning in roots, but they have the same biochemical characteristics (Bishopp, Help, *et al.*, 2011; Vaughan-Hirsch *et al.*, 2021).

There are indications, from a study aiming to characterise the AtHPts, that AHP4 might not be a canonical HPt protein despite its large sequence homology especially at the conserved domain where the phospho-accepting His residue is situated (Hutchison *et al.*, 2006). Phylogenetically, AHP4 does not cluster with any of the AtHPts or the AHP6 but it clades closely to monocots OsPHPs which act as CK inhibitors (Huo *et al.*, 2020; Vaughan-Hirsch *et al.*, 2021). Biologically, disruption of *AHP4* did not produce any comparable CK responses to the canonical HPts (Hutchison *et al.*, 2006).

Table 2.1: The number of genes in the TCS system in plants
Numbers include proteins involved in CK signalling and ethylene signalling.
Taken from (He *et al.*, 2016)

Species	HK	HPts	PHPs	Type-A RR	Type-B RR	Reference
Arabidopsis thaliana	17	5	1	10	12	(Hwang and Sheen, 2001)
Oryza sativa	11	2	3	13	13	(Pareek *et al.*, 2006)
Lotus japonicus	14	7	-	7	11	(Ishida *et al.*, 2009)
Glysine max	36	13	-	18	15	(Mochida *et al.*, 2010)
Zeas mays	11	7	2	16	9	(Chu *et al.*, 2011)
Triticum aestivum	7	3	-	41	2	(Gahlaut *et al.*, 2014)
Brassica rapa	20	7	1	21	17	(Liu *et al.*, 2014)
Solanum lycopersicum	20	4	2	7	23	(He *et al.*, 2016)

The aim of this chapter is firstly to better investigate the phylogenetic relationship of the AHP4 with homologous proteins from angiosperms, by including more dicot species than in phylogenies previously published (Huo *et al.*, 2020). This

will test whether AHP4 is unique to Arabidopsis or whether it is present throughout eudicots.

Alongside the phylogenetic relationship between HPts, it is important to understand whether AHP4 is functionally more related to monocot PHPs (i.e. acting as an inhibitor) or AtAHPs (i.e. acting as a positive regulator). This was demonstrated by *in-silico* analysis of the structural characteristics of AHP4 compared with the AtAHPs. The His residue is important for the phosphorelay to happen. AHP6 as a negative regulator, completes AHPs for the interaction with the AHKs and by doing so, blocks them from phosphorylating (Bauer *et al.*, 2013). Yeast-two-hybrid (Y2H) experiments showed that AHP4 is able to form interaction with some components of the TCS machinery, especially with the ARRs (Huo et al., 2020). Therefore, if AHP4 can bind components of the TCS machinery but the His residue cannot receive the phosphate due to structural obstructions, then it is logical to assume it would act as a negative regulator. To test whether the His residue is accessible in the same way as the other HPts the alignments of the proteins were compared *in-silico* based on their sequence as well as their final tertiary structure.

Furthermore, functional assays of AHP4 can give indications to its role in CK signalling by testing whether it can trans-complement the *ahp6* mutant. AHP6 has an important role in shaping the root vascular pattern by promoting protoxylem differentiation through inhibition of CK (Mähönen *et al.*, 2006) and resulting in a very distinct phenotype of the protoxylem tube. Therefore, if AHP4 is negatively affecting CK signalling, the same phenotype would be expected to occur in *ahp4* seedlings. However, there is the possibility of AHP4 not to be present in the protoxylem cells and therefore not affecting their differentiation naturally. To test whether AHP4 can act as AHP6, the distinct protoxylem pattern of *ahp6* plants was used. Consequently, ectopically expressed AHP4, driven by the AHP6 promoter in ahp6 plants would be expected to rescue any protoxylem defects caused by the loss-of-function of *AHP6.*

Lastly, in this chapter is demonstrated an optimised procedure of protein isolation *in-vitro* phosphotransfer assays to be performed in the future. Through these assays the ability of AHP4 to accept a phosphate from the HK *in-vitro* will be tested and they will shed light to whether AHP4 is or not a functional phospho-accepting protein despite the His residue at the crucial position.

2.2 AHP4 differs from the HPts phylogenetically and structurally.

2.2.1 Phylogenetic analysis reveals distinct clade for AHP4 and its orthologues

The phylogenetic relationship of HPts and PHPs have been investigated previously in many species (Tsai *et al.*, 2012; He *et al.*, 2020; Huo *et al.*, 2020; Vaughan-Hirsch *et al.*, 2021). These analyses suggested that the TCS proteins in monocots and dicots have evolved separately after the divergence of the two groups. In fact, this was proven experimentally as it was previously demonstrated that rice PHPs were not able to rescue the *ahp6* plants in *Arabidopsis,* but they function as negative regulators of CK signalling in rice (Vaughan-Hirsch *et al.*, 2021). While the function of PHPs from monocots and their divergence from the Arabidopsis PHP (APH6) was established, it was observed that AHP4, a His containing protein clusters closer to the monocot PHPs than to the other Arabidopsis HPts (Vaughan-Hirsch *et al.*, 2021). This raises the question if it also functionally more similar to those proteins.

Functionally, AHP4 is not yet understood, however there is some evidence suggesting it might be acting differently from its HPt homologues in *Arabidopsis* (Hutchison *et al.*, 2006). To understand the relationship of AHP4 with other HPt proteins a phylogenetic analysis was performed on HPt and PHP proteins from six species three dicots, two monocots and yeast: *Medicago truncatula*, *Solanum lycopersicum, Arabidopsis thaliana*, *Zea mays*, *Oryza sativa* and *Saccharomyces cerevisiae*. Similar to previous works, the analysis of the HPt and PHPs protein sequences revealed great homology in several conserved domains and especially at the region where the phospho-accepting His residue is situated across species. This

active site of the proteins consists of a series of very conserved aminoacid sequences and, in the case of HPts, the His residue (Fig 2.3 A). To perform this analysis, the Phytozome database was searched for proteins homologous to each of the *Arabidopsis* AHP1-6. Duplicates were manually removed, and sequences of the selected proteins were inserted in the MEGA software for calculating the phylogenetic relationship. This analysis was done using the maximum likelihood calculation method and bootstrapping of 1000 replicates. Branches resulting from values smaller than 50 were collapsed to achieve high confidence in the separation of the proteins. The phylogenetic tree illustrated in figure 2.1 is an unrooted tree and for the purpose of simplicity the bootstrap values were removed, however a detailed rooted tree is shown in the appendix.

Based on this analysis there are four distinct clades formed. The clades with the black branches represent the known HPts in the six species analysed here. The known HPts are defined as proteins which participate in the TCS machinery by being able to become phosphorylated and interact with both HKs and B-RRs. Apart from the subbranch on the top with proteins containing Leu, Arg or Tyr residue instead of His, all the other proteins have the canonical sequence. The proteins with the canonical form from dicot species cluster separately from those of the monocot species. Additionally, the AHP6 protein along with the other dicot PHPs have their His residue substituted with N. This seems to be conserved between the dicot species as all the species tested here had an AHP6 orthologue. At the opposite site of the tree, in the red branch, the monocots PHPs form a clade with a Gln as a substitution of the His.

Interestingly, AHP4 along with other proteins from tomato and *Medicago* form a separate clade which is closely related to the monocots PHPs (Fig 2.1, blue branch). Interestingly, there is at least one such orthologue in the three dicot species investigated here. Structurally, these proteins contain the phospho-accepting His in the conserved region of the protein however, they seem to have diverged from the well characterised monocot and dicot HPts (black branches).

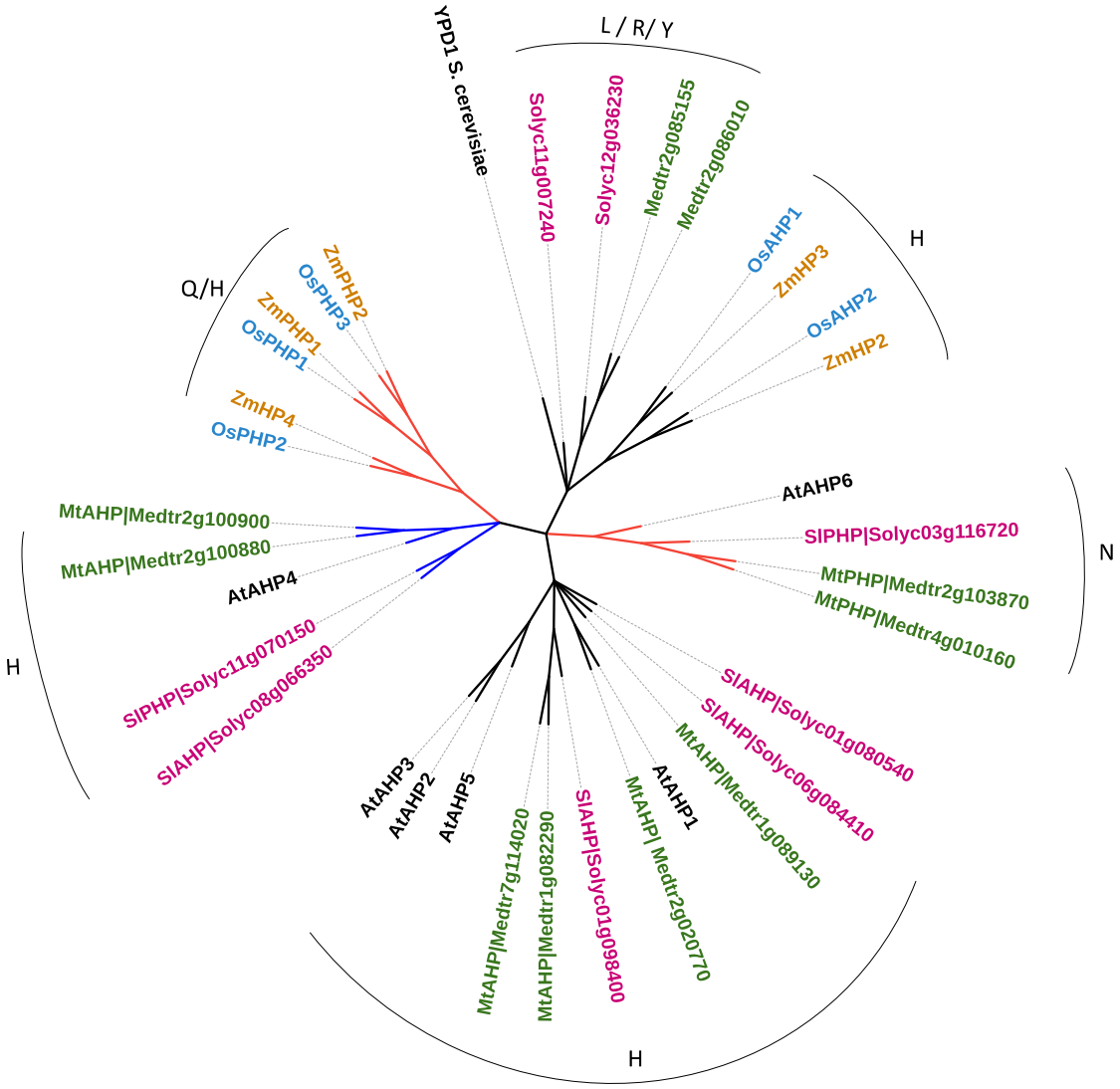


Fig 2.1 Phylogenetic tree revealing that AHP4 forms a clade of proteins in dicots species distinct from the monocot PHPs and known HPts. This unrooted phylogenetic tree shows the relationship of HPt and PHP proteins from *Medicago truncatula* (green), *Solanum lycopersicum* (pink), *Arabidopsis thaliana* (black), *Zea mays* (yellow), *Oryza sativa* (blue) and *Saccharomyces cerevisiae* (black) as determined by their aminoacid structure. Red branches represent the PHPs in dicots and monocots. Black branches show the HPts in those 6 species and blue branches indicate the distinct clade of AHP4 and its related proteins as compared with the other HPts and PHPs of monocots and dicots. This phylogenetic tree was contracted using the Mega and annotated using iTOL software.

To investigate a wider range of dicot plant genomes, gene loss trees were made for AHP4 (Fig 2.2) and AHP6 (Appendix). The tree analysis involved more than

60 species of plants belonging to the dicot clade. Aside from one, all other dicot species contain one or more orthologues of AHP4 (Fig 2.2), and all species contain one or more orthologues of AHP6 (Appendix). Therefore, these two proteins are highly conserved within dicots.

Additionally, it is striking how the HPts of monocots and dicots are so diverged (phylogenetically), but the monocot PHPs and AHP4-like are close together. This divergence could mean that these protein forms could be a result of an ancient angiosperm specific clade of negative regulators that predates the dicot-monocot divergence. On the other hand, since AHP6 is conserved between the dicots but clades so distantly from the monocot PHPs might suggest that this clade of AHP6-like proteins is lost in modern monocots; or even gained in dicots.

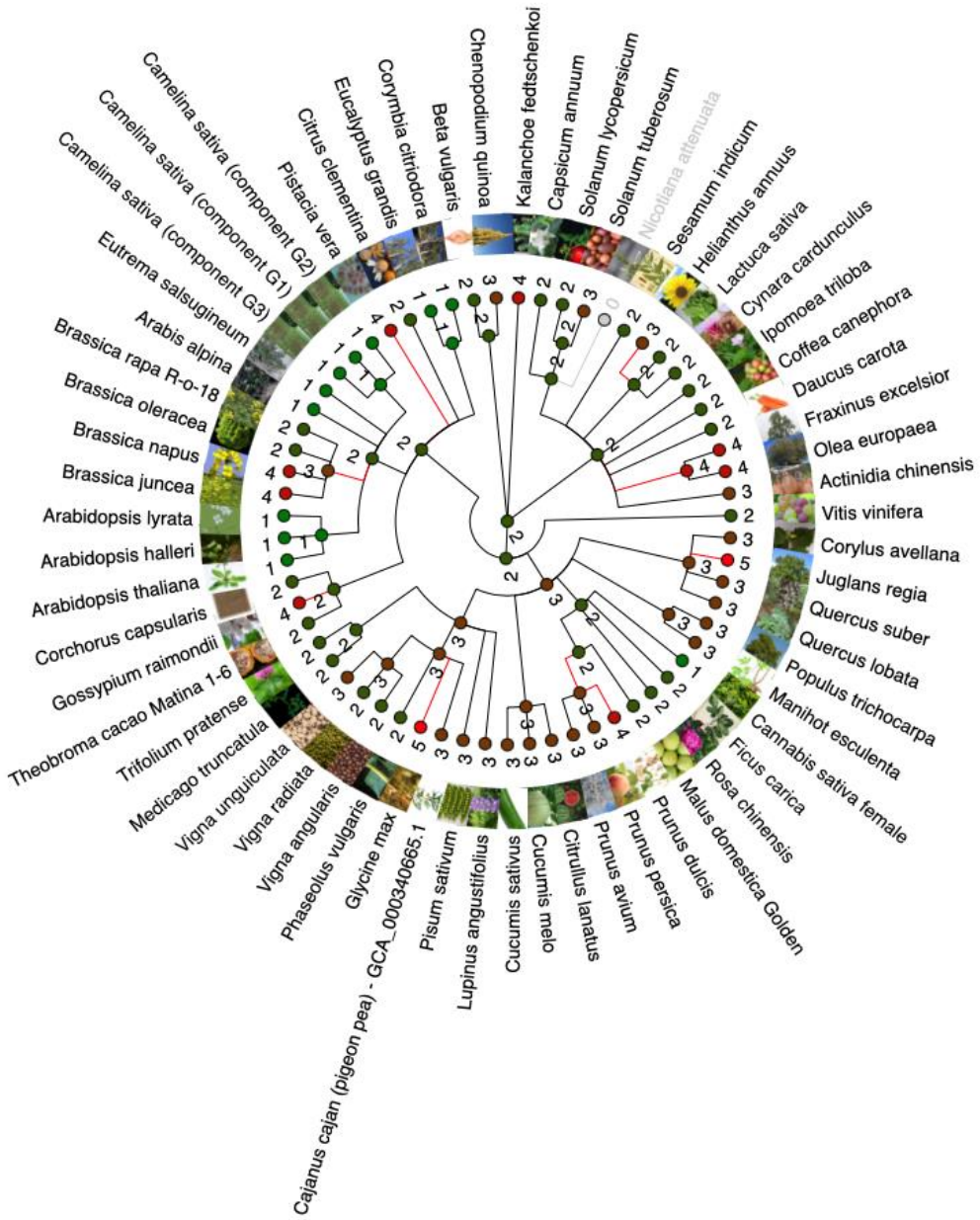


Fig 2.2 AHP4 is conserved in all dicot species. Gene loss tree demonstrating that AHP4 is conserved in virtually all dicot species and therefore is likely to have a conserved function. Numbers on the junction of the branches demonstrate the number of homologous gene members for each species. This tree was constructed using *EnsemblPlants* website.

2.2.2 Structural alignment modelling reveals the unique structure of AHP4

The above phylogenetic analysis suggests that AHP4 is evolutionary distant from the HPts and conserved in angiosperms. However, the consequence of this separation is not known. One possibility is that AHP4 evolved to be a negative regulator earlier than the divergence of monocots. Subsequently, somewhere in the evolutionary space, the conserved His residue of modern monocots mutated to give rise to monocot PHPs, and it has continued as a negative regulator in all species. Alternatively, another possibility would be that it was a positive regulator in angiosperms that became negative regulator only in monocots. But if this is the case it has persisted as a positive regulator (distinct from the other positive regulators) in virtually every dicot species.

To address the above questions is to find out the role of AHP4 in a number of dicot species, starting with *Arabidopsis*. To do this, deeper investigation of the structure of AHP4 and its common characteristics with other HPts were investigated further (Fig 2.3). As mentioned above, protein alignments reveal that AHP4 contains the His which would normally enable the protein to participate in the phosphorelay. However, having the His is not necessarily sufficient for phosphorelay, if the interactive surface is not exposed. Since AHP4 is closely related to monocot PHPs which do not have the phosphor-accepting ability (Vaughan-Hirsch *et al.*, 2021), it was hypothesized that there might be differences in the tertiary conformation of AHP4 and proteins alike that could alter their function.

To test this, the crystal structure of the AHK5-AHP1 complex (Bauer *et al.*, 2013)was used as a template to predict the 3D structures of the AHP2,3,4,5 and 6. This work was done by the PhD candidate Shannon Ward and her supervisor Dr Aditi Borkar (School of Veterinary Science, University of Nottingham). The tertiary structures of these proteins were then aligned with AHP1 as presented in fig 2.3 B. The H residue responsible for accepting the phosphoryl group in AHP1 is projected towards the AHK5. AHK5 contains the receptor sequences where the phospho-exchange is

happening between the receptor's Asn and the His of the AHP1 therefore the orientation and position of both these amino acids on the proteins is important to be accessible for the interaction to happen. A similar orientation of the His residue was observed for all the other proteins examined, apart from AHP6 which has an Asn in that region of the protein. However, the His residue in AHP4 had a small shift in the orientation compared to the other four proteins (Fig 2.3 B). Previous affinity experiments demonstrated that the AHK5-AHP1 is conserved amongst the HPts, apart from AHP6 which displayed a weaker interaction with AHK5 and AHP4 where no interaction was established neither *in vitro* nor *in planta* (Bauer *et al.*, 2013). The shift of the His residue observed from this analysis is possibly sufficient to prevent the protein from functioning like the canonical form of the HPts.

To further investigating the conformational structure of the AHP4 and understand the causalities of the His orientation shift, mutations in the protein's conserved region were imposed on the predicted structure of AHP4 (Fig 2.4). Like above, this modeling work was performed by Shannon Ward and Dr Aditi Borkar. The hypothesis was that nearby amino acids that are only different in AHP4 might be responsible for this shift of His residue. Initially, amino acids methionine (Met) and phenylalanine (Phe) from either side of the His residue were isolated because they only exist in AHP4 in that position (Fig 2.3 A). When Met was replaced with valine (Val) *in-silico*, no effect in the original orientation of the His residue was observe (Fig 2.4 A). The same effect was recorded when Phe was replaced with leucine (Leu, Fig 2.4 B) and when both those amino acids were replaced at the same time forming an *in-silico* double mutant (Met-Val and Phe-Leu, Fig 2.4 C). These results suggest that the difference in His orientation is not resulting from the presence of Met or Phe on either side of the His. This unique shape of AHP4 might be due to changes in other highly conserved regions of the protein. The investigation to pinpoint the exact location/amino acid of the protein which is responsible for this shift in His orientation was not performed because any possible targets would be based on speculations.

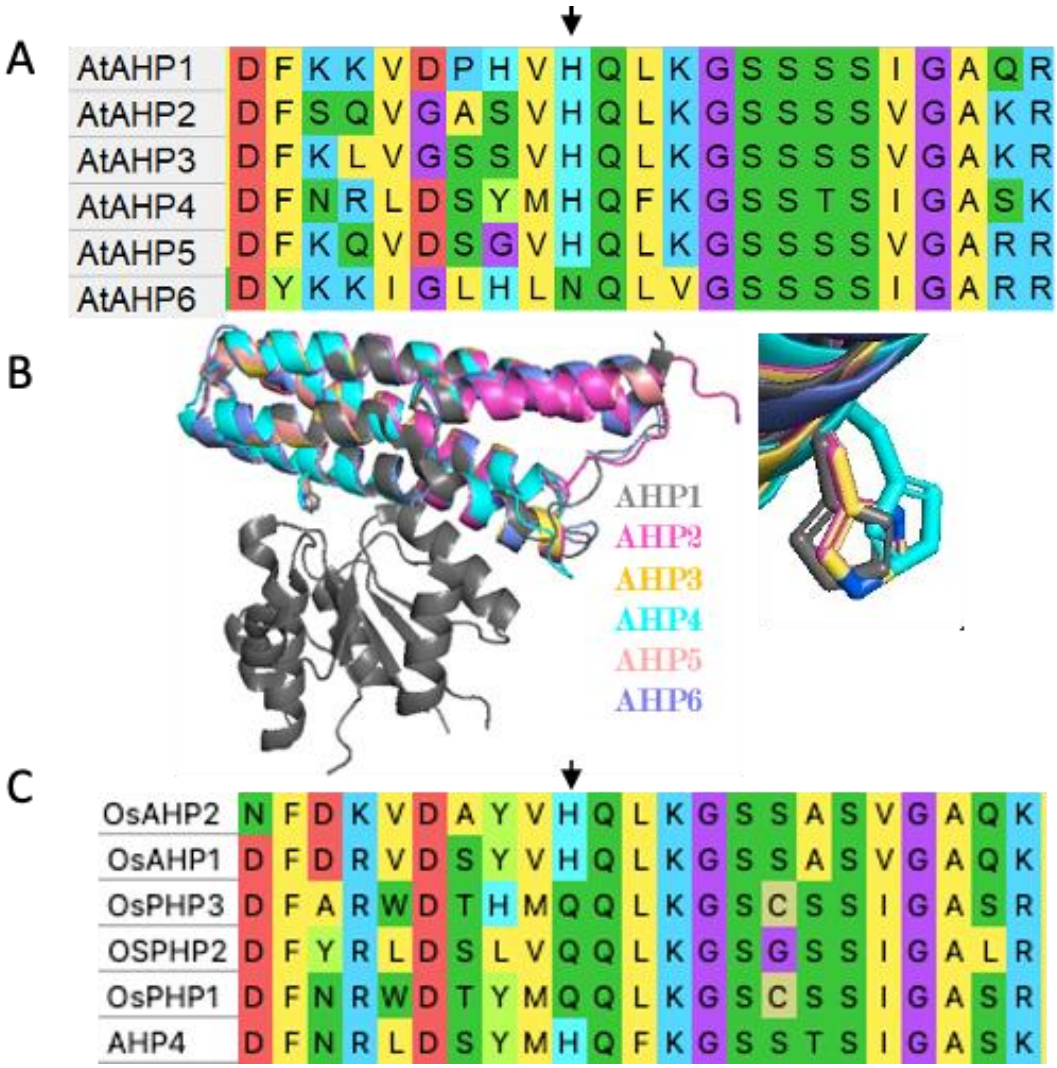


Fig 2.3 AHP4 retains the histidine residue in the conserved region of the protein but has different structure than the other Arabidopsis HPts. A) The conserved region of the HPts proteins as shown by this amino acid sequence alignment shows that AHP4 has a histidine (His) residue just like all the other HPts, unlike AHP6 which has an asparagine in the same position (black arrow). B) The tertiary structure of the *Arabidopsis* HPt proteins aligned with the model structure of AHP1. The grey protein below the HPts is the histidine kinase protein 5. The image on the right is a zoomed in view of the His residue showing the difference in the orientation of AHP4. C) Alignment of amino acid sequences from part of the rice AHPs and PHPs protein sequence. The arrow indicates the position of the His residue responsible for the phosphotransfer in AHPs and its substitution with glutamine (Gln/Q).

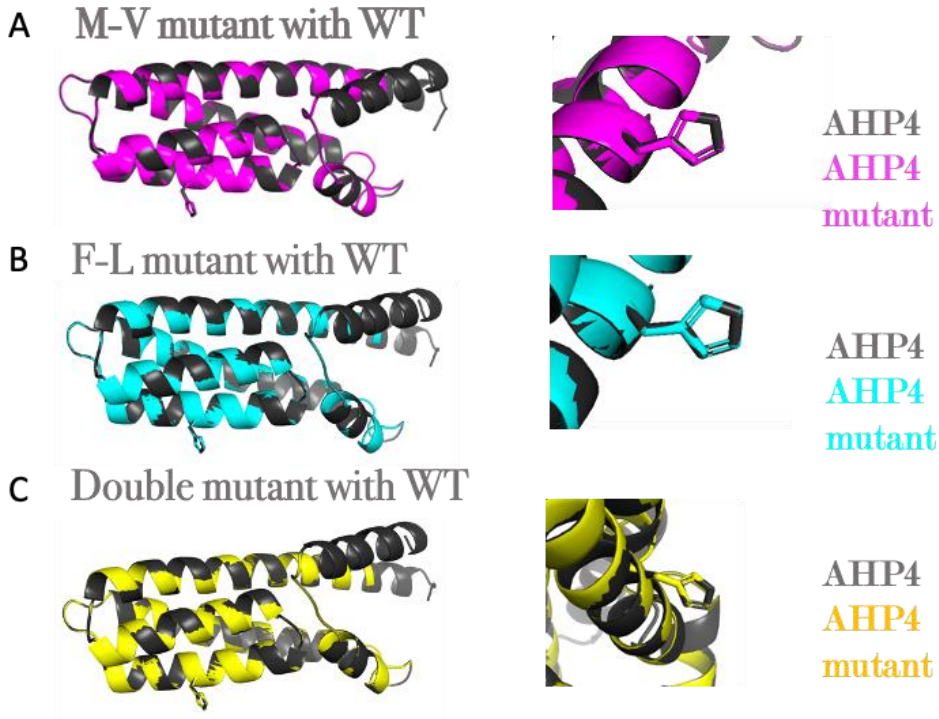


Fig 2.4 Single or double mutations of the aminoacids adjacent to His residue are not sufficient to cause a shift of the His residue to match the other HPts. A) Valine (Val) substitution to methanione (Met) is causing no change to the orientation of the His residue in AHP4, evident from the zoomed in image on the right showing the WT and mutant AHP4 3D alignment. Similarly, B) substitution of phenylalanine (Phe) to leucine (Leu) or C) double mutations Met-Val and Phe-Leu cause no effect to the structure of the WT AHP4.

2.2.3 Preliminary work for biochemical characterization of AHP4

Since AHP4 was found to be more closely related to rice and maize PHPs (Fig 2.1), and its conformation is predicted to be different from the Arabidopsis HPts (Fig 2.3 B) along with the fact that it cannot interact with AHK5 *in-planta* (Bauer *et al.*, 2013) suggests that it might not function as phopho-accepting protein despite having the His residue in the conserved domain of the protein. To investigate whether this change in the orientation of the His was enough to prevent the AHP4 from participating in the phosphorelay, *in-vitro* phosphotransfer assay was designed, similar to what was done to show that AHP6 and the rice PHPs were not able to accept the phosphoryl group (Mähönen *et al.*, 2006; Vaughan-Hirsch *et al.*, 2021). To do this *Arabidopsis* HPt proteins as well as the yeast HK SLN1 were expressed in the vector pGEX-6-1 with glutathione S-transferase (GST) tag and

purified using column purification. The yeast cytokinin histidine receptor was used instead of the Arabidopsis because plant HKs are very hard to express and isolate from heterologous systems due to the complexity of the plant protein. The construct for AHP1-GST was provided by a Dr John Vaughan-Hirsch (Vaughan-Hirsch *et al.*, 2021) and the SLN1-HK as well as the SLN1-HKRec were provided by Prof Ann H. West (University of Oklahoma) (Fassler and West, 2010). For construction of the remaining HPts and AHP6, the coding sequence (CDS) was isolated from *Arabidopsis* plants and cloned into pGEX-6p-1 vectors. Protein expression in bacteria cells was achieved by the addition of β-D-1-thiogalactopyranoside (IPTG) to induce gene expression. Inducible systems in protein expression are important to avoid high basal protein production which can lead to toxicity issues in the host cells affecting growth and resulting in poor strain viability. The pGEX-6p-1 is an IPTG inducible vector. It is expressing the *lacI* under the influence of *LacIq* promoter which represses the lac operator and the expression of downstream genes. Addition of IPTG or lactose, can remove the suppression and allow the expression of the inserted genes (NEB: Avoid Common Obstacles in Protein Expression). This way, the proteins are synthesized only after induction and the viability of host strain stocks is not affected because of the uncontrolled expression of foreign proteins. To ensure higher rates of solubility of the AHP proteins the IPTG induction was performed at low temperatures (16°C) over a period of 20 hours. Proteins were then extracted using the Pierce GST spin purification kit as per manufacturer's instructions and at each step of the purification a small fraction of the solution (50µl) was kept to run on acrylamide gel (Fig 2.5). For the proteins extraction DH5a *E. coli* cells were used apart from SLN1-HKRec which failed to produce any protein in those cells. Instead, the protein was able to be induced and purified as shown in fig 2.5 H from the Origami *E. coli* cells which have less background expression and fewer proteases to cause degradation of the protein.

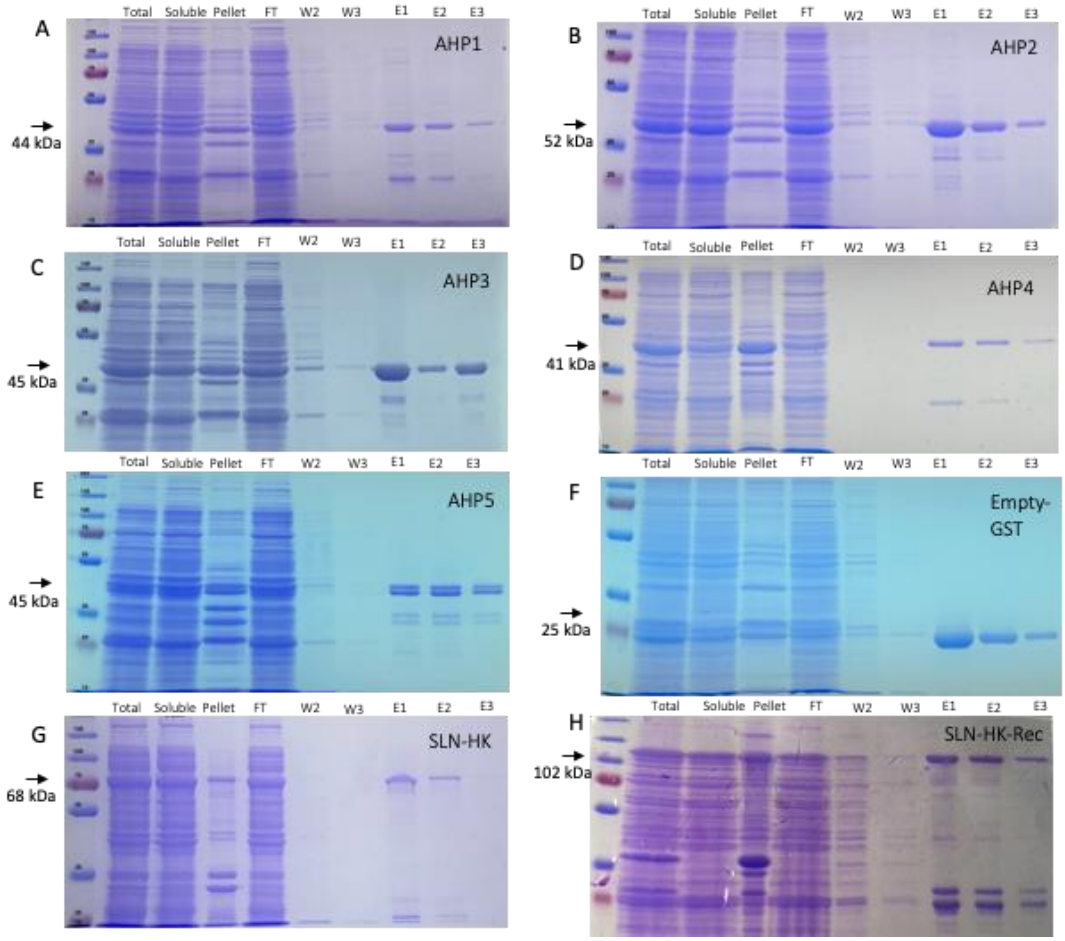


Fig 2.5 Expression and purification of the Arabidopsis HPts and the yeast SLN1-HK and SLN1-HKRec in a heterologous system. The proteins are fused with glutathione S-transferase (GST) protein and grown in DH5a cells or Origami cells. A) AHP1-GST, B) AHP2-GST, C) AHP3-GST, D) AHP4-GST, E) AHP5-GST, F) empty-GST, G) SLN1-HK-GST and H) SLN1-HKRec-GST. Protein A-G were induced and purified from DH5a cells and H) from Origami cells. Arrows indicate the size of the protein. Total: the total extract from cells, Soluble: the extract from the soluble fraction, otherwise known as “input”, Pellet: the amount of proteins trapped in the insoluble fraction, FT: the flowthrough after the GST tagged proteins run through the column, W2 and W3: the proteins that run through the column after the second and third wash, E1, E2 and E3: the proteins extracted at each elusion stage. Each elution solution was kept separately to preserve the highest concentration of the protein extract.

Despite growing the bacteria cells in low temperatures after IPTG induction, AHP6-GST was trapped in the insoluble fraction of the protein extracts and therefore was not able to be purified (Fig 2.6 A). To resolve this, the induced

bacterial pellet containing the AHP6-GST trapped in the inclusion bodies was incubated with 10% sarkosyl, a surfactant which disturbed the aggregated proteins and 3% Triton X-100 with 30mM CHAPS, to form micelles around the proteins moving them to the soluble fraction and facilitating the proper protein refolding (Tao *et al.*, 2010). By doing so, the AHP6-GST was able to be purified as seen in Figure 2.6 B. All the purified proteins resulting from the first elution (E1 in Fig 2.5 and 2.6) were quantified by Bradford assay and 1µg of those proteins was run on 10% acrylamide gels to verify the quantification (Fig 2.7).

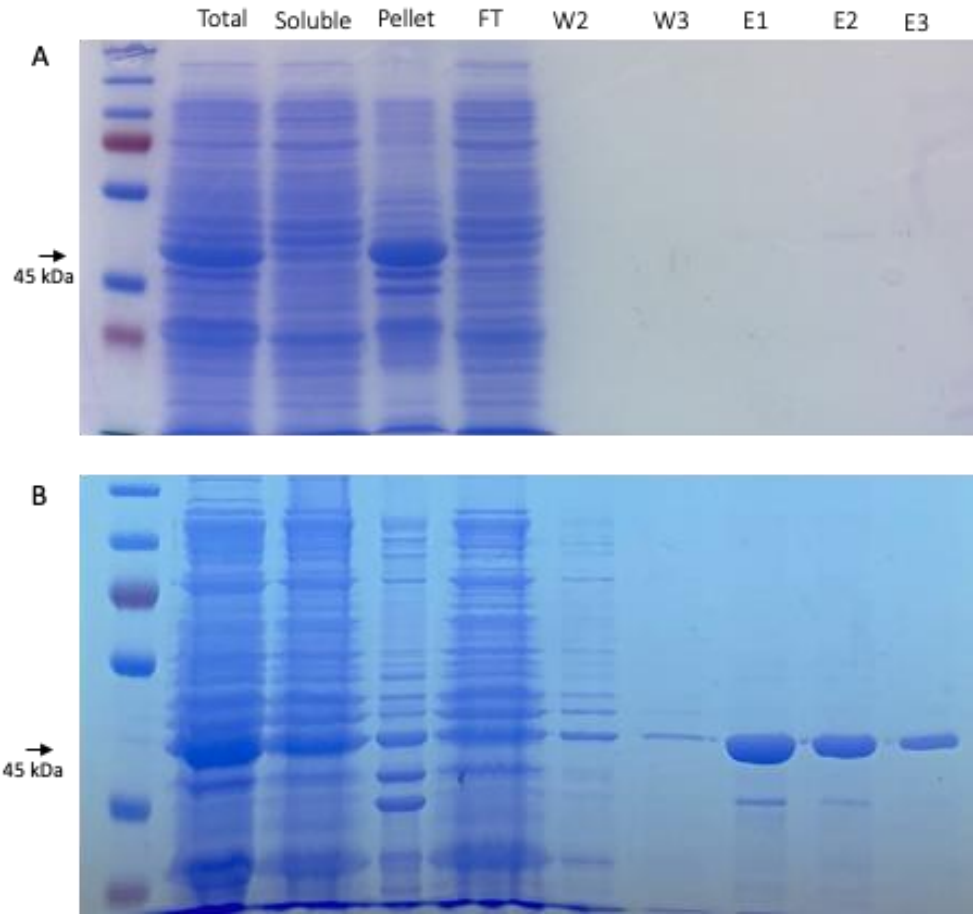


Fig 2.6 AHP6-GST purification was able only after treatment with 10% sarkosyl detergent. A) AHP6-GST was induced in DH5a cells but was trapped in the insoluble fraction probably due to formation of inclusion bodies resulting in no purification. B) Treatment of the pellet with sarkosyl, triton X-100 and CHAPS enabled the release of the protein from inclusion bodies and increased purification efficiency.

Even though the purification of the proteins to perform the *in-vitro* phosphotransfer assays were purified to high standards, due to time constrain, these assays were not able to be performed. However, it was demonstrated here that purification of such proteins by the optimisations presented above is feasible for such assays to happen in the future.

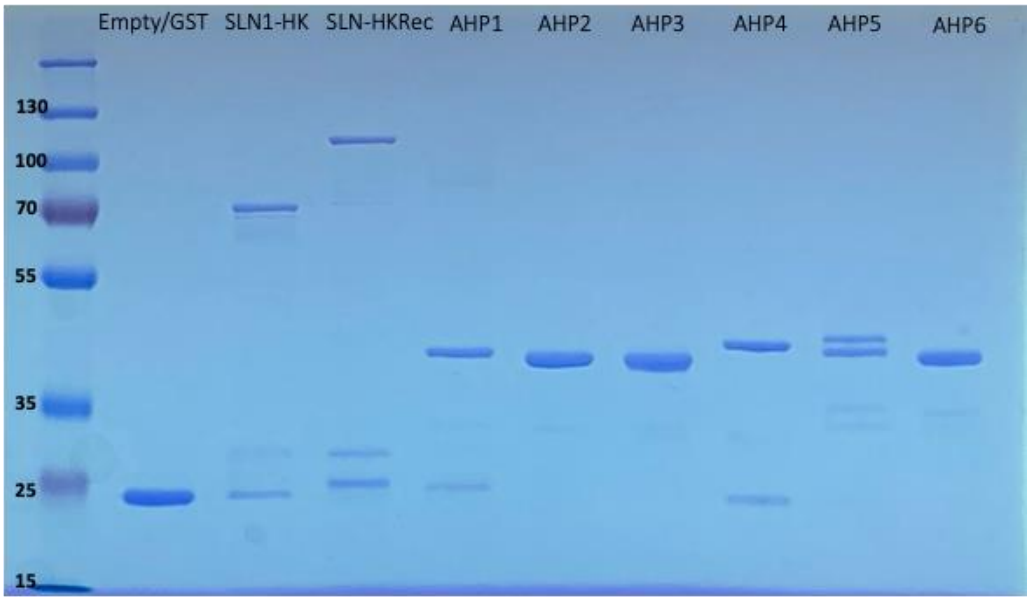


Fig 2.7 The purified proteins on 10% acrylamide gel after Bradford assay quantification. The proteins have been loaded for every well to contain 1μg of the first elution solution (E1).

2.3 AHP4 is not sufficient to trans-complement AHP6.

Suggestions that AHP4 might be different to the Arabidopsis HPts pre-existed this work (Hutchison *et al.*, 2006) and as demonstrated above, more evidence are accumulating in favor of this claim. Phylogenetically AHP4 is more related to PHPs of monocot species rather than the AtHPts or AtPHP (Fig 2.1) and the tertiary structure of the protein differs from that of the other four AtAHPs despite the large homology in the region where the phospho-accepting aminoacid His is situated (Fig 2.3). If this shift is enough to cause a His containing protein to function like PHPs then it is likely that knock-out *ahp4* plants would also exhibit some of the phenotypes observed in plants defective in *AHP6*. The *ahp4* plants were generated using the CRISPR-Cas method and are described in Chapter 3, section 3.2.

It was shown that AHP6, as a cytokinin signalling inhibitor and auxin inducible gene, plays a central role in regulating the boundaries of auxin and cytokinin signalling for vascular patterning in roots amongst other phenotypes (Mähönen *et al.*, 2006; Bishopp, Help, *et al.*, 2011). Specifically, in protoxylem cells, were *AHP6* is expressed, cytokinin signalling is inhibited and therefore protoxylem differentiation is activated. In *ahp6* plants, the protoxylem differentiation occurs sporadically in the root because CK inhibition is reduced (Mähönen *et al.*, 2006).

Since there are indications that AHP4 might be behaving differently to the AtHPts, investigation of its role in the CK signalling pathway was important. In this section, the root vascular patterns of *AHP6* were used to examine if AHP4 has a similar role in defining the formation of protoxylem in the roots. By doing so it would be clearer to determine its position in CK signalling.

To test this, the protoxylem differentiation patterns were scored in WT, *ahp4* and *ahp4 ahp6* plants and compared with the patterns observed in *ahp6* plants (Fig 2.8). The scoring was done based on whether the protoxylem appeared

on both poles for the entire length of the 5-day old root (=both), whether it appeared with defects in either one of the xylem poles (=mild phenotype) or whether protoxylem failed to differentiate at both of the xylem poles (= strong; Fig 2.8 A). The *ahp4* single mutant and *ahp4 ahp6* double mutant plants used for this assay are CRISPR generated alleles which were developed for this work and will be discussed in Chapter 3. Based on these scorings, the protoxylem differentiation in *ahp4* plants across all alleles tested were showing no defect in protoxylem differentiation. Furthermore, the protoxylem pattern for the double *ahp4 ahp6* plants did not appear to differ from the pattern observed in *ahp6* plants (Fig 2.8 B). These data suggest that *AHP4* does not ordinarily play a role in modulating CK perception for the differentiation of protoxylem in *Arabidopsis* roots.

However, AHP6 function can be substituted by expressing other cytokinin inhibitors at the protoxylem position. For example, expressing CKX2 under the AHP6 promoter is sufficient to rescue the loss of protoxylem phenotype of ahp6 mutants (Mähönen *et al.*, 2006). If AHP4 can act as a negative regulator, then it would be a reasonable assumption that if it was also expressed under the AHP6 promoter then it too may rescue the *ahp6* phenotype (Fig 2.1).

To examine this, *AHP4* was ectopically expressed under the influence *AHP6* promoter in *ahp6* background. To do this, the AHP4 CDS was isolated and cloned into C-module cassette of the Green-Gate cloning system. The A-module was previously designed by Dr Britta Kuempers and the destination vector was assembled as described in Chapter 5 Materials and Methods which was then used to transform *ahp6* plants. The protoxylem differentiation pattern was analysed in these trans-complementation lines as mentioned above, and based on those analyses it was observed that the ectopic expression of *AHP4* under the AHP6 promoter was unable to rescue the *ahp6* phenotype (Fig 2.8 B). This suggests that AHP4 is either not as strong an inhibitor of cytokinin signalling as AHP6, or that it only acts as an inhibitor under specific circumstances, or unlike both AHP6 and the rice PHPs it does not inhibit cytokinin.

Such trans-complementation experiments were done in the past to analyse the response of rice OsPHPs in shaping the vascular development. The authors demonstrated that even though OsPHPs negatively influence CK signalling in rice, the expression of those proteins under the influence of *AHP6* promoter in Arabidopsis *ahp6* background was unable to rescue the *ahp6* protoxylem phenotype. This phenotype was independent of the amino acid they were carrying in the critical position (N or Q) (Vaughan-Hirsch *et al.*, 2021). Based on these observeations the authors concluded that the *Arabidopsis* and rice TCS machinery was functionally diverged. However this explanation is not valid for AHP4 as it is an *Arabidopsis* protein. Given that AHP4 is phylogenetically more related to OsPHPs than to any of the other *Arabidopsis* protein (Fig 2.1) it is possible that mechanistically the OsPHPs and AHP4 cannot inhibit CK signalling in the same way as AHP6, i.e., the binding affinity of those proteins belonging to the same phylogenetic branch with the AtHKs might be affected because of changes in the sequence elsewhere in the protein that have not been investigated. Even though data for about the binding capacity of rice proteins are not available, it has been shown that AHP6 interacts with AHK5 albeit with lower affinity than the other AtHPts whereas, AHP4 failed to produce any interaction with the same protein (Bauer *et al.*, 2013).

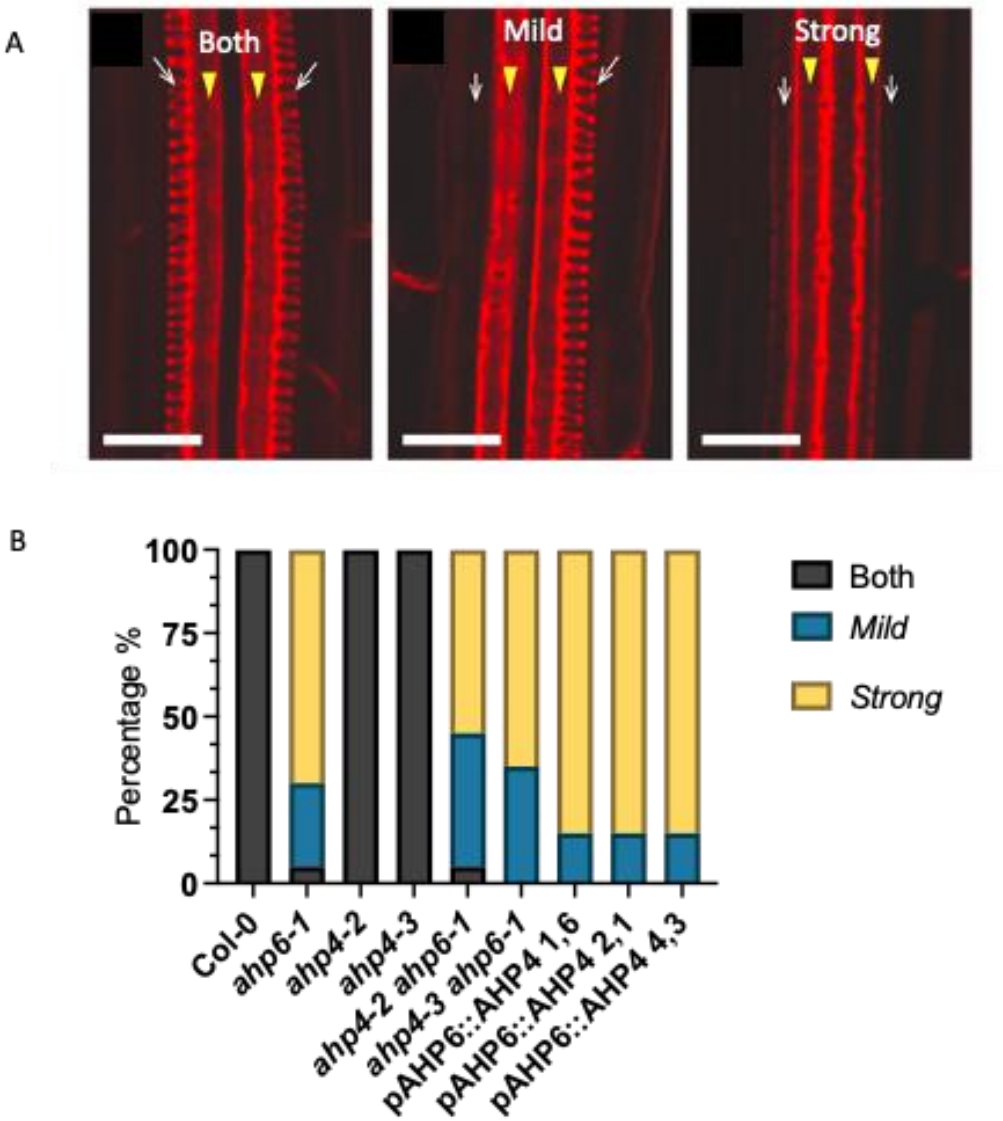


Fig 2.8: AHP4 cannot rescue the loss of protoxylem phenotypes caused by *ahp6*. A) Confocal images of roots stained with fuchsin showing the metaxylem (yellow arrowheads) and the protoxylem (white arrows) in ahp6 plants. The first image indicates the WT phenotype with two protoxylem and two metaxylem poles (both), while the next images are showing the mild phenotype where protoxylem is formed at only one pole (mild) or the strong phenotype where no protoxylem is observed at parts of the root (strong). These images have been taken from (Vaughan-Hirsch *et al.*, 2021). B) Scoring of the protoxylem formation phenotype in the indicated plants. n= 20.

2.4 Discussion

In the work presented here, AHP4 forms a distinct clade with other HPt proteins of tomato and *Medicago* but collectively, this clade is more phylogenetically related to monocot (rice and maize) PHPs than the clades containing AtHPs and AtPHP (Fig 2.1). This raised the question whether AHP4 could be a negative regulator of cytokinin signalling.

When expressed under the AHP6 promoter, AHP4 could not trans-complement the *ahp6* phenotype (Fig 2.8), even though other inhibitors of cytokinin (e.g. CKX proteins) can. This data does not support a role for AHP4 as an inhibitor of cytokinin signalling. It also does not disprove one, as it is possible that AHP4 may be a weaker inhibitor or only inhibit under certain conditions.

The same response was observed for the OsPHPs when expressed under the influence of the *AHP6* promoter (Vaughan-Hirsch *et al.*, 2021). The fact that AHP4 is closely related to OsPHP and their genes cannot replace the role of AHP6, may suggest that like OsPHPs AHP4 might be an inhibitor of the CK signalling with a different mechanism of action. For example, AHP6 as an inhibitor, even though it cannot accept or transfer the phosphoryl group, it can interact with the AHK5 competing with AtHPts. But AHP4 does not form such interaction (Bauer *et al.*, 2013) and therefore it is possible that the inhibition is happening through a different mechanism. AHP6 and AHP4 are not only functionally different, but they also differ structurally. APH4, like its homologues AtAHPs has the His residue in the crucial region of the protein which is highly conserved. Having this phosphor-accepting His residue in that specific region would mean that AHP4 can potentially get phosphorylated. However, *in-silico* analysis of the tertiary structures indicated that there is a shift in the orientation of the amino acid which could be enough to block the phosphorylation process. Another possible scenario would be that AHP4 has the ability to accept the phosphoryl group, but the structure of the protein is altered to a degree enough to cause such change where it cannot bind to any of the HKs (just like it cannot interact with AHK5) or that it lost its ability to form high

affinity bonds with any of the RRs in the nucleus. Based on the so far indications it is hard to know the mechanisms of AHP4 action in the CK signalling however, successful protein purifications of the AtAHPs and AtPHP done for the purpose of this work would enable future experiments to help determine the phosphorylation status of the AHP4 and provide the much-needed proof.

Chapter 3: AHP4 does not regulate global cytokinin response

3.1 Introduction

CKs are phytohormones and as such are linked to many aspects of plant growth and development. They often work antagonistically with auxins, a second group of major plant hormones (D'agostino, Deruè and Kieber, 2000). Post-embryonic accumulation of auxin at the root meristem (RAM) is essential for maintaining its activity and promoting differentiation of cells in the RAM (Rahman *et al.*, 2007). On the other hand, elevated CK levels in the RAM result in root growth inhibition by reducing the differentiation rate of meristematic cells (Kuderova *et al.*, 2008). For example, when the CK biosynthesis *ADENYLATE-ISOPENTENYLTRANSFERASE (IPT)* is ectopically overexpressed, both the primary root length and the number of meristematic cells were decreased in a dose dependant manner (Kuderova *et al.*, 2008). Similarly, when WT plants are grown on increasing levels of exogenously applied 6-Benzylaminopurine (BA), a synthetic form of CK, the root elongation was decreased (Hutchison *et al.*, 2006).

The function of the HPt class of proteins in the CK signalling pathway has been clearly described over the last years however, the role of individual HPts is not clear. In *Arabidopsis*, CK responsiveness differs between cell types, and this is likely due to the complexity in genes regulating both CK signalling and catabolism. Many of those genes have overlapping functions (Hutchison *et al.*, 2006; Nishiyama *et al.*, 2013). To be precise, there are three HKs, five HPts (AHP1-5), one PHP (AHP6) and 22 RRs: 10 type-A RRs and 12 type-B RRs (Hutchison and Kieber, 2002; Heyl and Schmülling, 2003). From a biochemical point of view each class of these components performs broadly similar functions. The HKs all bind cytokinin (Takei *et al.*, 2004b; Riefler *et al.*, 2006) albeit with different affinity to each isoform, and the type-B RRs bind a similar response element within the promoters of cytokinin induced genes (Hosoda *et al.*, 2015). The HPts proteins perform an essential role of relaying the phosphor-signalling from HK to RRs via their movement between the

cytoplasm and the nucleus translocating the phosphorylation signal from the HKs to the ARRs (Hwang and Sheen, 2001; IMAMURA, YOSHINO and MIZUNO, 2001; Suzuki *et al.*, 2002; Mähönen *et al.*, 2006). These proteins sharing a high degree of homology as seen in phylogenetic analysis (Fig 2.1) and they have redundant functions. Single mutants for each of the HPts (AHP1,2,3 and 5) do not have any obvious phenotypes whereas higher order mutants decrease the CK sensitivity dramatically and have redundant functions regulating abiotic stresses (Hutchison *et al.*, 2006; Jeon and Kim, 2013; Nishiyama *et al.*, 2013).

Contrary to HPts, the *Arabidopsis* PHP, AHP6, is well characterised. It is unable to participate in the exchange of the phosphoryl group as it lacks the important His residue in the conserved domain of the protein (Mähönen *et al.*, 2006). Further, AHP6 interacts with the phosphorylation machinery to inhibit phosphorelay as it was demonstrated to inhibit the phosphorylation between ARR1 and AHP1 establishing its negative role in the CK signalling pathway (Mähönen et al., 2006). It also has distinct functions as single mutants display a wide range of phenotypes (Besnard et al., 2014; Mähönen et al., 2006; Moreira et al., 2013).

AHP4 is a misfit amongst HPts. When the conserved phospo-accepting His residue is considered it is most like the canonical group of AHP1,2,3 and 5. However, when it's phylogenetic relationship with other HPts are considered, it is most closely related to the monocot group of cytokinin signalling inhibitors. The AHP4 orthologues in rice (OsPHP1, OsPHP2 and OsPHP3) are negatively influencing CK signalling by inducing a variety of *A-ARRs* and *CKXs* resulting in phenotypes affecting shoot morphological traits, like changes in panicle architecture and seed size (Vaughan-Hirsch *et al.*, 2021). In the AHP6 case, it is known that it has an inhibitory action in the CK signalling, regulated by auxin to shape the vascular pattern of the root in Arabidopsis (Mähönen *et al.*, 2006). Even though the rice PHPs (which are AHP4 orthologues) have an inhibitory action in CK signalling, they do not have the same effect on root vascular pattern as AHP6 has in *Arabidopsis* roots (Vaughan-Hirsch *et al.*, 2021) and they are not auxin inducible (Tsai *et al.*, 2012). Therefore, there are functionally diverged as compared with AHP6. In fact,

supporting the above findings, the OsPHPs fail to rescue the disturbed protoxylem pattern of *ahp6* plants (Vaughan-Hirsch *et al.*, 2021).

Accumulating evidence, along with what was shown in Chapter 2, suggests that AHP4 might have a different role than the remaining HPts, i.e., by acting as a negative regulator. For example, *ahp4* plants were found to have reduced numbers of lateral roots when seedlings were treated with exogenous CK but there was an observed insensitivity to CK when using single or multiple magnitude HPt mutants (Hutchison *et al.*, 2006). Additionally, increased root growth was observed from plants with higher order mutations (i.e., the triple *ahp1,2,3* mutant) when grown on various BA concentrations compared with the WT but interestingly, the quadruple *ahp1,2,3,4* plants responded in a similar way to *ahp1,2,3* suggesting that AHP4 has no role in this response (Hutchison *et al.*, 2006).

This work aims to elucidate the role of AHP4 in CK signalling pathway by investigating the CK responses of the newly generated CRISPR alleles under CK treatments. To do this, three possible scenarios will be considered.

The first scenario is that AHP4 acts as a negative regulator of CK signalling. In this scenario the *ahp4* alleles would be expected to have enhanced CK sensitivity to the CK treatments in growth assays. Aside from the *ahp4* allele, the *ahp4 ahp6* double mutant will be investigated for the same CK responses. This is important to establish the redundancy degree of the two genes. If for example, the phenotypes in CK responses increase in the double mutant plant that would establish that AHP4 has an inhibitory action in CK signalling and acting redundantly with AHP6. Furthermore, if indeed AHP4 is a negative regulator then its function should be investigated in relation to OsPHPs (since these proteins are phylogenetically related).

The second scenario is that AHP4 acts as a positive regulator of cytokinin. In this case, *ahp4* plants would behave in the opposite way to what was described above, and the *ahp4 ahp6* double mutants would be expected to be less responsive

to CK compared to what *ahp6* plants are. If, however, AHP4 is a positive regulator of CK signalling, the fact that HPts will be present might mask any effects due to possible redundancy. Based on the investigation of the structure of AHP4 and its phylogenetic relationship with other components of the CK singalling, this second scenario is less likely to be the case. However, *in-planta* AHP4 cannot substitute AHP6 role in modulating protoxylem development and therefore the possibility of AHP4 being a positive regulator cannot be excluded.

Finally, the third scenario would be that AHP4 has lost its function and can no longer participate in the cytokinin response pathway. However, this scenario is less likely because AHP4 is so well conserved in angiosperms (Chapter 2). If though such function is not retained anymore then AHP4 is likely to have a different function other than participating in CK signalling pathway.

Collectively, it is intended that these data will elucidate: 1) whether AHP4 is a positive or negative regulator *in-planta* 2) whether AHP4 is involved in any specific processes independently of the other HPts and 3) whether it shares a conserved function with the monocot PHPs in regulating shoot architectural phenotypes and controlling seed size.

3.2 Available mutant alleles and CRISPR

The characterisation of a gene usually involves the generation of several knock-out mutants where the protein fails to be expressed; or knock-down mutant plants where a truncated form of the protein is probably present with reduced function. Presently, the most frequently used publicly available mutant alleles have been created using T-DNA insertions (O'Malley and Ecker, 2010). These sequences are inserted randomly in the plant genome resulting in loss or disruption of function of a gene (Krysan, Young and Sussman, 1999). Because the level of the disruption of each gene is uncertain and such mutants often contain multiple insertions at different loci, several independent alleles must be used to characterise genes in an unbiased way ruling out any positional effects the t-DNA insertion

might present. The publicly available alleles for AHP4 have been created with T-DNA insertions (Hutchison *et al.*, 2006). The insertions are positioned in the third intron, the fourth exon and near the 3'end of the gene (Fig 3.1A). All three t-DNA insertions are located downstream of the conserved domain that contains the coding sequence for the putative phosphate-accepting histidine and therefore a truncated form of the protein may retain function or partial function in these mutant plants. The phenotypes associated with AHP4 and especially to its response to CK, including lateral root numbers, length of primary root, chlorophyl concentration were found to be very weak and this could be due to the published lines being only weak alleles (Hutchison *et al.*, 2006).

To date, the function of AHP4 remains to be determined, however it is not clear that the uncertainty arises from the quality of available mutant alleles or whether the role of AHP4 in the tested responses in marginal. To resolve this ambiguity, the CRISPR technology was utilized, aiming to generate independent loss-of-function mutants by inducing a premature stop codon to the translation of the protein or a frameshift upstream the conserved site, resulting in stronger mutant alleles. The CRISPR-Cas9 targeted mutagenesis was done in Col-0 and *ahp6-1* backgrounds. Any mutations arising from Col-0 plants would result in single *ahp4* plants and the mutations of *AHP4* in the *ahp6-1* background would allow the creation of *ahp4 ahp6-1* double mutants. AHP6 being the only know PHP in the *Arabidopsis* genome might mask any phenotypes caused by disruption of *AHP4*, therefore a mutation of *AHP4* in the *ahp6-1* background was decided to be essential to establish any redundancy effects caused by a possibly similar function of *AHP4* and *AHP6*.

Clustered regularly interspaced short palindromic repeats (CRISPR) is a method employed by bacteria and archaea to target viral infections, essentially constituting an adaptive and hereditary immune system (Brouns *et al.*, 2008). CRISPR, as the name suggests, consists of various short repeated sequences located between fragments of DNA with unique and specific sequences. The latter are called protospacers and are derived from viral DNA and foreign plasmids which

were incorporated into the bacterial genome upon first contact (adaptation stage). When the viral DNA enters the bacteria cell, transcription of the CRISPR RNA (crRNA), a small transactivating crRNA (tracrRNA) and other CRISPR associated (Cas) genes take place to direct, recognize and cleave the target sequence which is complementary to crRNA (interference stage) introducing a double strand brake (DSB).

As expected, there is a lot of variation in the CRISPR-Cas systems found in bacteria, which were categorized into two classes (Class I and II) and six types (I- VI) based on the structure and the molecular function of the Cas proteins involved. Specifically, type I, II and IV CRISPR-Cas system use various Cas nucleases to form a complex to bind crRNA which will guide it to the target. On the other hand, type II CRISPR-Cas system has a simpler structure as the crRNA:tracrRNA duplex recruits a single Cas protein (Cas9) at the interference stage to cleave the target sequence (Doudna and Charpentier, 2014; Makarova *et al.*, 2015). For the cleavage to occur, a short sequence need to be present near the crRNA:tracrRNA targeted sequence usually a few nucleotides long and it is recognized by Cas9. This is called protospacer adjacent motif (PAM) and it is essential for the interference stage as it only exists on the target sequence, allowing for identification between self and non-self, thus preventing the host for attacking its own CRIPR locus (Rath *et al.*, 2015). In the case of Cas9, the PAM sequence is NGG (N = any nucleotide). As the technique developed over the years, the crRNA:tracrRNA duplex was simplified into one single guide RNA (sgRNA) containing a 20 bp sequence customizable to any target sequence near a PAM size at its 5'end, and a double stranded linkage of crRNA and tracrRNA binding Cas9 (Jinek *et al.*, 2012). When gRNA is attached to the target sequence, Cas9 cleaves the DNA resulting into a DSB. At the DSB site the homology-directed repair (HDR) or the error-prone non-homologous end joining (NHEJ) pathway of the donor is activated to introduce a deletion, an insertion, or a substitution, resulting in a genetic change. Due to the system's simplicity, type II CRISPR-Cas9 system is being widely used in biotechnology to precisely edit, regulate, and mark genes of many organisms (Doudna and Charpentier, 2014).

In plants, CRISPR editing technology has been widely used to generate knock-out or knock-down plants in basic *Arabidopsis* research and in research regarding agriculturally important crops. In rice CRISPR-Cas9 assisted mutations are proving to be a very successful in producing plants resistant to biotic and abiotic stress. For example, a mutation of the ethylene responsive factor *OsERF922*, targeted using CRISPR-Cas9 contributed to the resistance of blast disease (Krysan, Young and Sussman, 1999; O'Malley and Ecker, 2010).This is particularly important for crops because CRISPR editing enables the creation of stable heritable mutations in the absence of the transgene.

The generation of CRISPR mutants for this work was done using Agrobacterium mediated transformation to insert the CRISPR cassette from pKAMA-ITACHI_KIR1.1 vector into the *Arabidopsis thaliana* Col-0 and *ahp6-1* plants. The vector drives Cas9 expression under the influence of ribosomal protein S5 (*RPS5A*) promoter and the red fluorescent tagged (*TagRFP*) Oleosin 1 (*OLE1*) gene driven by its native promoter (Tsutsui and Higashiyama, 2017). This enables constitutive expression of Cas9 in plants containing the transgene. Furthermore, the presence of the OLE1::OLE1:TagRFP facilitates the selection of plants with the transgene at T1 after the plant transformation by screening for seed with fluorescent seed coats. Upstream of RPS5A::Cas9 is the hygromycin selection gene driven by nopaline synthase (NOS) promoter allowing additional selection of the transgenic plants. In addition to the Cas9, the TagRFP and the hygromycin gene, the vector holds the sgRNA cassette which consists of the sgRNA scaffold driven by U6.26 promoter (U6.26p::sgRNA) with two AaRI restriction sites between them. Digestion of these sites allows insertion of the 20 bp fragment which is complementary to *AHP4* and will direct the Cas9-gRNA complex at the desirable position (Fig 3.1 A; Tsutsui & Higashiyama, 2017).

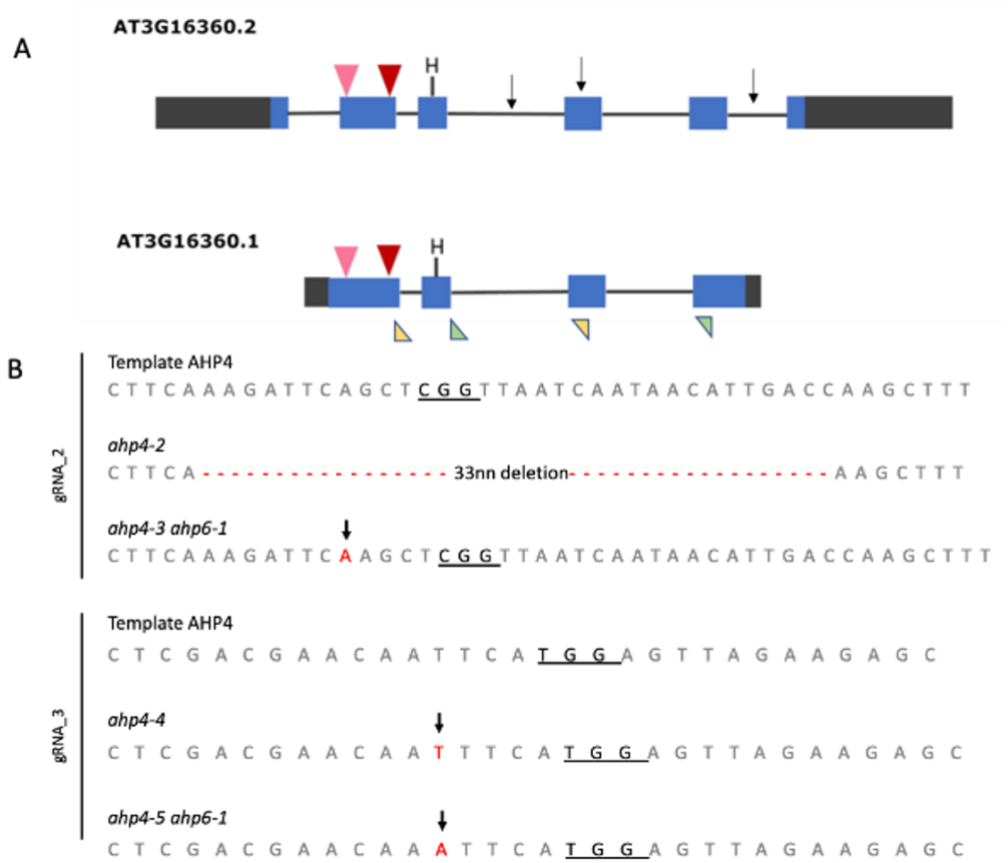


Fig 3.1: Generation of the CRISPR-Cas mutants. (A) Schematic representation of *AHP4* gene showing the sgRNA_2 (pink triangle) and sgRNA_3 (red triangle) targeting sites. Black arrows indicate the insertion area of T-DNA in the publicly available SALK lines. The position of the conserved region where the histidine sequence is located is indicated on the schematic by the letter H. Black boxes indicate 3'UTR and 5'UTR, blue boxes indicate exons and black lines are introns. Yellow triangles show the position of the qPCR primer pair E14-E15 and green triangles showing the position of the qPCR primer pair AHP4end (B) Showing the editing events of the two independent CRISPR alleles. Underlined sequences indicating the PAM recognition sites, black arrows showing the position of the insertion, letters in red indicating the inserted nucleotide and red dotted line showing the 33-nucleotide deletion of the *ahp4-2* allele.

In *Arabidopsis* there are two alternative splice variants with different exons as shown in figure 3.1. The region of *AHP4*, where the 20bp gRNA would bind, was selected based on four important parameters and taking in account the two variants of the gene: 1) the edited region needed to be upstream of region expressing the conserved histidine, therefore coding sequence deriving from exon 1

and exon 2 of the gene was selected for the AT3G16360.1 and AT3G16360.2 respectively. This is to ensure that any resulting editing event encode a protein with a disrupted phospho-accepting domain. 2) The 20bp gRNA must be near a PAM site (NGG) for Cas9 and the scaffold proteins to be able to bind. 3) Specificity: The gRNA selected, being only 20bp long, must be unique to the sequence of *AHP4.* The gRNA-Cas9 complex will be targeting any sequence matching the 20bp gRNA, therefore, any genomic sequence that has large similarity to the gRNA sequence will be edited too as an off-target. 4) Lastly the efficiency of the binding between the target genomic sequence and the gRNA was maximized. This is assessed by a variety of factors, spanning from the sequence composition to accessibility of the target DNA as determined by chromatin structure. gRNAs with high efficiency are more likely to perform the cleavage. All four parameters have been considered to design the appropriate gRNAs with the help of an online software (CRISPOR) which provide values for specificity and efficiency while suggesting sequences near a PAM site (Concordet and Haeussler, 2018).

Based on the software's recommendation, three gRNAs were selected for meeting the above criteria. The efficiency and specificity of gRNA as well as the possibility of binding to any off-target target sequences was determined *in-silico*. However, it is important to select multiple gRNAs to establish gene edited plants arising from independent editing events to ensure that there are not any off-target effects. These gRNAs were hybridized and cloned into pKIR1.1 CRISPR-Ca9 vector described earlier. However, cloning was successful only for gRNA2 and gRNA3 and therefore, gRNA1 was discarded.

The successfully cloned gRNAs were then used to transform plants in Col-0 and *ahp6-1* backgrounds using *Agrobacteria* mediated transformation. The selection process of primary transformants was facilitated by the red seed color resulting from the OLE1::OLE:TagRFP which was visible under the fluorescent screening microscope. The presence of RFP indicated that the seeds were transformed with the construct which also includes the gRNA-Cas9 cassette. The transgene was removed from the T2 generation by selecting for seeds that do not

express RFP and therefore not expressing Cas9 anymore. This selection ensures the removal of the transgene; however, these plants were likely to have an editing event at the *AHP4* locus as they originated from plants expressing Cas9. To identify the presence of an editing event, PCR based genotyping followed by sequencing was performed. The plants that contained a possible edit were identified and propagated in T3 where homozygous edited plants would be available. The resulting CRISPR lines originated from gRNA2 transformations produced *ahp4-2* plant line with 33bp deletion and the *ahp4-3 ahp6-1* line with an adenine inserted 5bp upstream of PAM site (Fig 3.1 B). The CRISPR lines resulting from transformation involving gRNA3, produced *ahp4-4* line with a thymine added 5bp upstream of the PAM site and the *ahp4-5 ahp6-1* line with an adenine added in the same position (Fig 3.1 B).

The 33-nucleotide deletion created in *ahp4-2* plants resulted in 11 amino-acid deletion and the production of a truncated form of the AHP4 which retained the histidine residue but was no longer in the same position. The *ahp4-3 ahp6-1* with an adenine insertion 5bp upstream of the PAM sequence resulted in frameshift and a stop codon was introduced at about 15 bp downstream of the PAM sequence. In the case of *ahp4-4* and *ahp4-5 ahp6-1*, there was an insertion of T and A respectively at the same position (5bp) upstream of the PAM site resulting in a premature codon about 50 bp downstream of that change. Overall, *ahp4-2* encodes a truncated version of AHP4 retaining the histidine residue, albeit in a different position. This may represent a weak allele. However, the editing events of the remaining alleles all produced premature termination codons (PTC) and therefore are likely to be null alleles.

In CRISPR-Cas9 edited lines the effects are often observed at the translational level where the changes in the sequences of transcripts result in non-functional proteins. However, at the mRNA level, the transcripts are still synthesized unless the mRNA produced is not of sufficient quality to produce a protein. The quality check is based on the position of PTC in relation to exon-exon junctions or the length of the 3’ UTR. In the event which the mRNA fails to pass the

quality check, it is degraded before reaching the translational machinery by the translation depended, nonsense- mediated mRNA decay (NMD) pathway (Lykke-Andersen and Jensen, 2015). Most of the alleles created with CRISPR-Cas9 technology in this work have a nucleotide addition which results in a PTC and therefore may be targets of the NMD pathway for degradation. Furthermore, the *ahp4-2* plants with the 33-nt deletion have the possibility to produce a truncated AHP4 protein if its mRNA reaches the translational machinery. However, the deletion might be enough to cause the activation of NMD pathway. With the degradation of the mRNA before the translation, this allele might not be as weak as initially thought.

Quantitative real-time PCR (qRT-PCR) was performed to identify the levels of transcripts present in the CRISPR edited plant lines (Fig 3.2). Two sets of reactions were performed, one with primers spanning the edited positions resulting from gRNA2 editing event (Fig 3.2 A) and the second with primers attaching near the 3' end of the gene (Fig 3.2 B). No transcript was detected in the *ahp4-2* allele for the first pair of primers (Fig 3.2 A) but it was present and equal to the WT control for the second pair (Fig 3.2 B). This is because primers of the one primer pair bind in the area deleted by the editing event in *ahp4-2* in the first primer set. However*, AHP4* mRNA levels produced in this mutant are likely to be equivalent to those in WT, as evident from the second 3' end binding pair (Fig 3.2 B). This indicates the possibility of a truncated protein being produced raising the possibility that the *ahp4-2* might not be null. Contrary to *ahp4-2*, the *ahp4-4* allele showed reduced transcript levels for both the primer pairs, suggesting that the insertion resulting from the CRISPR-Cas method, which is introducing a premature termination codon (PTC), may be enough to cause the activation of the nonsense-mediated mRNA decay (NMD) machinery (Lykke-Andersen and Jensen, 2015).

The transcript levels for the CRISPR mutants in the *ahp6-1* background were also reduced when the second pair of primers was tested, attaching near the 3' end of *AHP4* (Fig 3.2 B). This indicated that both double mutant alleles were expressing reduced levels of *AHP4* and therefore they could be used for further characterisation experiments.

Based on the above analysis of the mutants it was determined that all the alleles were suitable null alleles to be used for further analysis, with the exception of *ahp4-2* which suggest the presence of a viable protein. However, the mutant was not excluded from all the analyses of this work.

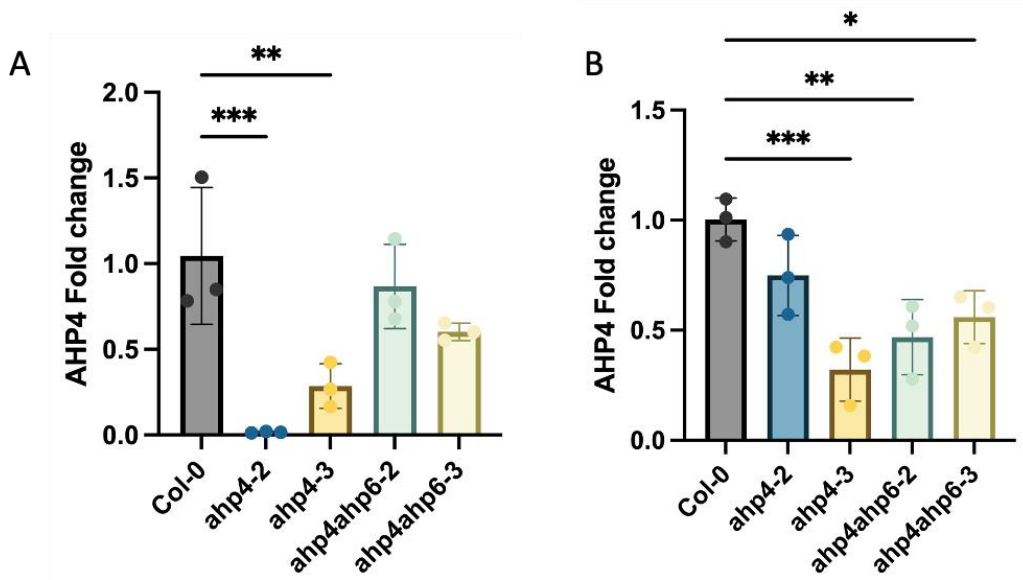


Fig 3.2: AHP4 expression in CRISPR edited plants. (A) AHP4 transcript levels of the four CRISPR edited lines using exon 1-2 spanning forward primer (E14) and exon 3 reverse primer (E15). (B) AHP4 transcript levels in the same CRISPR plant lines using primers spanning the exon 2-3 and exon 4 (AHP4endF and AHP4endR). Significance is calculated by one-way ANOVA and Brown-Forsythe statistical tests. Multiple comparisons to the control were corrected using the Dunnett's test to test the significant differences as compared to Col-0 control. (*P≤ 0.05, **P≤ 0.01, ***P≤ 0.001).

3.3 Constitutive ectopic *AHP4* expression does not translate into increased protein levels

One method commonly used for characterising the function of a gene is creating lines able to over express the gene, which consequently express the protein, ectopically and in a constitutive manner. Further, *AHP4* expression levels are very low and in a small subset of cells (discussed in chapter 4), therefore any effects the A HP4 might have on the responses tested might not be able to result in

observable changes. However, if AHP4 is a negative regulator, its constitutive expression would result in visible reduction in the CK responses tested.

To ensure the overexpression, the gene must be inserted in the genome in the form of a transgene driven by a promoter that is constitutively active throughout the development of the plant. Even though there are various native plant promoters that can be used to drive such expression pattern, the heterologous promoters, deriving from non-plant organisms, are more preferably used. This is because native plant promoters, such as actin (Act) or ubiquitin (Ubi) produce transformed plants with lower levels of protein expression, when compared with viral-derived promoters such as Cauliflower Mosaic Virus (CaMV) 35S promoter, as demonstrated in maize transformations (Beringer *et al.*, 2017). In Arabidopsis, the native *ACT2* promoter was found to drive strong expression of genes under is control, but not necessarily in all plant tissues (An *et al.*, 1996; Potenza, Aleman and Sengupta-Gopalan, 2004). Such a problem is bypassed when using a heterologous promoter, i.e., from a virus, because plant viruses developed a strong constitutive activity in regulating their gene expression as they rely on their host's gene expression machinery to express and synthesise their infectious proteins (Reviewed by Amack & Antunes, 2020).

Currently, the most widely used viral promoter for transgenic expression in plants is the CaMV 35S promoter which meets the above requirement of expressing the transgene in all plant tissues (Odell, Nagy and Chua, 1985). An additional advantage of using 35S promoter comes with the resources available. Because of its common usage, it is available in many commercial vector cassettes allowing for easy subcloning of the gene of interest.

For the generation of the overexpressing lines, the CaMV 35S promoter was used to drive *AHP4* expression in Col-0 background. The vector was created using the modular GreenGate technology, using the prefabricated plasmid pGGA_002 expressing the 35S promoter (Lampropoulos *et al.*, 2013). The final vector was used to transform plants and these were selected by growing T1 seeds on Kanamycin

containing ½ MS medium. Similarly, kanamycin containing medium was used to establish single transgene insertion of T2 plants by ensuring a 3:1 segregation ratio. Homozygous lines were selected in the T3 generation and plants were tested for *AHP4* transcript levels using qRT-PCR.

The resulting two independent lines generated, p35S::AHP4 7-2-1 (OEx1) and p35S::AHP4 10-5-6 (OEx2), which expressed 1000-fold and 500-fold more *AHP4* as compared with the WT respectively (Fig 3.3 A). To test these OE lines further, the transcript levels of three *ARR*s (*ARR3*, *ARR4* and *ARR5*) were tested using qRT-PCR. These are type-A ARRs and are upregulated in elevated CK conditions to negatively affect the expression of type B ARRS and therefore are negative regulators of CK signalling (D'agostino, Deruè and Kieber, 2000; Hutchison *et al.*, 2006). If AHP4 inhibited cytokinin response, it could be predicted that the levels of these ARRs would decrease. The *ARR*s tested were expressed at levels not significantly different from the WT in both independent OE lines (Fig 3.3 B) indicating that increased *AHP4* expression is unlikely to affect cytokinin response.

To test that the increased transcript levels observed in the OE lines (Fig 3.3 A) correspond to elevated protein levels, protein extract was isolated from OEx1, OEx2 and OEx 3 (line p35S:AHP4 10-7-1) seedlings and western blotting was performed using antibodies against AHP4 (aAHP4). The aAHP4 is a monoclonal antibody raised in mouse hybridoma cells and was commercially sourced from Central European Institute of Technology (CEITEC).

The qRT-PCR experiment above and the localisation experiments discussed in Chapter 4, indicate that AHP4 is expressed at very low levels. Therefore, it was assumed that it would be extremely challenging to visualize the AHP4 protein in wild-type plants. As the antibody had not been used previously, protein extracts from E. coli expressing AHP4 (described in Chapter 2, 2.2) were used as a control.

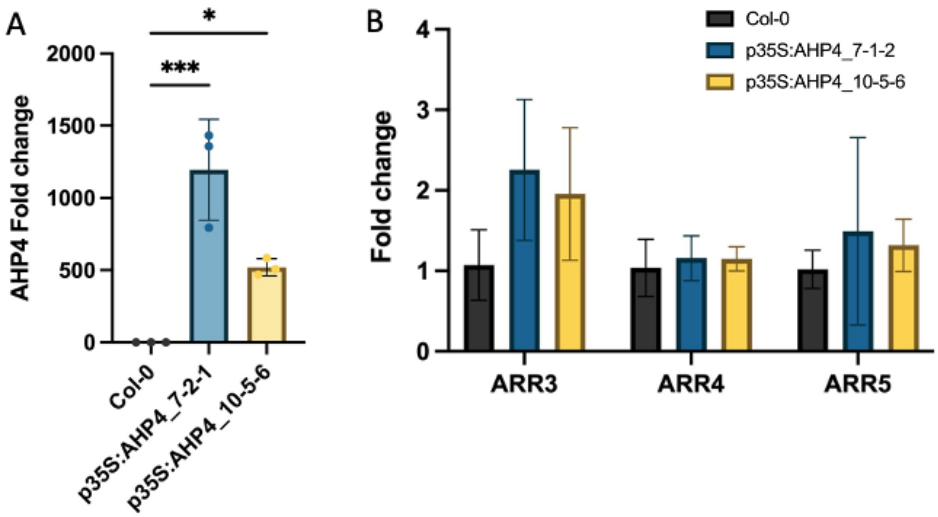


Fig 3.3 p35S::AHP4 lines show increased *AHP4* expression but do not affect the expression of type-A ARRs. A) Expression of *AHP4* in WT and the two independent OE lines as determined by qRT-PCR using the E14 and E15 primer pair. B) Transcript levels of *ARR3*, *ARR4* and *ARR5* in the OE lines. One-way ANOVA and Brown-Forsythe statistical tests were used to calculate significance. Multiple comparisons to the control were corrected using the Dunnett's test to test the significant differences as compared to Col-0 control. Error bars represent standard deviation (n=3, *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001).

AHP4 could not be detected in WT, *ahp4* plants or the over-expressing lines (Fig 3.4 A). However high levels of AHP4 were detected in material taken from bacteria. These findings firstly suggest that the antibody against AHP4 is able to recognise the appropriate protein, evident from its ability to bind to the bacterial expressed AHP4. Secondly the OE lines are not able to synthesise AHP4 at high levels (Fig 3.4 A) despite the high transcript levels produced (Fig 3.3 A).

This phenomenon may be caused by epigenetic gene silencing at the post transcriptional level (PTGS) (Jauvion *et al.*, 2012). This often occurs when a transgene is introduced at high levels, with its endogenous homologue present in the genome too (homology- dependent gene silencing-HDGS). For example, when *Petunia chalcone synthase* (*CHS*), an enzyme in flavonoid biosynthesis facilitating the anthocyanins accumulation, was overexpressed in an effort to produce more vibrant colours, the opposite effect was achieved and flowers appeared partially or completely white due to the loss of expression of both the transgene and the

endogenous gene (Napoli, Lemieux and Jorgensen, 1990). A second possibility as to why the gene failed to get translated in the OE lines is that AHP4 translation might require some additional sequences not present in the CDS such as the UTR sites or the introns. UTRs were found to control the transportation of mRNAs to the cytoplasm affecting the translation efficiency of certain genes, the localisation of the mRNA subcellularly and its overall stability in the cytoplasm (Reviewed in Mignone et al., 2002).

Due to time constraint, generation of better OE lines was not possible, however, given the fact that AHP4 is expressed in very low levels and its expression is restricted in a small subset of cells (discussed in Chapter 4), these lines are valuable to determining the CK regulation status of AHP4. To do this, the AHP4 CDS could be expressed under the 35S promoter in *ahp4* background to eliminate the possibility of HDGS caused by the presence of the endogenous gene. Another way to work around this problem is the use of genomic DNA instead of just the CDS to incorporate the introns and/or the UTRs.

From the results described in this section, it is evident that the overexpressing lines generated for the purpose of this thesis are not able to produce the protein in enhanced levels and therefore they will not be used for further analysis and the mutants will be prioritised.

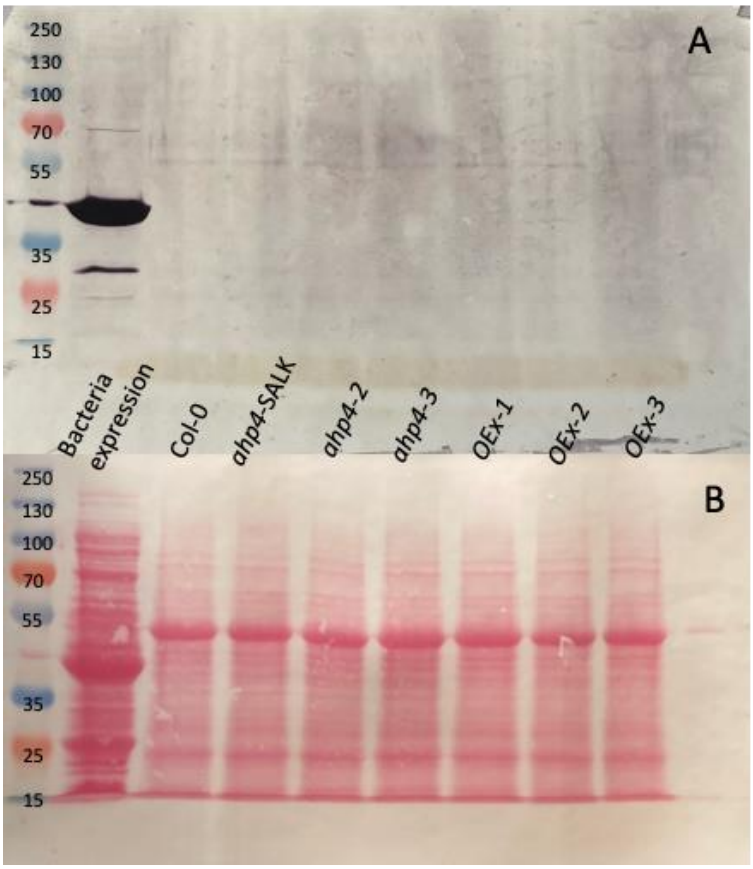


Fig 3.4 AHP4 was not detectable in WT, *ahp4* and OE lines. Western blotting for detecting AHP4 levels in the CRISPR edited plants and the OE lines using aAHP4 as the primary antibody, targeted by AP-conjugated anti-mouse IgG. A) Membrane showing the AHP4 level for the indicated lines and B) The ponceau staining of the same membrane to indicate equal loading of the protein samples. OE 1 is p35S::AHP4_7-2-1 line, OE 2 is p35S::AHP4_10-5-6 and OE 3 is p35S::AHP4_10-7-1.

3.4 AHP4 exhibits a subtle response to CK, and does not work redundantly with AHP6

As mentioned in the previous section (3.2) the investigation of the role of AHP4 in CK signalling was performed mainly using the SALK t-DNA insertion lines which are likely to be weak alleles. Development of the new *ahp4* alleles using CRISPR gene editing technology allowed the re-assessment of the role of AHP4 in CK signalling using a reverse genetic approach. To do this, the response of the *ahp4-2*, *ahp4-4*, *ahp4-3 ahp6-1* and *ahp4-5 ahp6-1* CRISPR alleles to various CK treatments were compared with WT and *ahp6-1* plants. A range of responses were investigated to include both below and above ground developmental aspects for

which CK is known to be involved. For example, the investigation of root length was chosen for examining the role of AHP4 in the root using the new CRISPR lines and chlorophyl content, shoot branching and seed size were used to represent some of the CK related responses in the above ground tissues. The response of *ahp4* mutants to CK was measured using these assays because they are easily measured and the involvement of CK in these pathways is strong.

Plants were grown on media containing increasing concentrations of CK, and root length was recorded between the third and fifth day post germination (Fig 3.5 A). Consistent with the literature, CK inhibited root growth (Hutchison *et al.*, 2006; Kuderova *et al.*, 2008). Furthermore, *ahp4-2* and *ahp4-4* showed a subtle insensitivity to low and medium concentrations of CK compared with the WT, while *ahp6-1* responded the same as WT to all levels of CK despite having significantly longer roots in control conditions. When double mutant *ahp4-5 ahp6-1* plants were examined, there was no observable change in the CK response as compared to the one *ahp4-4* had (Fig 3.5 B).

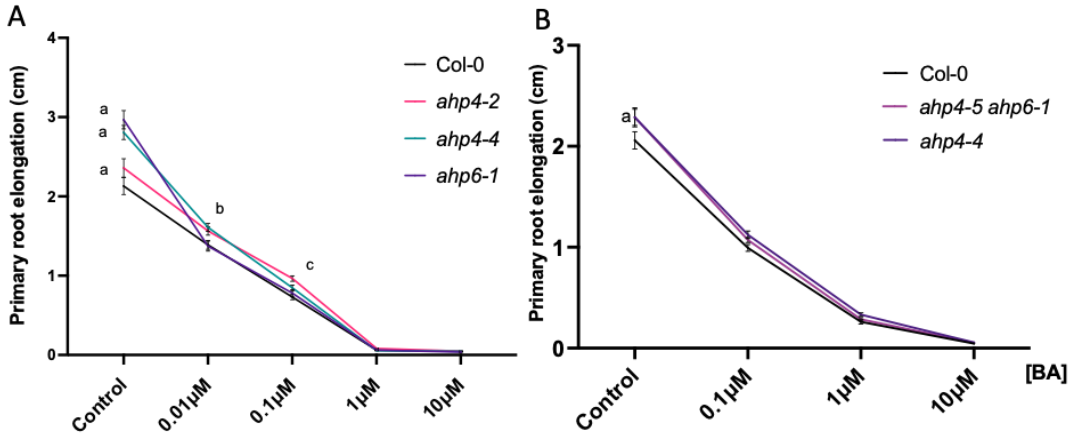


Fig 3.5 *ahp4-2*, *ahp4-4* plants have marginal insensitivity to BA treatment in root growth. Root elongation between 3rd and 5th day post germination of WT, *ahp4-2*, *ahp4-4*, *ahp6-1* and *ahp4-5 ahp6-1* plants in increasing concentrations of BA. Measurements of the roots were performed using ImageJ and statistical significance was determined using two-way ANOVA and Tukey's multiple comparison test (n=20, P≤ 0.0001). a: significance between WT in control, b: significance between 0.01 µM BA and c: significance between 0.1 µM BA. This set of data is a representative of 3 independently executed experiments.

For the above the ground tissues, chlorophyl content was one of the assays chosen to investigate the effects of AHP4 in response to increasing CK. Chloroplast develop from stem cells which contain proplastids. In the dark protoplasts develop into etioplasts but in light conditions the thylakoid membrane develops, and they transform into becoming fully functional chloroplasts. This is termed the deetiolation or greening process because chlorophyl biosynthesis occurs (Reviewed by von Arnim & Deng, 1996). Light is the major agent driving the chlorophyl biosynthesis but cytokinin was found to partially regulate chloroplast development by inducing thylakoid membrane differentiation (Chory *et al.*, 1994). Specifically, total chlorophyl (Chl) content of the *ahk2 ahk3* mutants, which have reduced CK sensitivity, was significantly reduced as compared to the WT while application of CK increased Chl concentrations in WT plants (Cortleven *et al.*, 2016).

Therefore, *ahp4* mutants were assayed for the cytokinin-mediated accumulation of Chl to determine whether AHP4 played a role in this aspect of ck response. The Chl content of *ahp4-4* plants was measured in fourteen-day old seedlings along with WT and *ahp6-1* plants for positive and negative control respectively. This assay was done on seedlings grown in control media as well as low (25nM BA) and medium (100nM BA) CK containing media (Fig 3.6). As expected, the Chl content in *ahp6-1* plants was significantly increased as compared to the WT in control conditions and in the 25mM BA treated plants. However, *ahp4-4* plants responded the same way to WT in those two conditions suggesting that AHP4 has no role in regulating the chloroplast development in response to CK. Under control conditions, the total Chl content in *ahp4-5 ahp6-1* double mutants was found to be comparable to the *ahp6-1* plants, but when CK was present in the growing medium plants had less Chl than both *ahp6-1* and WT plants. The above results suggest that AHP4 and AHP6 do not act redundantly in the CK pathway to promote Chl production when intrinsic CK levels are elevated but they might act antagonistically at increased exogenously applied CK. AHP4 increases CK perception levels only in elevated CK environments and in the absence of AHP6.

Chlorophyl concentration was reduced significantly but equally in all genotypes tested when seedlings were grown on 100 mM BA (Fig 3.6). These plants were chlorotic and significantly smaller than those in the other two treatments. At this concentration, growth was affected so severely, it was not possible to interpret the chlorophyl data with confidence.

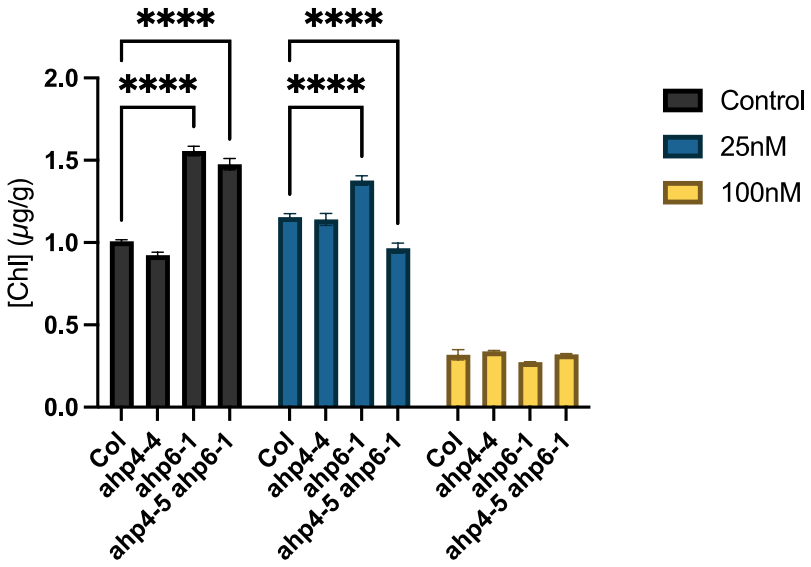


Fig 3.6 Single *ahp4* mutants do not show differences in the accumulation of chlorophyl but may act antagonistically to AHP6 when plants are exogenously supplied with high levels of cytokinin. Chlorophyl (Chl) content in 14-day old plants grown on control medium and on the indicated concentrations of BA. Two-way ANOVA and Tukey's multiple comparison tests were used to calculate significance (n=3, *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001). This set of data is a representative of 3 independent experiments.

A second shoot related assay to test the role of AHP4 involved shoot branching. Cytokinin has central role in regulating shoot growth and architecture (Reviewed in Hwang et al., 2012). The shoot apical meristem (SAM) activity is increased in environments of increased CK concentrations (Bartrina *et al.*, 2011b). A second meristematic zone situated in the leaf axils, the axillary meristems (AM) are essentially mimicking the SAM activity and, when activated, are responsible for the formation of branches. Branching increases the reproductive organs produced and it is a complex process influenced greatly from environmental conditions such as

nutrient availability, light conditions, and temperature but orchestrated by the interaction of the various phytohormones. For example, auxin and strigolactones inhibit shoot branching, however CK works antagonistically to regulate the activation and outgrowth of AMs (Muller and Leyser, 2011; Wheeldon and Bennett, 2021).

To investigate the effects of AHP4 in branching the total number of inflorescence stems was measured in 25 day-old plants in *ahp4-4*, *ahp6-1* and *ahp4-5 ahp6-1* double mutant plants. On average, more stems were recorded only in *ahp4-4* plants as compared to WT (Fig 3.7 B). Though AHP6 had no significant effect on the total number of stems, *ahp4-5 ahp6-1* plants had significantly fewer stems than WT. Furthermore, the length of the primary inflorescence stem was longer in *ahp4-4* plants than wild-type but significantly shorter in *ahp4-5 ahp6-1* plants, whilst *ahp6-1* plants had similar primary inflorescence stem length to WT (Fig 3.7 C). For both shoot assays, AHP4 appears to negatively regulate CK signalling but only in the presence of a functional AHP6.

In the reproductive tissues, increased CK levels results in larger flowers and larger siliques carrying more seeds (Hutchison *et al.*, 2006; Riefler *et al.*, 2006; Bartrina *et al.*, 2011b). This is observed across a variety of plants especially those of agronomical importance. For example, a defective *OsCKX2* gene in rice resulted in plants with increased endogenous CK levels and increased grain yield (Ashikari *et al.*, 2005b). To investigate the effects of AHP4 in shoot growth and development seed size was determined by measuring the length, the total number of inflorescences formed and the length of the primary inflorescence (Fig 3.7). Seeds were larger across all genotypes tested as compared with the WT, but *ahp6-1* and *ahp4-5 ahp6-1* plants produced equally larger seeds than *ahp4-4* (Fig 3.7 A).

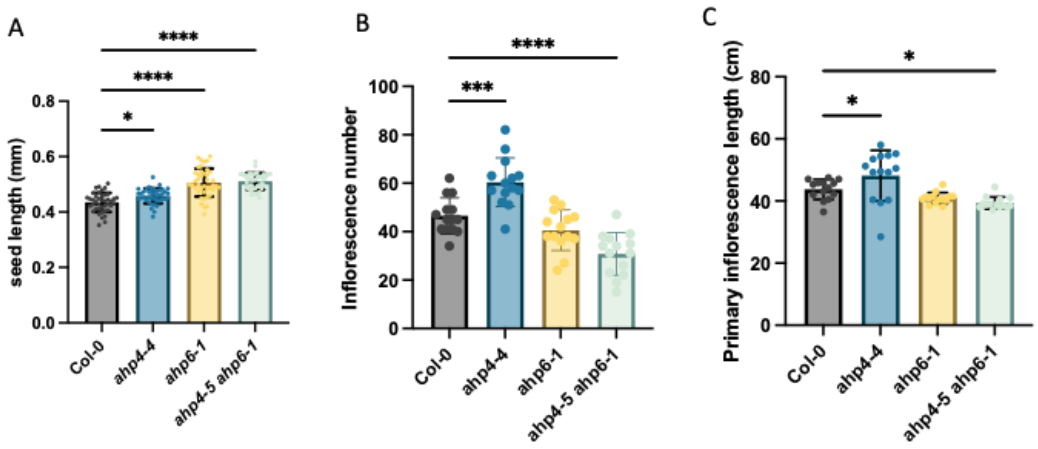


Fig 3.7 AHP4 increases CK perception in shoots but does not act redundantly with AHP6. Investigation of shoot and seed phenotyping traits related to CK signalling mutants. (A) Mature seed size was determined by measuring the length of the longest side of each seed (n=41). (B) The total number of inflorescence stems observed in 25-day-old plants for each genotype was recorded (n=14) and (C) the length of the primary inflorescence was measured for each of the four genotypes shown (n=14). For all the three experiments, significance was calculated by one-way ANOVA, Brown-Forsythe, and Bartlett's statistical tests. Multiple comparisons to the control were corrected using the Dunnett's test to test the significant differences as compared to Col-0 control (*P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001).

Based on the CK responses investigated the AHP4 appears to have different functions in the shoot and the roots and does not act redundantly with AHP6, the known negative regulator of CK signalling in *Arabidopsis*. Furthermore, its role appears to be minor in both organs as evident from the small effects observed in the responses investigated in this report. In the roots, AHP4 exhibits reduced sensitivity to exogenously applied CK, suggesting that it may act as positive regulator of CK signalling. In the shoots however, while no redundancy with AHP6 is observed, AHP4 appears to be working to inhibit CK signalling but only in the absence of a functional AHP6.

Some differences of phenotypes observed for the aforementioned CK responses between mutants as compared with the WT were significant however,

relatively mild. Furthermore, CK is a hormone regulating a myriad of distinct biological processed in plant growth and development. Due to this pleiotropy, the examination of each process separately to understand where AHP4 plays an important role, would not be productive. Therefore, to pinpoint the specific process in which AHP4 is involved, changes in the transcriptome between mutants and WT plants were investigated as a high throughput approach. This would be able to be seen if, for example, genes associated with a specific GO term were DE between *ahp4* and WT then it would make sense to focus future phenotyping on processes associated with that term.

3.5 Analysis of transcriptome reveals AHP4 has no impact on the whole plant transcription

For this analysis, plants have been grown on agar medium supplemented with ½ MS for seven days and the total RNA was extracted from 7-day-old WT and *ahp4-2* shoots or roots. This age of plants was chosen so that it does not coincide with the onset of the secondary development of the plant to avoid the transcriptional changes that come with this period of development (Oh, Park and Han, 2003). Prior to harvesting, plants were treated by submersion in 100 nM BA containing ½ MS solution with 0.1% DMSO or in ½ MS solution containing only 0.1 % DMSO for a total period of 2 hours. The quantity of DMSO in the mock was equal to the DMSO concentration present in the BA containing medium. Previous experiments investigating the expression of *ARRs* in response to exogenously applied CK were done using 45 min treatments (To *et al.*, 2004; Mason *et al.*, 2005; Hutchison *et al.*, 2006), however the treatment for this transcriptomics experiment was decided to be 2-hours to include more targets but no longer than that to only target the genes that are directly affected by CK. The examination of the transcriptome was done in shoots and roots separately because the expression profile of the two organs is massively different. In addition, CK has different often opposing roles between the shoot and roots (Kuderova *et al.*, 2008). In addition, it was shown earlier (Chapter 3, 3.4) that AHP4 acts differently in the shoots from the roots. Therefore, the two tissues were separated in order to be able to see the

differences in gene expression ensuring that the differentially expressed (DE) gene pool would not be diluted from including both tissue types. The CK treatment was done by the addition of a synthetic form of CK, BA. The comparison between BA treatment and mock was important because comparison between CK inducible genes is essential to understand the function of AHP4 in the CK signalling pathway.

The mapped sequencing reads were then analysed using a principal component analysis (PCA) for assessing the reproducibility of the DE genes between the replicates within a sample type (Fig3.8). The above PCA suggests high reproducibility between replicates since they are clustering together. In addition, PCA suggest that there is a clear separation between the two treatments in roots but not between the WT and *ahp4-2* (Fig 3.8).

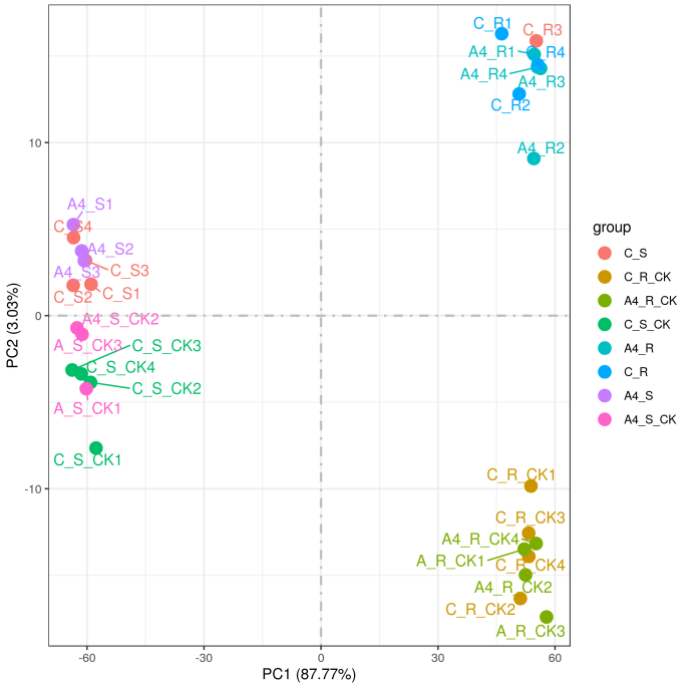


Fig 3.8 Principal Component Analysis showing clustering analysis the separation between the treatments in roots but not between the mutant and the WT. In the shoots, the two treatments are clustering closely but the separation exists. There is no separation between the *ahp4-2* (A4) shoots and WT (C). PCA was performed by the commercial RNASeq provider (Novogene). C_S: Col shoot, C_S CK: Col shoot CK+, C_R: Col root, C_R_CK: Col root CK+, A4_S: *ahp4* shoot, A4_S_CK: *ahp4* shoot CK+, A4_R: *ahp4* root, A4_R_CK: *ahp4* root CK+.

The initial analysis of the transcriptome was done commercially by Novogene and subsequent analysis converted raw counts into fragments per kb mapped reads (fpkm). A cut-off value of < 1 was set to filter out the genes that are very lowly expressed. Gene values that made the cut-off were expressed in as the logarithm of 2-fold change (log2FC) and the threshold to define DE genes was set to 0.05 (p-value). This analysis was done by Dr Rahul Bhosale and revealed that only a very small number of gene were DE in comparisons between WT and *ahp4-2* plants irrespective of the tissue (Fig 3.9). Specifically in the roots, only 20 genes were found to be DE in WT versus *ahp-2* plants from the mock treatment, 5 of which were upregulated and 15 of them downregulated; and only 7 DE genes found when these two genotypes were treated with CK (Fig 3.9 A). Some of those genes involved ribosomal proteins, glutamate decarboxylase 4 (GAD4), Jacallin related lectin 41 (JAL41), flavin-monooxygenase glucosinolate S-oxygenase 2 (FMO GS-OX2), vegetative storage protein 2 (VSP2) and a lot of putative or unknown proteins which are not normally associated with CK response. 15/20 DE genes from tissue derived from roots were unique to the mock treatment and 2/7 DE genes were only DE in roots treated with CK (Fig 3.9 B).

Similarly, in WT versus *ahp4-2* shoot tissues only 2 DE genes were identified in plants treated with the mock solution and 1 DE gene was isolated from the analysis in CK treated plants (Fig 3.9 A). 1/2 DE genes found in the comparison between WT and *ahp4-2* shoots were unique to the control/mock treatment. Consequently, no unique DE genes were isolated in the analysis of shoots from plants treated with CK (Fig 3.9 C).

Taken together, these findings suggest that there is very little difference in the transcriptome of tissues originating from WT or *ahp4-2* plants, at the specific timepoint examined. Furthermore, treatment with exogenous CK did not reveal any DE gene which would possibly be implicated with the function of AHP4 in CK signalling.

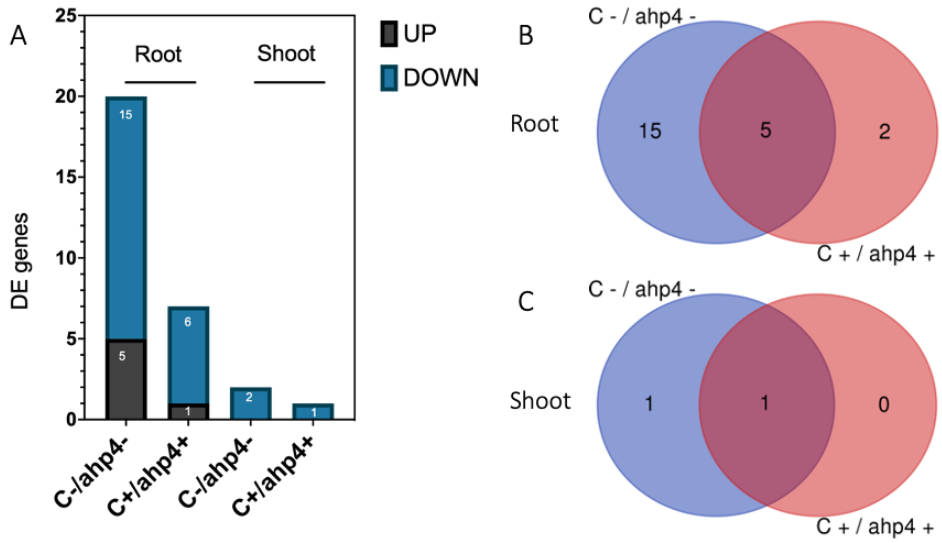


Fig 3.9 Transcriptomic analysis of WT shoots and roots vs *ahp4-2* shoots and roots treated with CK or with a mock solution. A) The DE genes found in roots and shoots when WT tissue is compared with tissue obtained from *ahp4*-2 plants in situations where plants were treated with BA or a mock solution. The numbers on each block represent the number of upregulated or downregulated genes. B) Venn diagrams showing the number of unique DE genes in roots of WT vs *ahp4-2* plants treated with BA or a mock solution. C) Venn diagrams showing the number of unique DE genes found in shoot tissue of WT vs *ahp4-2* plants with the same treatment. C: Col-0, ahp4: *ahp4-2*, -: treated with mock DMSO solution, +: treatment with BA.

Comparison between treatments of the same genotype revealed many DE genes in both the root and the shoot tissues (Fig 3.10). Specifically, 288/1140 genes are DE in WT roots due to CK treatment and 348/1200 DE genes found in *ahp4-2* roots (Fig 3.10 A). Likewise, in the shoots, 117 genes were DE only in WT as a response to CK treatment and other 117 DE genes were unique to ahp4-2 (Fig 3.10 B). This indicates firstly that CK treatment induced several genes and secondly that many of those genes are unique to either the WT control or the *ahp4-2* mutant.

To understand the function of the DE genes identified in the roots of WT and *ahp4-2* plants treated with mock or CK solution as well as to identify processes in which AHP4 might function, a functional enrichment analysis was performed (3.11). The functional enrichment analysis was done using Gene Ontology (GO) terms for the category Biological Process (BP) using the PANTHER

overrepresentation tool and revealed a variety of significantly enriched processes. The terms identified were mainly universal for both WT and *ahp4-2* roots, specifically the terms were relevant to development, abiotic stress, responses to stimuli, and metabolic processes. The only unique GO terms for BP were observed in C-/C+ root tissues and included responses to hormones, organ senescence and defense response (Fig 3.11 A).

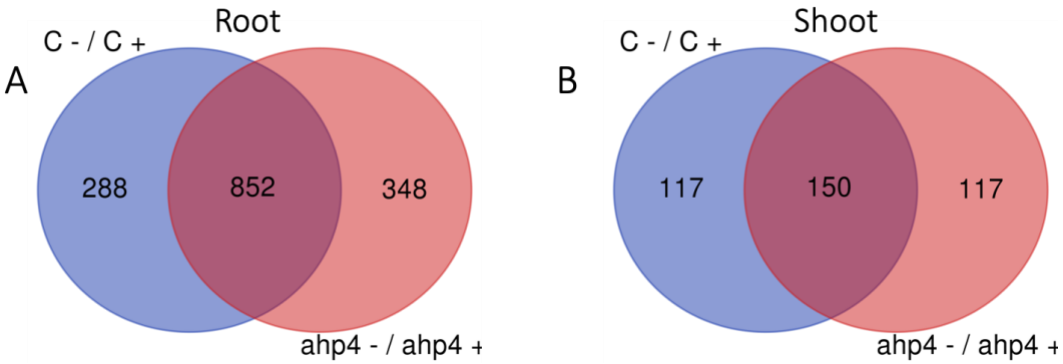


Fig 3.10 Venn diagrams form transcriptomic data showing the DE genes of WT and *ahp4-2* plants treated with BA vs treated with a mock DMSO solution in A) root tissues and B) shoot tissues. C: Col-0, ahp4: ahp4-2, -: treated with mock DMSO solution, +: treatment with BA.

These data allow for a few inferences. Firstly, the 2-hour CK treatment, as expected, induced a variety of genes as expressed in both plant tissues examined, irrespective of the genotype (Fig 3.10). This indicates that the experiment was technically sound. Secondly, there are no observable changes regarding gene expression; evident from the comparison of DE genes between WT and *ahp4-2* (C/*ahp4*) regardless treatment (Fig 3.9). This indicates that AHP4 does not have a significant impact on the whole plant transcription.

Further functional enrichment of GO terms of the unique DE genes resulting from the comparison between CK and mock treated root tissues (C-/C+ and ahp4-/ahp4+; Fig 3.10 A and Fig 3.11), revealed great similarities in DE genes as a result of the treatment and irrespective of the genotype, consistent with the data in Fig 3.9.

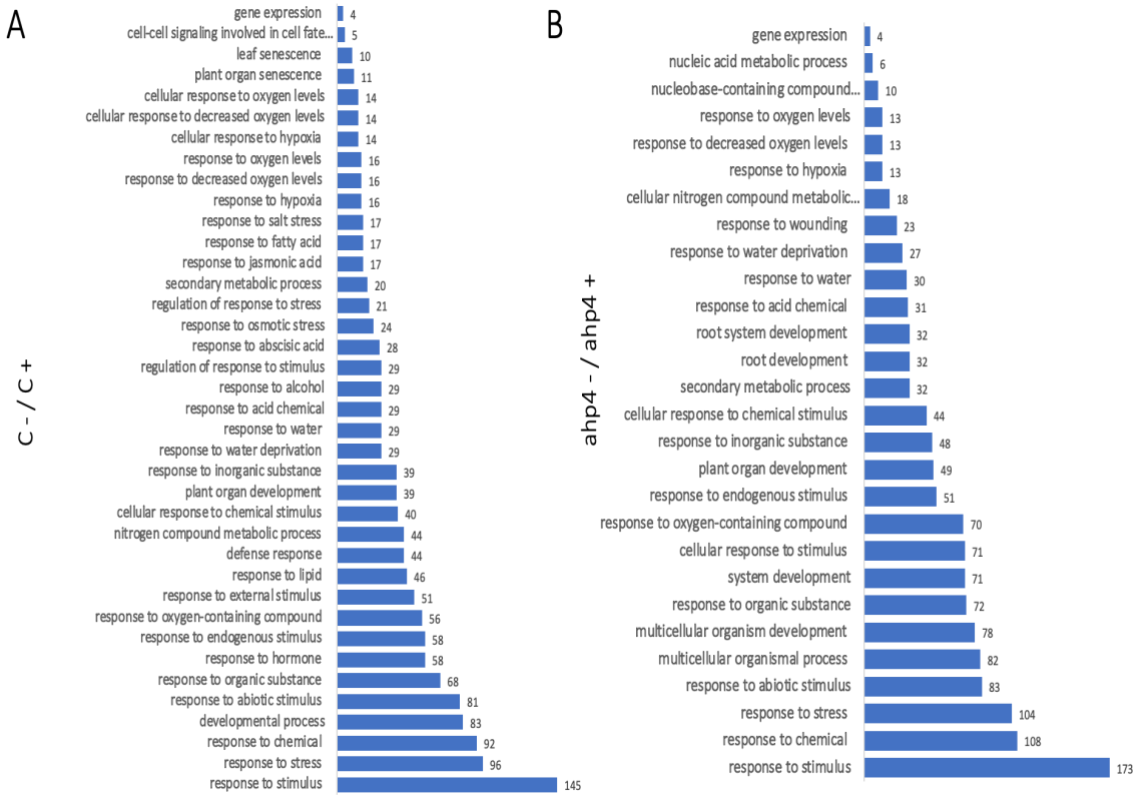


Fig 3.11 Gene Ontology (GO) enrichment analysis of DE genes for of Biological Process (BP). A) GO of DE genes isolated from comparing BA vs mock DMSO treatment in WT roots and B) in *ahp4-2* roots. Numbers next to the bars indicate the number of genes found in the indicted processes. Analysis was done using PANTHER Overrepresentation Test against the *Arabidopsis thaliana* database as a reference list and using Fisher statistical test corrected with False Discovery rate (FDR) to determine significance with p-value < 0.05.

In the CK responses investigated above (3.4), it was found that AHP4 has a subtle effect on development. However, this is not represented by the root or the shoot transcriptome of the 7-day-old plants. It is possible that changes are too small that a more sensitive analysis would be able to show more gene, however changing the cutoff point to a less stringent can result in a lot of noise in the data. Another possibility is that the changes in gene expression occur in a subset of cells and their expression is diluted in the whole organ transcriptome or the genes regulated by AHP4 may be only relevant at a specific developmental stage of the plants that is not included here. The latter is less likely as changes are observed in root length over the period of 5 days (Fig 3.5).

ARABIDOPSIS RESPONSE REGULATORS (ARRs) are the direct targets of HPts, and are transcriptionally upregulated in response to CK. In Arabidopsis there are two types of *ARRs*, the *type-B ARRs* (11 genes) which drive the expression of cytokinin signalling related genes acting as a positive regulator (Mason *et al.*, 2005) and 10 *type-A ARRs* which are transcriptionally activated by type-B ARRs and act as negative regulators signal transaction (To *et al.*, 2004). To test whether AHP4 has any effect on the expression of A-AARs directly, their expression was plotted using the fpkm values from the transcriptomics analysis.

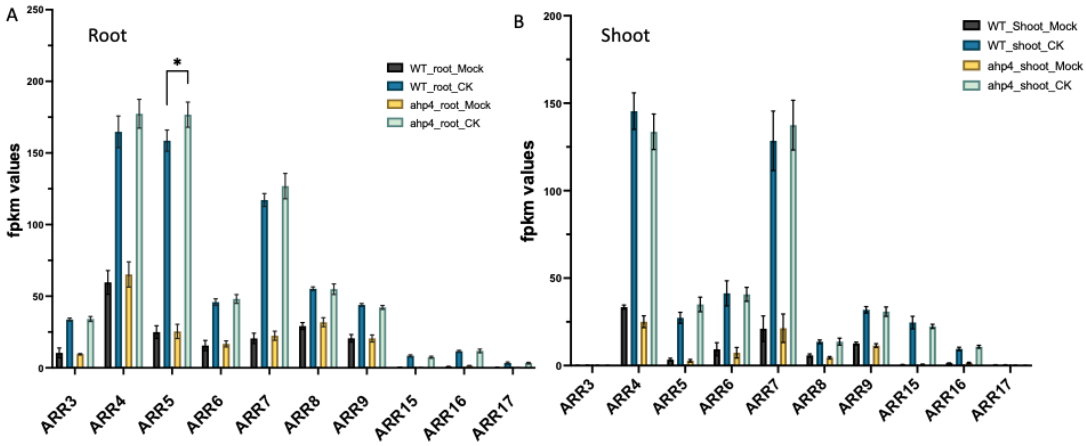


Fig 3.12 Expression of the type-A *ARRs* measured by the number of fragments per kilobase of transcript per million (fpkm) mapped reads. (A) The expression of type-A *ARRs* in the root tissues of WT and *ahp4-2* plants treated with mock or BA solution. (B) The expression of *A-ARRs* in the shoot tissues of the same plants treated with mock or BA solution. Two-way ANOVA and Tukey's multiple comparison tests were used to calculate significance between WT and *ahp4-2* within the same treatment (n=3, *P $\leq$ 0.05).

Consistent with the literature (D'agostino, Deruè and Kieber, 2000), the expression of *A-ARRs* is significantly increased in high CK environments both in the shoot and in the roots based on the fpkm values found in the transcriptomic analysis done in root and shoot tissue of WT and *ahp4-2* plants (Fig 3.12). However, apart from ARR5 in the roots, there was no significant difference in the expression levels of *A-ARRs* between WT and *ahp4-2* plants regardless of the treatment. These results agree with the data described above and indicate that AHP4 involvement in

regulating the expression of *A-ARRs* is not significant at a global level and suggest either a minor role in the signal transaction mediated by CK or a role in a very specific subset of cells.

Overall, the transcriptomic experiments did not indicate any differences in gene expression that could lead to conclusions on the involvement of AHP4 in the CK signalling. This might also be due to the choice of *ahp4-2* as the mutant allele. This allele, has larger part of the protein unaffected due to the editing event occurred from using the CRISPR-Cas method, compared to the *ahp4-4* allele which results in virtually no detectable transcripts. The choice of the allele was only enforced by the timeline of the experiment, as this was the first allele to become available followed by *ahp4-4* later in the project.

3.6 Discussion

This chapter aimed to investigate the role of AHP4 in the CK signalling pathway and determine whether AHP4 acts as a negative or positive regulator. As discussed earlier, AHP4 is one of the very few components in the CK signal transduction pathway, which have not been extensively studied and therefore, its role remained a mystery. To do this, new CRISPR edited null alleles were generated to enable a better comparison with the WT plants in CK relevant responses as opposed to analysis done previously using the pre-existing SALK alleles.

Several phenotypic traits have been analysed as part of this work to include both above and below ground tissues. CK concentration and perception found to be associated with the traits examined previously (Ashikari et al., 2005; Bartrina et al., 2011; Chory et al., 1994; Cortleven et al., 2016; Hutchison et al., 2006; Kuderova et al., 2008; Mller & Leyser, 2011; Riefler et al., 2006), therefore *ahp4* mutant alleles were examined for those traits too. The phenotypic assays suggested that *AHP4* had different function in the roots and the shoots and does not act redundantly with the AHP6 as the known negative regulator of CK signalling (Mähönen *et al.*, 2006). In the roots, *AHP4* exhibits reduced sensitivity to exogenously applied CK,

suggesting that it may act as positive regulator of CK signalling (Fig 3.5). In the shoots however, *AHP4* appears to be working to inhibit CK signalling (Fig 3.6 and Fig 3.7).

Transcriptomic analysis of the rice AHP4 orthologues (*OsPHP1*, *OsPHP2* and *OsPHP3*) revealed induction in the expression of a variety of *A-ARRs* and *CKXs* (Vaughan-Hirsch *et al.*, 2021). These are gene upregulated by CK and its induced expression in the triple mutant plants suggest enhanced CK signalling, therefore suggesting strong association with the negative regulation of CK signalling by *OsPHP1*, *OsPHP2* and *OsPHP3.* On the contrary, the analysis of the transcriptome of WT and *ahp4-2* plants revealed that AHP4 had no impact on the transcriptome of the organs examined (Fig 3.9 and Fig 3.11). This can be due to *AHP4* only affecting a small subset of cells and therefore any changes in gene expression were masked by whole organ sampling.

Furthermore, *AHP4* might be responsible for changes occurring in specific developmental stages or under specific environmental conditions which are not examined here. For example, it may act as either a positive or a negative regulator in a temperature- or pH-dependant manner. This possibility is not unlikely as its predicted structure suggests a small shift in the orientation of the important phospho-accepting His, when aligned to other HPts (Chapter 2). One explanation would be that AHP4 has no function at standard conditions, but specific (possibly stress inducing) conditions could inflict changes on the protein's structure to shift the His residue back to the position where the His is positioned for the other HPts. This could explain the subtle changes seen in all the assays investigating CK responses as these were only be done on standard, not abiotic stress inducing conditions.

Additionally, evidence from the phylogenetic relationship of APH4 with monocot PHPs and the fact that is conserved in over 60 dicot species indicates a conserved function. Whether this function is associated with CK signalling is yet to be determined as the results presented here do not rule out any of the three

possible scenarios (1st being a positive regulator, 2nd being a negative regulator or 3rd not participating in CK signalling).

Therefore, to understand AHP4 function, further investigation of the *AHP4* must concentrate on examining its expression pattern as well as investigating whether it has a role on plant development under specific environmental conditions.

Chapter 4: AHP4 promotes tolerance against osmotic stress.

4.1 Introduction

Plants respond to stresses to ensure their survival. CKs are among the phytohormones involved in responses to both biotic and abiotic stresses, however this thesis focuses on abiotic responses. Several components of the two-component phosphorelay system modulate CK responses to temperature changes, salt stress, drought and generally stresses responsible for changing turgor pressure (Kieber and Schaller, 2014). For example, many type-A *ARRs* are induced when plants are exposed to cold; a process in which AHP2, AHP3 and AHP5 which act redundantly to positively mediate their expression (Jeon *et al.*, 2010; Jeon and Kim, 2013). The redundant action of these *HPts* was demonstrated in response to drought and salt too. Triple *ahp2,3,5* plants have increased tolerance to drought, which is achieved by maintaining the cell membrane integrity and thus preventing electrolyte leakage (Nishiyama *et al.*, 2013). Plants subjected to drought or salt stress also show reduced levels of cytokinin and plants defective in several CK biosynthesis genes (i.e., *IPT* genes; Nishiyama et al., 2011) show enhanced drought tolerance.

While the redundant nature of most of the HPts was determined in respect to their function in CK signalling pathway (Hutchison *et al.*, 2006) and for their role in the responses in abiotic stresses (Nishiyama *et al.*, 2013), the role of AHP4 still remains a mystery. In a recently published work taking place the same time as this project, AHP4 negatively regulated responses to drought. Loss-of-function *ahp4* mutant plants were shown to be more tolerant to drought, had longer primary roots, higher water content in the leaves, improved cell membrane integrity, higher Chl content in the leaves, decreased stomatal aperture and increased accumulation of ROS scavenging enzymes under drought conditions (Ha *et al.*, 2022). While all these are adaptations for drought tolerance, some of those responses were not reproduced in our analyses. For example, *ahp4-4* plants had no change in the Chl content when compared to WT (Fig 3.6) and the transcriptomic analysis of shoots and roots presented earlier did not reveal any differences in gene expression that could better explain adaptation to abiotic stresses (Fig 3.9). Furthermore, if AHP4

acts as a negative regulator of drought, this would suggest that it would act like the canonical form of HPts in CK signalling and our data suggest otherwise. Given its great structural homology with the HPts, AHP4 was predicted to act in a similar way to the canonical group of HPts (Fig 2.2 A). However, phylogenetically it was found to be closely related to CK signalling inhibitors of monocot plants and putative CK signalling inhibitors of other dicot plants (Fig 2.1).

Additionally, the expression of *AHP4* was found to be 2 to 3-fold upregulated in plants exposed to a variety of abiotic stresses based on a meta-analysis of publicly available microarray data. These stresses included osmotic stress, heat stress, drought, wounding, salinity and more (Singh *et al.*, 2015). Therefore, its function might be conditional i.e., it might have a specific function under certain environmental conditions. More specifically, it is possible that AHP4 is inactive under optimal conditions due to its shape. However, the protein can be modified under specific stress conditions (like drought or high temperature) and this modification is possible to result to an active form of protein through the non-covalent posttranslational protein modification mechanism. Click or tap here to enter text.

This chapter further characterises the role of AHP4 in drought responses. To do this it is important to determine its basal expression level and localisation in both the roots and the shoots. Moreover, some of the responses examined by Ha et al. (2022) were reanalysed using both the old *ahp4-SALK* line and the newly developed CRISPR-Cas alleles, *ahp4-2 and ahp4-3*. By doing so, a function of AHP4 in response to water limiting conditions was determined.

4.2 AHP4 is expressed at low levels in both roots and shoots.

The analysis of CK related phenotypes comparing WT and *ahp4* plants (in chapter 3) revealed minor differences in CK response due to *AHP4*. It was not possible to infer the function of *AHP4* from the comparison of transcriptomes (described in chapter 3) as the changes were so small. These results need to be

considered in connection with information regarding the expression of *AHP4*, as if it is expressed in only a small number of cells or under certain environmental conditions or in a different developmental stage, this would mean that it may have highly specific phenotypes. This could explain the modest changes seen in the previous chapter when looking at broad root and shoot phenotypes.

The RNASeq data from the previous chapter was re-analysed to compare the levels of AHP4 mRNA with the other HPts to understand the lack of striking physiological differences described in the previous chapter (Chapter 3, 3.4). Based on that analysis, the expression of HPts in the shoot was relatively lower than their respective expression in the roots (Fig 4.1). Furthermore, the *AHP4* levels in the shoots were relatively low but not lower than the majority of HPts (Fig 4.1 A). On the other hand, *AHP4* in WT roots was expressed very weakly compared to most of the HPts (Fig 4.1 B). Difference in expression level is observed in all HPts both in the roots and shoots suggesting that each protein has an organ specific role (Hradilová and Brzobohatý, 2007) even though mutant analysis shows that most of them act redundantly (Hutchison *et al.*, 2006; Jeon and Kim, 2013; Nishiyama *et al.*, 2013).

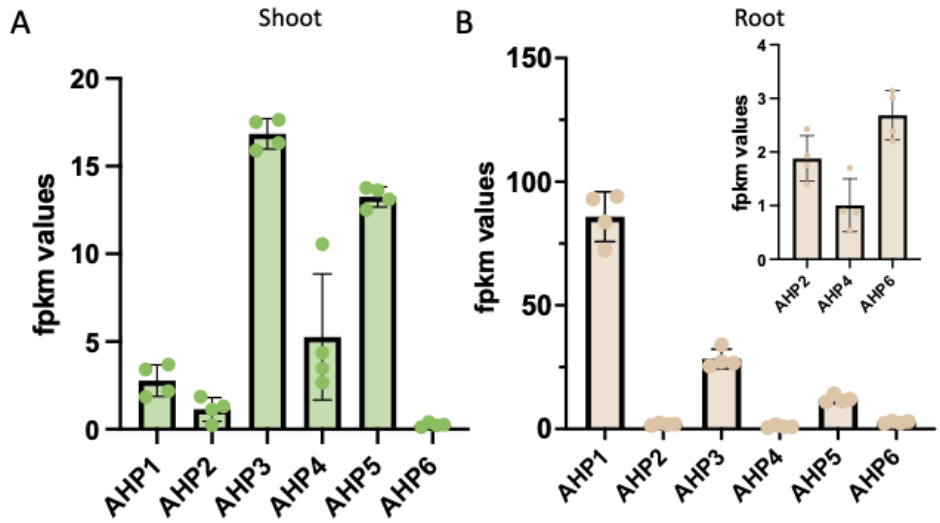


Fig 4.1 *AHP4* is expressed at low levels in roots. Values are extracted from the transcriptomics data and represent the number of fragments per million kb mapped reads (Fpkm) normalized to the length of the gene. A) Expression of HPts in the shoots of WT seedlings and B) Expression of HPts in the roots of WT seedlings. Error bars: Standard deviation, n=4.

Interestingly, the expression levels of *AHP4* are in a similar order of magnitude to those of *AHP6* in root tissues (Fig 4.1 B). In roots, *AHP6* has a specific expression pattern as it is expressed in a very small subset of cells in the protoxylem and adjacent pericycle cells near the root tip (Bishopp, Help, *et al.*, 2011). This spatially restricted expression allows the formation of protoxylem by inhibiting the cytokinin signalling in those cells (Mähönen *et al.*, 2006). In the case of *AHP4* such information is lacking. The low expression levels observed from the transcriptomics data as well as other studies (Hradilová and Brzobohatý, 2007; Hradilová, Malbeck and Brzobohatý, 2007) suggest that *AHP4* is either expressed at exceedingly low levels ubiquitously or, like *AHP6*, it might have a highly specialised role with a restricted expression pattern. In the former case, it may have a function under specific conditions if its expression levels are changed relative to the other HPts. To address these questions, it is critical to determine the expression pattern of *AHP4* and to do this reporter lines were developed.

4.3 AHP4 expression is limited to mature phloem companion cells.

To investigate the expression pattern of *AHP4*, transcriptional reporters were created driving the expression of green fluorescent protein (GFP) with a nuclear localised signal (NLS). The GFP expression was driven by the 2.6 Kb long native *AHP4* promoter containing the 5' UTR sequence with a nuclear localisation signal attached to the GFP. This approach was used because as it was expected that the signal was low, and therefore targeting GFP to the nucleus would concentrate the signal making it easier to visualise.

Promoters are genomic regions that are upstream of the transcription start site which enable the gene expression. These allow the spatial and temporal expression specificity of the gene they activate. This is achieved by the recruitment of cis- and trans- elements on the promoter regulatory elements to activate or supress the transcription of the gene. The sequence upstream of the *AHP4* used in this experiment was chosen to include the core promoter region which is usually a few bp upstream of the transcription start site, the proximal promoter extending to

about 300 bps away from transcription start site and contains sites where transcription factors bind and finally, the distal promoter region which can extent several kbps away from the transcription starting site and contains regulatory elements (Spitz and Furlong, 2012).

The construct was created using Green Gate technology which is an adaptation of the Golden Gate technology using type II restriction endonucleases for plant biotechnology (Lampropoulos *et al.*, 2013). The destination vector used was a modified Green Gate vector pGGZ003 expressing "tdTomato" chromophore protein as a membrane marker (Kümpers *et al.*, 2022). The destination vector was used to transform WT plants using *Agrobacteria* mediated transformation and single insertion, homozygous plants were selected using antibiotic resistance. Using laser confocal microscopy (LCM), the expression of *AHP4* was investigated in shoot and root tissues. The expression of GFP driven by the *AHP4* promoter, was observed in the vasculature of the plants in the cotyledons, roots, and hypocotyls (Fig 4.2 A). In cotyledons, *AHP4::GFP* covered the length of the midvein and the secondary veins around the leaf margins (Fig 4.2 C). The *AHP4* expression pattern in these tissues was limited to a few cells within the vascular bundle of the organ. Similarly, in the roots, the AHP4::GFP signal was present in a few adjacent cells in the vasculature of the transgenic plant (Fig 4.2 B, D). By visualising the position of the two cells alongside a visible LRP (Fig 4.2 B) it is possible to infer that from the focal plane that they are expressing in cells that are situated approximately 90° to the LRP. As the LRP are formed at the xylem pole, this is indicative that these cells are in the phloem pole. Furthermore, the fact that they appear in pairs with one cell between them suggests that these cells are most likely to be phloem companion cells (CC).

Furthermore, the protein is not expressed in the meristematic region of the root which is the area where phloem identity is assigned to undifferentiated cells, therefore AHP4 is unlikely to affect phloem patterning. However, as it is only expressed above the meristematic regions, where phloem is already differentiated, then it is more likely AHP4's function to be associated with phloem function.

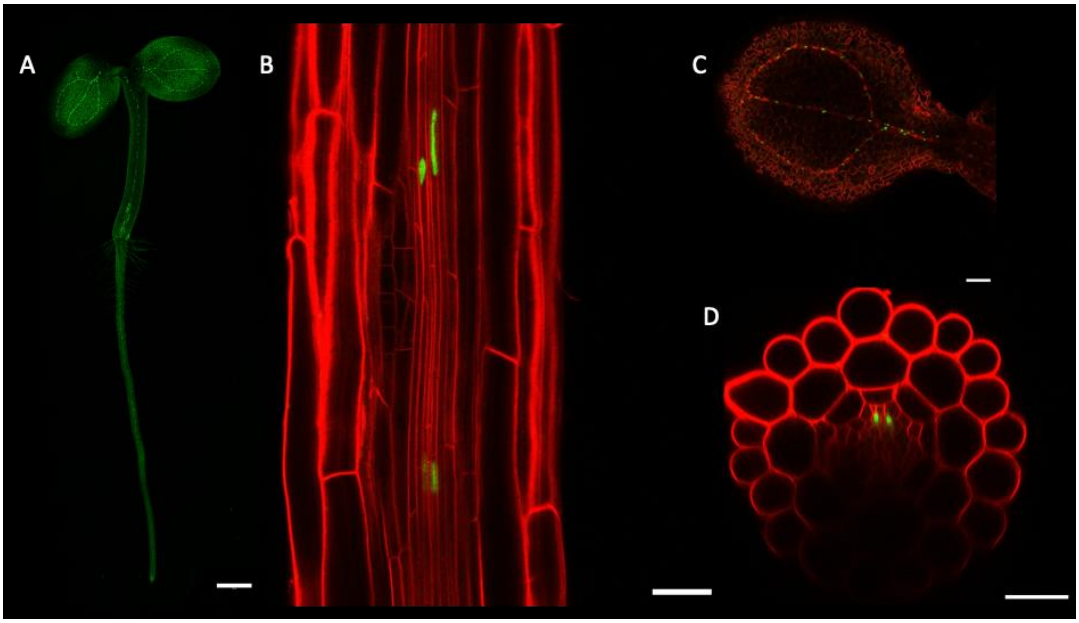


Fig 4.2: pAHP4::GFP-NLS expression is restricted in phloem companion cells in the vasculature. A) Maximum projection of a z-stack of a 3-day old seedling showing the expression of the transcriptional *AHP4* reporter. Imaged with x10 objective; Scale bar=500 µm. B) Longitudinal section of the root of 5-day old plants showing the expression of AHP4 in two adjacent cells the vasculature. Objective: x63; Scale bar:25 µm. C) Section of a cotyledon from a 3-day old plant expressing pAHP4::GFP in the vasculature. Objective: x10, Scale bar:100 µm. D) Cross section of a root of 5-day old plants showing the expression of AHP4::GFP in two adjacent cells in the vasculature. Objective x63, Scale bar: 5 µm. These images were obtained using the Confocal Laser Microscope Leica SP8. Green channel: GFP, red channel: red membrane marker-DEAL.

To verify the exact cell type in the vasculature in which *AHP4* is expressed the single cell atlas online software was used. This was developed from VIB institute and Gent University from single cell sequencing (Wendrich *et al.*, 2020). Single cell sequencing (scRNAseq) allows for a better resolution on the gene expression in each cell type. Furthermore, it allows detection of native mRNA levels and removes the certain biases present in reporter genes. For example, the length of the chosen native promoter or whether it includes certain regulatory element from the intrinsic areas of the gene can affect the reporter gene expression pattern and therefore present a bias. The individual cells are separated with various techniques in a reaction mixture and the transcripts of each cell are marked with unique barcodes which are converted into cDNA and sequenced using a deep sequencing

platform. Results are then analyzed to create cell type annotations and identification of cell clusters (Jovic *et al.*, 2001). The single cell atlas database contains clusters for all cell types found in root (Fig 4.3 C) and trajectory analysis to show the developmental transitions within each cluster (Wendrich *et al.*, 2020). For example, phloem initials develop into phloem procambium, sieve elements and phloem companion cells (Fig 4.3 B). Based on a search for *AHP4* expression in the single cell atlas database, the mRNA was expressed in very small subset of cells, predominantly in the phloem companion cells but additionally in a small number of the sieve elements (Fig 4.3 A).

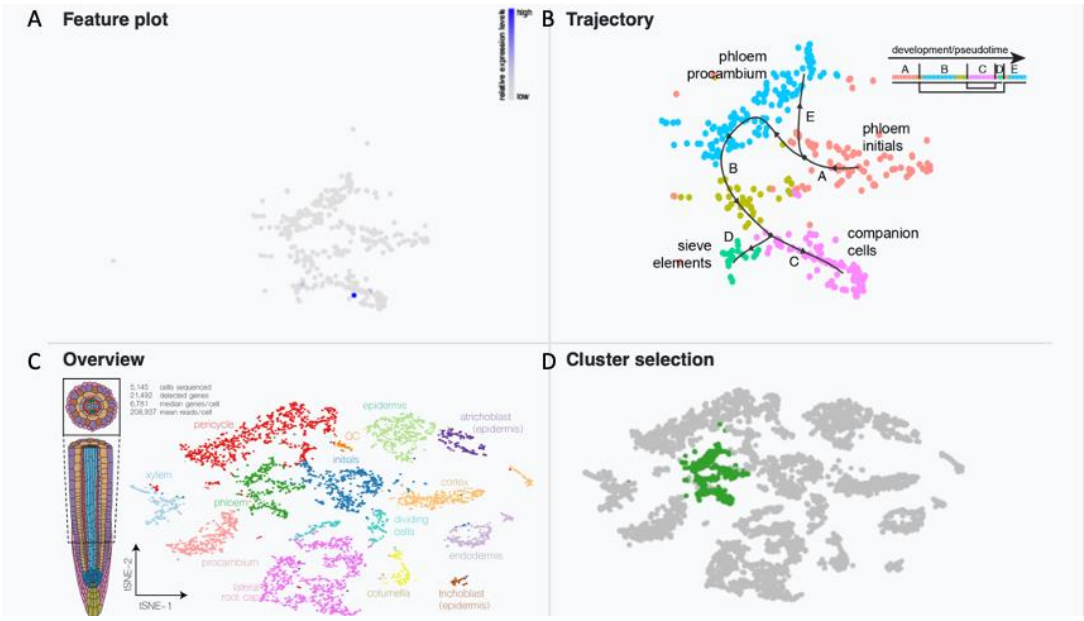


Fig 4.3: AHP4 was found in phloem companion cells in single cell sequencing analysis. A) The expression of AHP4 is restricted in a subset of cells. B) The trajectory of developmental sequence from phloem initials to procambium to sieve elements and companion cells. C) The cell clusters identification resulting from the separation analysis. D) The cluster of cells in which AHP4 was found to be expressed- phloem cells. This graph was taken from the Plant sc-Atlas of Gent University (https://bioit3.irc.ugent.be/plant-sc-atlas/) (Wendrich et al., 2020).

The images obtained from the transcriptional *AHP4* reporter lines as well as the single cell atlas database, confirmed the location of *AHP4* to be in the phloem companion cells. This observation is consistent to what the transcriptomic data

(Chapter 3, 3.5) suggested; that AHP4 is expressed in a subset of cells and the effects were masked when total organ transcriptome was investigated.

4.4 AHP4 has no role in drought stress responses in the whole plant level.

As described in section 4.1, CK levels were implicated in the tolerance of plants under drought conditions. In summary, CK negatively regulates drought responses. This has been observed by the responses of several CK biosynthesis and signalling mutants to drought (Nishiyama *et al.*, 2011, 2013; Huang *et al.*, 2018; Nguyen *et al.*, 2018; Ha *et al.*, 2022).

Furthermore, the observations regarding the localisation of *AHP4* described above (section 4.3, indicated that *AHP4* expression is restricted in the vasculature (Fig 4.2) and coupled with closer analysis using previously published scRNAseq data determined that AHP4 was strictly localised in the phloem (Fig 4.3).

Unlike xylem, which is responsible for water transport, phloem is the main tissue translocating photosynthetic products and other organic substances in plants through an extensive tubular network. Phloem consists of two main cell types which ensure the sap translocation efficiency: the sieve elements (SE) and companion cells (CCs). In mature phloem, SE loose most of their cytoplasmic components and the plasmodesmata connections become wider forming a network of cells assembled longitudinally and resembling the structure of a sieve (Oparka and Turgeon, 1999; Schulz and Thompson, 2009; Otero and Helariutta, 2016). Because the SE lack many cytoplasmic components, their vitality is largely depended on the CCs which have a very dense cytoplasm rich in mitochondria and ribosomes. Both cell types are connected with plasmodesmata, from which large molecules can travel through enabling the CCs to support the attached SE (Schulz and Thompson, 2009; Otero and Helariutta, 2016). Phloem translocation begins in tissues with high photosynthetic activity, termed source tissues, i.e., the leaves. Assimilates from cells at the source are actively loaded in the adjacent SE of the phloem resulting in osmotic difference between the plasma membranes of the cells

(VAN BEL, 2003; Gould *et al.*, 2005; Schulz and Thompson, 2009). The environment within the SE cells has high viscosity due to the amount of solutes it contains and it is referred to as the sap. The osmotic difference created in SE near the source stimulates the influx of water from the xylem resulting in the increase of turgor pressure locally. As the phloem is a long continuous transport system, the solutes are directed towards the sink tissues; areas where turgor pressure is lower. The solutes are then unloaded from the phloem sap in the sink tissues with the help of transporters (De Schepper *et al.*, 2013).

While the role of xylem in water limiting conditions has been studied extensively, many of the phloem functions under water stress are limited to mathematical models due to the complexity of measuring phloem transport. When water availability is limited, water in the xylem tube reduces causing stomata to shut to avoid water loss (Brodribb, McAdam and Carins Murphy, 2017; Martin-StPaul, Delzon and Cochard, 2017; Venturas, Sperry and Hacke, 2017). Stomatal closure leads to decline in photosynthesis which in prolonged periods could lead the plant to carbon starvation and to ultimately die (McDowell and Sevanto, 2010). It has been suggested that carbon starvation in drought conditions might not exclusively depend on the reduction of available photosynthetic materials being produced, but it could be exacerbated by the failure of phloem transport. Phloem transport can be affected by either reduction in loading and unloading as a result of the reduced assimilate availability or by the reduction in phloem conductance resulting from the increased viscosity of the phloem sap (Hölttä, Mencuccini and Nikinmaa, 2009; McDowell and Sevanto, 2010). Because phloem and xylem are hydraulically connected, water can travel in either direction through the semipermeable walls separating the two structures. In drought conditions there is less water passing up the xylem tube and consequently this water tension change is sensed by the phloem. To prevent water flowing towards the xylem, the environment in the phloem must become denser than normal and therefore solutes are actively transported in the phloem in higher rates. This results in reduction of osmotic potential and ensures waterflow to the phloem is maintained along with the turgor pressure (Hölttä, Mencuccini and Nikinmaa, 2009; Turgeon,

2010; Sevanto, 2018). The higher the water stress, the more solutes transferred in the phloem, this increases the viscosity of the phloem sap limiting transport by the sap resistance instead of the water availability (Hölttä, Mencuccini and Nikinmaa, 2009; Stanfield and Bartlett, 2022).

It is known that *HPts* have negative and redundant role in the responses against drought (Nishiyama et al., 2013).This, in combination with the restricted expression of *AHP4* in the phloem, and the fact that phloem transport is affected by water limiting conditions, has leads to the question on AHP4 involvement in drought responses. To investigate this connection, two approaches were employed. Firstly, the effect of drought was observed in mature plants grown on soil, which will be discussed in this section. This was done to investigate effect of drought to the plant responses at the whole-plant level. The second approach was based on plants grown on plates to look at root anatomical changes. This second approach will be discussed in the following sections of this chapter.

To do this, *ahp4-2* and WT plants were subjected to moderate drought conditions using the gravimetric methodology which is widely applied for such type of experiments (Nishiyama *et al.*, 2011; Ouwerkerk and Meijer, 2011; Ha *et al.*, 2022). Furthermore, this experimental design was based on the work done on *AHP4* by another group which published their findings during the time of this study (Ha *et al.*, 2022). They investigated the effects of *AHP4* in drought responses and were able to observe significant reduction in biomass in *ahp4-SALK* plants under drought conditions (Ha *et al.*, 2022).

Based on those observations it was decided to verify their results with the CRISPR alleles generated as part of this work. Briefly, plants were germinated on agar plates supplemented with ½ MS for 10 days under a short-day regime and the transferred in pre-weighed pots with the soil watered at field capacity (100%). Field capacity is defined by the maximum amount of water the soil is able to absorb while maintaining the air spaces between the soil particles. This was achieved by allowing the waterlogged pots containing a set amount soil to drain for 2 days

before planting seedlings. *ahp4-2* and WT plants were placed in two trays corresponding the two treatments with the pots placed at random locations within the trays (Fig 4.4 C). The fist condition was “well-watered”; water was constantly added to well-watered plants so that the soil was kept at field capacity throughout the experiment. The “dry” condition involved plants subjected to water limitations. These plants were not watered, and the soil was left to get down to 30% field capacity. Once it reached this level, this was maintained for 7 days before rewatering them to their 70% field capacity for 5 days. Either of the given field capacities of soil was maintained by daily weighing of pots, so that only the correct amount of water was provided. The fresh weight of harvested well-watered and water stressed shoots was then recorded. This material was left to dry for 5 days in 70°C before the dry weight was measured. This last step was done to allow water absorption in the organs of the plants which was important to ensure that the differences observed in biomass were due to the effects caused by the lack of water and not due to the water content in the plant system itself. Furthermore, plants were grown under a short-day light regime to prevent them from flowering. This experimental set up avoided changes in the physiology and gene expression of the plant caused by the transition to flowering.

Based on the average biomass change calculated, there was no observable difference between the WT and *ahp4-2* plants (Fig 4.4 A) indicating that, under moderate drought conditions, *AHP4* does not affect shoot development. Moreover, the number of leaves and the leaf area of WT and *ahp4-2* plants were severely affected due to drought, but no change was recorded between the two genotypes (4.4 B and D). The change in biomass and the leaf measurements together indicate that *AHP4* does not have a role in these responses under drought conditions, or that any effect is not observable in the shoots.

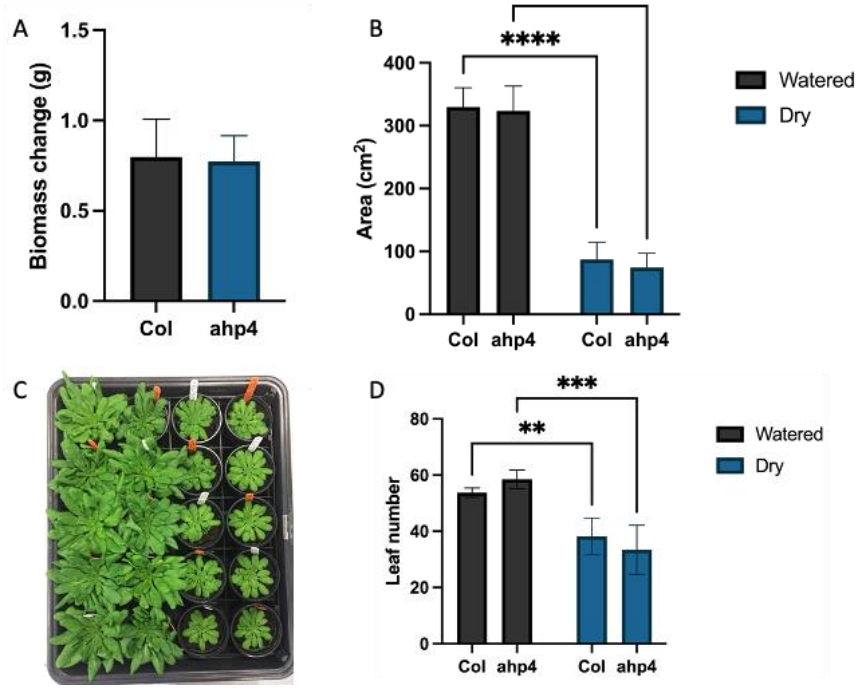


Fig 4.4 Drought treatment did not affect the *ahp4* plants differently than the WT plants. Plants were grown in short day conditions for 47 days before harvesting. They were well-watered for 15 days and then subjected to moderate drought for 7 days. Before harvesting, the plants were watered to maximum capacity for 3 days. A) Reduction in biomass in WT (Col-0) and *ahp4-2* plants as calculated by the following formula: (dry weight of well-watered –dry weight of droughted) / dry weight of well-watered. B) The average area of all leaves harvested from WT and *ahp4-2* plants grown under well-watered or moderate drought conditions. Leaf area was measured using ImageJ using the 'analyze particles' function. C) A representation how the plants looked like at the end of the experiment. Well-watered plants on the right and plants subjected to drought on the left. WT and *ahp4-2* plants were randomized in the same trays during the growing period, however plants on different water regimes were growing on separate trays. D) The average number of leaves found in WT and *ahp4-2* plants under the two water regimes. Two-way ANOVA and Tukey's multiple comparison tests were used to calculate significance (*P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001; n=20 for biomass analysis and number of leaves, n=4 for leaf area).

Even though there were no changes in general physiological effects observed between *ahp4-2* and wild-type (Fig 4.4), it was important to investigate whether moderate drought had any subtle changes in the physiology of the shoot tissues. Due to the nature of the *AHP4* expression pattern (Fig 4.1), it was hypothesised that in water limiting conditions the changes in phloem transport

might affect physiological changes at an elemental level which produce no visible effects under the conditions analysed but might enhance or repress aspects of the plant survival and nutrition. Such analyses were used in the past to uncover mutants with subtle changes in the root anatomy but no obvious phenotype in the shoots which had different ionome profile. For example, MYB36 transcription factor is responsible for the expression of an array of genes necessary for the formation of the Casparian strip in the root endodermis in a spatially specific manner. Naturally, its expression is very high in root tissues but very low in shoots without any obvious phenotype in these tissues. However, changes in the leaf trace minerals were able to be detected in *myb36* plants (Kamiya *et al.*, 2015) and consequently link the Casparian strip formation with a specific ionome composition. Therefore, the ionome can act as a proxy for below ground changes in anatomy. To test whether there was a specific ionome signature that could be associated with a specific process, leaf material taken from plants grown under both water regimes and were analysed for their mineral composition.

The sampling was done in young leaves of plants grown on moderate stress as described above and their ionome composition was analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). For this analysis the concentration of 16 elements was measured and compared between plants exposed to well-watered and moderate drought conditions (Fig 4.5). Water limitation had an impact to the concentrations of most elements analysed and only a few were found at different levels between WT and *ahp4-2* plants. Specifically, at well-watered conditions, the accumulation of copper (Cu), calcium (Ca) and strontium (Sr) was higher in WT plants than in *ahp4-2,* but no change was observed on the levels of those elements in drought conditions between the two genotypes (Fig 4.5), suggesting that when water conditions are optimal *AHP4* affects the solubility or mobilisation of those elements.

Minerals are transported through the xylem stream from the root tissues to the shoots and specifically to tissues with high transpiration activity. This flow is unidirectional and high transpiration areas are not always the areas where mineral

nutrition is needed. Therefore, some minerals are taken-up by the phloem sap to facilitate the distribution (Marschner, 2011). Even though many elements can be found in the phloem sap, not all of them are able to travel long distances due to the different mobility each mineral has within the phloem sap. Specifically, calcium (Ca) and strontium (Sr) which were found to accumulate to a lesser extent in the *ahp4-2* leaves under control conditions have very low phloem mobility (Kuppelwieser and Feller, 1991). However, copper (Cu) can be redistributed to the roots via the phloem with the help of metallothionein proteins (MTs), but its main transportation route is through the xylem (Guo, Bundithya and Goldsbrough, 2003). Even though AHP4 expression is restricted to the phloem CC, the changes observed in these trace elements in young leaf tissues are dependent on xylem transport.

These results together suggest that moderate drought induces many physiological and developmental effects such as differences in leaf development, biomass and mineral composition. However, the data presented here do not indicate a clear involvement for *AHP4* in the shoot, since the effects observed in *ahp4-2* are very subtle and only observable in the ionomics analysis for some trace elements. The above observations are in contrast with what was observed in a previously published work investigating *AHP4* responses to drought (Ha *et al.*, 2022). The authors showed that *ahp4* (*ahp4-SALK*) plants had better survival rates when grown under the same drought conditions investigated as part of the work presented here and lower biomass reduction than WT. Overall, these authors suggested that AHP4 has an important negative role in drought tolerance, since the *ahp4* plants performed better under those conditions (Ha *et al.*, 2022).

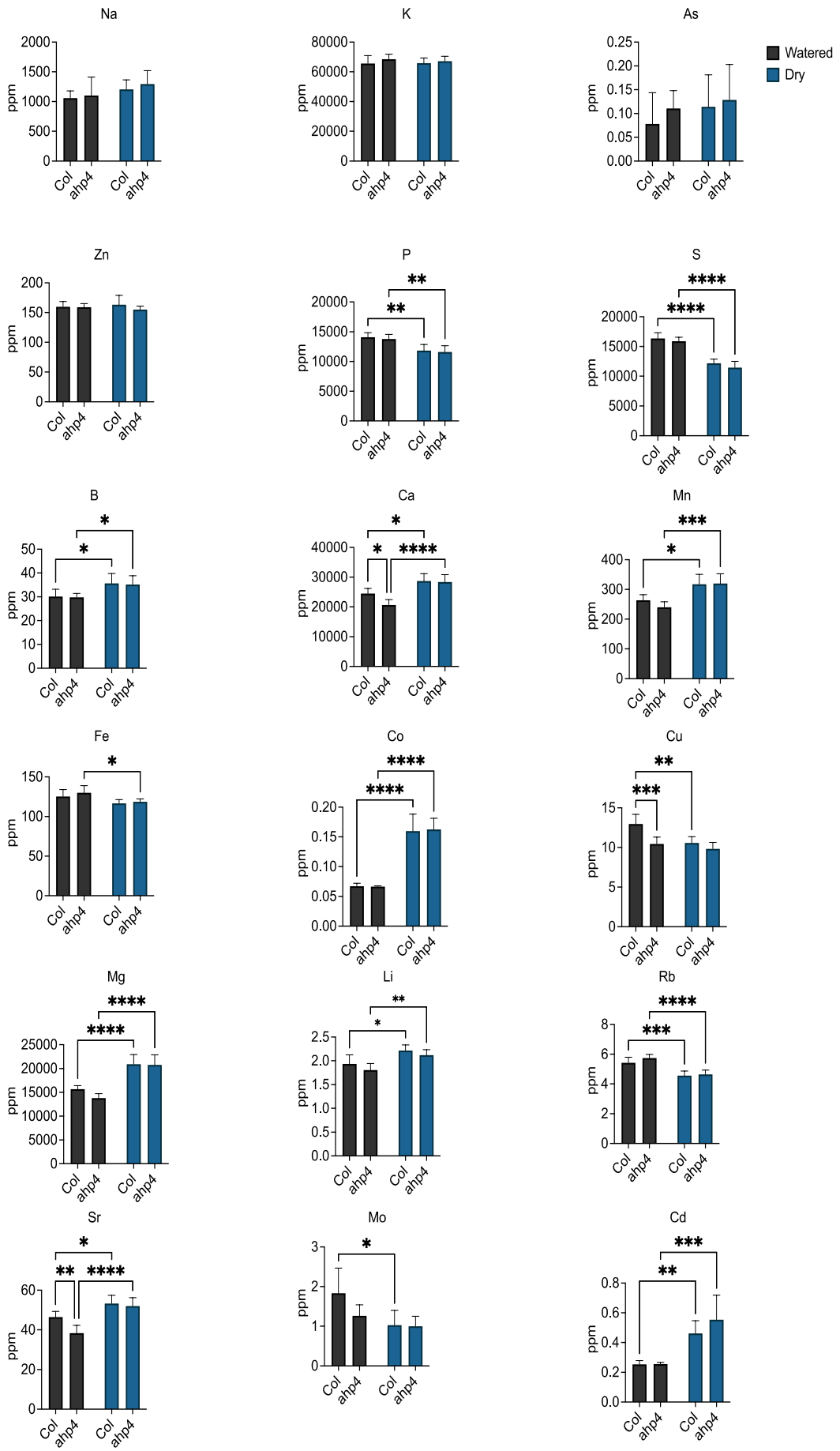

Na
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As
Watered
Dry
Zn
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S
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Ca
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Fe
Co
Cu
Mg
Li
Rb
Sr
Mo
Cd
ppm
Col
ahp4

Fig 4.5 Water availability impacts the ionome composition, but only minor differences are observed between WT and *ahp4-2*. Graphs represent mineral nutrients and trace element composition found in WT and ahp4-2 plants grown in either well-watered or moderate drought conditions. Tissue from young leaves, approximately 150mg of fresh weight, from a random selection of plants were used for the ICP-MS analysis. Minerals analyzed with order of presentation: Sodium (Na), Potassium (K), Arsenic (As), Zinc (Zn), Phosphorus (P), Sulfur (S), Boron (B), Calcium (Ca), Manganese (Mn), Iron (Fe), Cobalt (Co), Copper (Cu), Magnesium (Mg), Lithium (Li), Rubidium (Rb), Strontium (Sr), Molybdenum (Mo), Cadmium (Cd). Significance was calculated using two-way ANOVA and Tukey's multiple comparison tests (n=6, *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001).

4.5 Osmotic stress is inducing AHP4 expression.

Several abiotic stresses induce *AHP4* expression as discussed in 4.1, however, very little physiological changes observed in the leaves from plants subjected to moderate drought as a result of *AHP4*. This might suggest that in shoots AHP4 may not play a role in modulating these responses, leaving the question open as to whether it has an impact on roots development. Since drought treatments are mainly done with mature plants grown in soil, the root systems are hard to analyse because of their fragile nature which make cleaning the soil around them very problematic leading to root damage. To avoid this, plants were grown on plates and an osmotic stress applied.

Root physiological and anatomical assays are normally done on agar plates supplemented with ½ MS as it is easier to observe any physiological changes without damaging the root. To examine the role of *AHP4* in water limiting conditions, plants were germinated and grown for 5 days on the agar media with ½ MS as mentioned above and then transferred to plates with media containing mannitol for 5 more days. Mannitol was used as it is a membrane impermeable sugar and when inside the growing medium, plants are exposed to osmotic stress. Osmotic stress limits the water available for the plants and therefore it is a good approximation of drought conditions for tissue culture media. The transcriptional reporter lines were first used to examine the expression of *AHP4* in medium (150

mM) and high (300 mM) osmotic stress conditions using mannitol as the osmotic agent. *AHP4::GFP* expression in the roots was observed using confocal laser microscopy. It was found that *AHP4::GFP* was upregulated with increasing mannitol concentrations (Fig 4.6 A). The images were obtained in z-stacks to enable better coverage of the root in the z-axis and these were quantified using the selection tool of a specified area of the root and the 'sum of slides' function in ImageJ. The specified area was selected to concentrate the mean grey values in the vascular area of the root and avoid the small amount of signal derived from the red membrane marker. The quantification of the GFP signal indicated that AHP4 was significantly increased with the increasing mannitol concentrations (Fig 4.6 B), consistent with the literature (Singh *et al.*, 2015). The greater effect was observed in the high osmotic stress (300 mM) while there was a marginal difference concerning the 150 mM mannitol concentration. To quantify this further, the levels of *AHP4* were determined of WT plants growing in the same conditions described above using qRT-PCR. The level of transcripts in this case were increased by nearly 10-fold in 300 mM mannitol as compared to the control conditions and no significant change was observed in *AHP4* expression in plants grown on 150 mM mannitol (Fig 4.6 C). Moreover, in the 300mM mannitol treatment, the change in the expression level was not just due to the increased AHP4::GFP signal but also because of the expansion of the number of nuclei expressing the reporter gene in comparison to the control conditions (Fig 4.6 A).

While it was not possible to observe any effects on development as a result of *AHP4* in plants grown under moderate drought stress at the whole plant level, *AHP4* expression was significantly increased in roots grown in environments inducing osmotic stress. The latter, indicates that *AHP4* might have a role under water limiting conditions in the roots and that any changes caused by its action in the roots are not observable in the shoot tissues when the whole plant is assayed because of a compensation mechanism that might be in place. Or it might be that *AHP4* only affects plant development under extreme water limiting conditions. This interpretation is further supported as the 150 mM mannitol had such a small effect on *AHP4* expression.

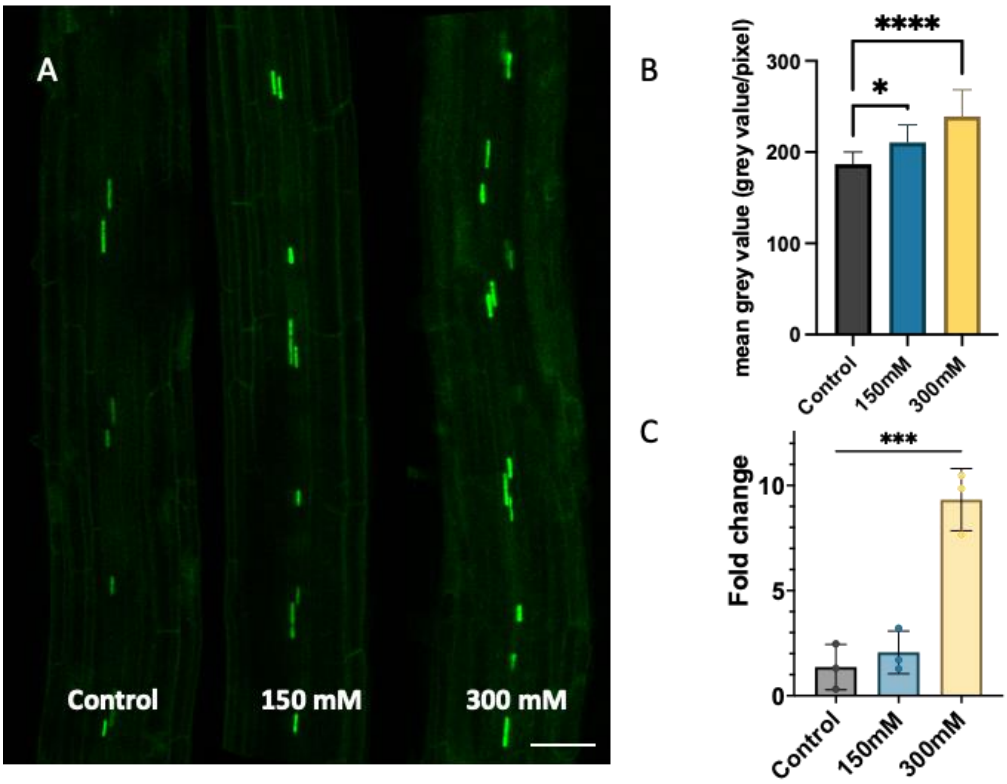


Fig 4.6 Expression of *AHP4* was induced under osmotic stress conditions. A) Confocal images of 10-day old plants expressing GFP driven by the *AHP4* promoter grown under the indicated amount of mannitol to apply osmotic stress. The images represent the sum of a z-stack from each root taken using identical settings for all roots and treatments. B) The mean grey value of the fluorescence observed in the roots expressing *pAHP4::GFP* was calculated from root z-stacks taken with identical settings. C) *AHP4* transcript levels of 14-day old seedlings as determined by qRT-PCR in plants grown on the indicated concentrations of mannitol. Significance for both tests was calculated by one-way ANOVA and Brown-Forsythe statistical tests. Multiple comparisons were corrected using the Tukey's test to test the significant differences as compared to WT control (n= 10 for B and n=3 for C; *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001).

4.6 AHP4 is responsible for maintaining primary root growth during water limiting conditions through maintaining phloem transport.

Root developmental changes under various stress are difficult to observe in experiments done in soil and therefore any changes that might have occurred to the root architecture during the moderate drought treatments described in section

4.3 were not monitored. Because *Arabidopsis* roots are very thin and fragile, agar plates are used for physiological measurements to allow better access to any root traits. To mimic drought conditions on agar plates just like described in section 4.4, mannitol was used as a membrane impermeable sugar to increase the osmotic pressure around the root growing environment. Plants were first germinated on control medium containing agar supplemented with ½ strength MS for 7 days before being transferred to the treatment medium containing agar supplemented with ½ MS and the indicated concentration of mannitol. For most assays the SALK line, which is the same line used by previous studies, was included in the comparison to obtain a better understanding of the AHP4 role in water limiting conditions as this has been used for a variety of studies to study root and shoot traits in the past (Wook Jung *et al.*, 2007; Deng, Dong, Mu, Ren, Zheng, Ji, W. C. Yang, *et al.*, 2010; Liu *et al.*, 2017; Ha *et al.*, 2022).

The *ahp4* mutant plants grown on control media had on average a slightly longer primary root compared with the WT (Fig 4.7 B), consistent with what was described in Fig 3.5. Osmotic stress significantly decreased the root length across all genotypes tested but the *ahp4* plants continued to have longer roots when treated with low mannitol concentration (150 mM; Fig 4.7 B). However, in 300mM mannitol treatments, primary root length was severely affected in all *ahp4* plants as they were significantly shorter than WT (Fig 4.7 B) indicating that *ahp4* mutants were extremely sensitive to high mannitol concentrations. Looking closer at the growth pattern of those plants growing on 300 mM mannitol treatment, it was observed that primary root growth was inhibited in *ahp4-2*, *ahp4-4* and *ahp4-SALK* plants immediately after their transfer to the treatment medium, while roots continued to grow after transfer in WT plants (Fig 4.7 A). In fact, when scoring the root growth after the transfer, it was found that 90% of the seedlings had their root growth inhibited in *ahp4-2* and *ahp4-SALK* plants, 70% in *ahp4-4* roots as opposed to the WT in which only 30% of seedlings showed root inhibition was observed (Fig 4.7 C). As *AHP4* expression is increased in roots exposed to 300 mM mannitol (Fig 4.6) and the root growth on mannitol is inhibited in *ahp4* mutants more strongly that in WT, then AHP4 must play a role in maintaining growth of the primary root in conditions

where water availability is decreased. These results were In contrast with what was reported previously about AHP4 in mannitol (Ha *et al.*, 2022). The authors of this work, under the same conditions and experimental setting, found that primary root grew longer in *ahp4-SALK* plants growing in mannitol. However, the results presented here are based on multiple independent lines whereas the study above only looked at the SALK mutant.

The plasticity of the plant tissues, especially the root enables the plant to survive and overcome certain constraints. Roots can sense the soil moisture and grow towards it through a process called hydrotropism. Water limiting conditions inhibits root growth and drought tolerant plants are able to survive because of their longer roots which can access the deeper layers of the soil. Osmotic stress induced by the addition of mannitol was found to significantly reduce the lateral root (LR) density (Xiong *et al.*, 2006). Consistent with this, in the experiment described above, LR numbers were reduced as the concentration of mannitol was increased across all genotypes (Fig 4.78 A). However, the reduction was more severe in *ahp4-2* and *ahp4-4* plants because initially these plants had more lateral roots than WT. Interestingly, when the length of the existing LRs was measured it was found that *ahp4-4* plants had longer LRs than the WT at high osmotic conditions (Fig 4.8 B) even though the number of LRs remained the same (Fig 4.8 A).

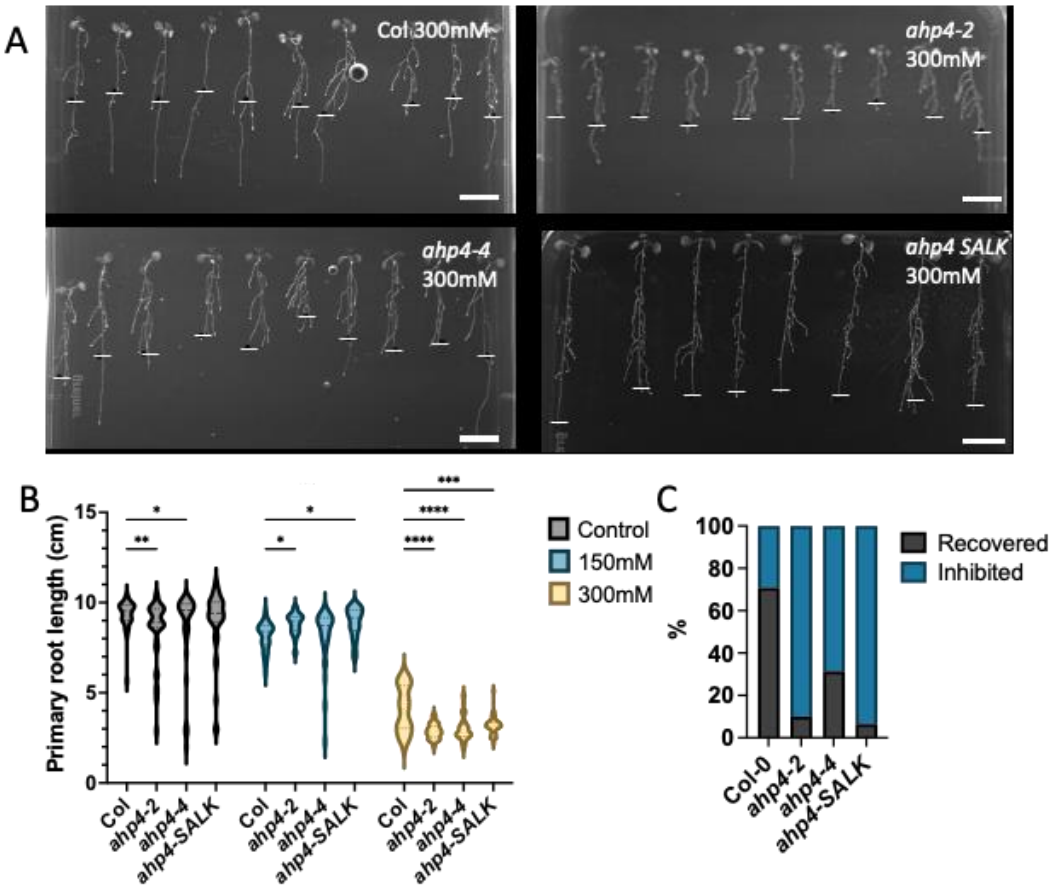


Fig 4.7 AHP4 sustains primary root growth in high osmotic stress environments. A) WT, *ahp4-2*, *ahp4-4* and *ahp4-SALK* plants germinated and grown for 7 days in agar supplemented with ½ MS media as the control medium and transferred to agar media containing ½ MS and 300 mM mannitol for additional 7 days. The white lines on the images indicate the length of the primary root at the point of transfer. Scale bars: 1cm. B) The primary root length on plants grown on control medium for 7 days and transferred to control, 150 mM or 300 mM mannitol containing media for 7 additional days. The data documented here are representatives of at least 3 independent experiments. C) Percentage of the plants grown on media with 300 mM mannitol which had their roots scored based on whether the primary root continued growing after the transfer to the treatment medium (recovered), or whether the root has stopped growing completely (stunted). Significance was calculated using two-way ANOVA and Dunnett's test to test multiple comparisons (n= 50; *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤0.0001).

These data together with the primary root length data in fig 4.7 indicate that AHP4 is essential in maintaining the integrity of primary roots in conditions where the water availability is decreased. Due to the inhibition of primary roots in the *ahp4* mutants, the LRs grow longer most likely as a compensation mechanism.

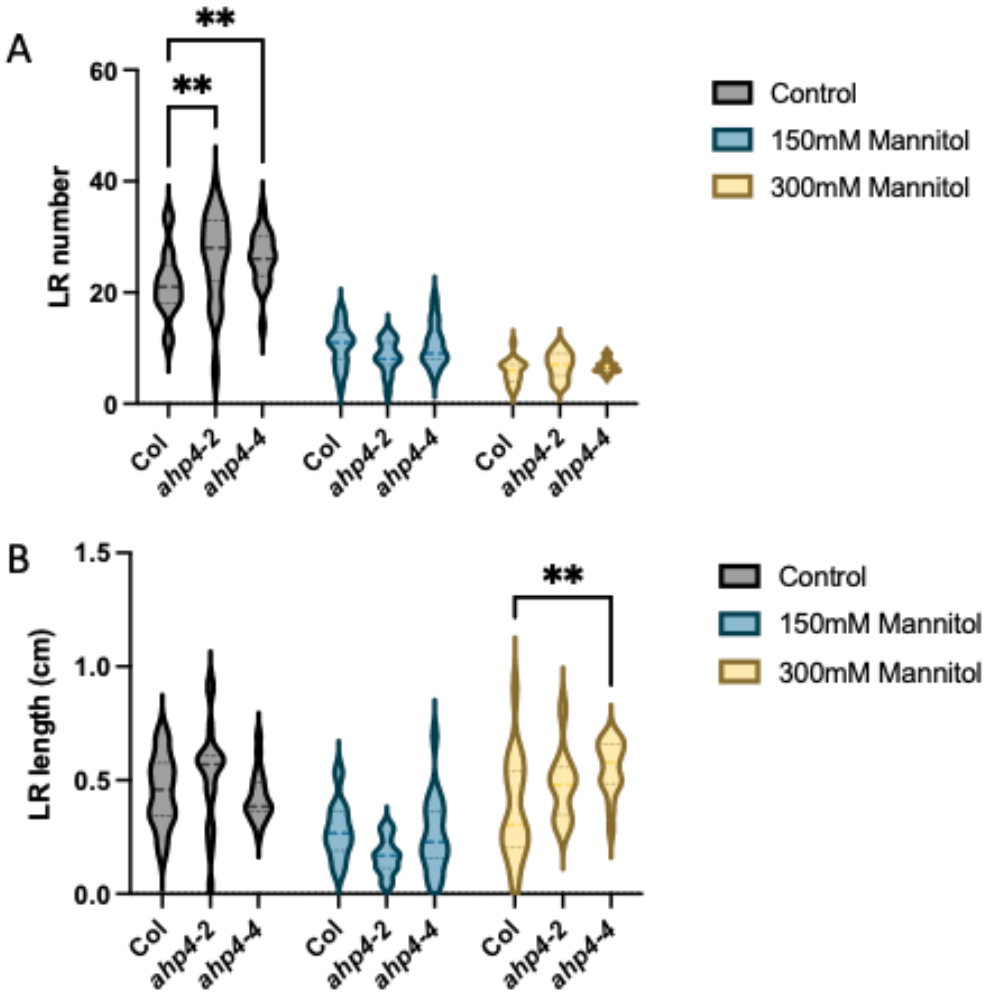


Fig 4.8 AHP4 affects lateral root length in plants grown under high osmotic stress conditions. A) The mean number of lateral roots found on *ahp4-2* and *ahp4-4* plants grown for 7 days on control media and transferred to the indicated media for 7 additional days. B) The mean lateral root length of the lateral roots found on plants growing in the above conditions. Significance was calculated using two-way ANOVA and Tukey's test to test multiple comparisons (n= 20; *P≤ 0.05, **P≤ 0.01).

The expression of *AHP4* is restricted in the phloem and it was found to be upregulated in various abiotic stress conditions (Singh *et al.*, 2015) but most specifically it was found to be induced in water limiting conditions in the roots (Fig 4.6 A). This, coupled with the fact that root growth is inhibited almost immediately after the plants were transferred onto plates containing mannitol, suggests that AHP4 may affect phloem transport under high osmotic stress. If this phloem transport is limited, then one could presume that fewer photosynthetic accumulates would reach the root. If so, then the phenotype observed on mannitol would be partially due to loss of sugars in the root. To test this hypothesis, plants were exposed to osmotic stress in an identical way as above (7 days on control medium and 7 days on the treatment medium) but along with the osmoticum, sucrose was added. Normally, sucrose is transported via the phloem from the sources, such as the leaves, to the sink tissues such as shoot and root apical meristems. If phloem transport is affected in *ahp4* plants, and this is the main reason of the primary root inhibition observed in fig 4.7 A, then supplementation of the sucrose in the treatment medium would be expected to rescue the reduced root growth phenotype.

In fact, primary root inhibition in *ahp4-2* seedlings was rescued in 100% of seedlings by the addition of sucrose in the medium, indicating that the inhibition observed in water limiting conditions was due to reduced sucrose availability in the root tips (Fig 4.9).

To investigate further the above rescue of the inhibition of primary root, the meristematic region of the roots was screened to look for differences in the presence or absence of sucrose. This was done to establish if the inhibition of the root tip observed under osmotic conditions, and its rescue by the addition sucrose, was due to the maintenance of the primary root as a result of the presence of AHP4.

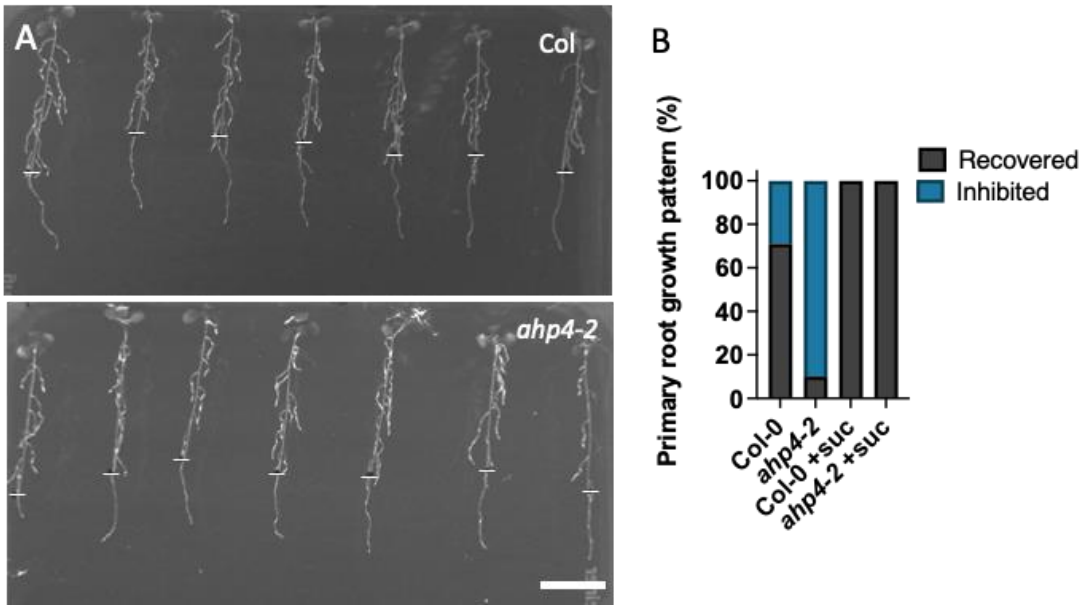


Fig 4.9 AHP4 maintains primary root growth by facilitating sucrose translocation in plants exposed to high osmotic stress. A) Plants were grown on control media for 7 days and then transferred to 300mM mannitol and 1% w/v sucrose. The white line on the primary root of the plants indicates the length of the root at the point of transfer. B) The percentage of the plants with their primary root recovered or inhibited in 300 mM mannitol or in 300 mM mannitol + 1% w/v sucrose (n=40).

Therefore, using confocal microscopy, the meristems of plants exposed to mannitol were examined after stained with propidium iodide. WT plants grown on mannitol had a very short root meristem, but all the cells within this zone were intact as indicated with by the propidium iodide staining being restricted to membranes only (Fig 4.10). On the other hand, the *ahp4-2* plants, growing under the same conditions showed significantly shorter meristems (Fig 4.10). In some cases, these were completely terminated, with all cells being differentiated. However, *ahp4-2* plants grown in mannitol and sucrose conditions had an intermediate meristem size with many viable cells (Fig 4.10) suggesting supplying sucrose alone is sufficient to lessen the effect of root growth inhibition.

The above results indicate that AHP4 is essential in maintaining the growth of primary root in high osmotic conditions. This is through maintaining a stable meristem zone that is supplied with sufficient sucrose. In this work, it is

hypothesized that this is mediated by maintaining a proper flow of sucrose into the root tip through the phloem (Fig 4.9). Phloem is the major transporter of sucrose, however, the difference in the growth pattern observed in plants with added sucrose in the media might be, to some extent, as a result of the reduction of the environmental osmotic pressure rather than by resupplying missing components transported by the phloem. Sucrose is metabolized by plants, and roots can absorb monosaccharides from the growing environment through monosaccharide/proton symporters (Sherson *et al.*, 2008). Even though monosaccharide root absorption takes place under optimal growth conditions, abiotic stresses are shown to cause an increase in the expression and activity of the monosaccharide/proton symporters. In fact, *STP13*, a monosaccharide/proton symporter in *Arabidopsis*, was found to have an enhanced activity in roots from plants growing in high salinity and drought conditions (Yamada *et al.*, 2011).

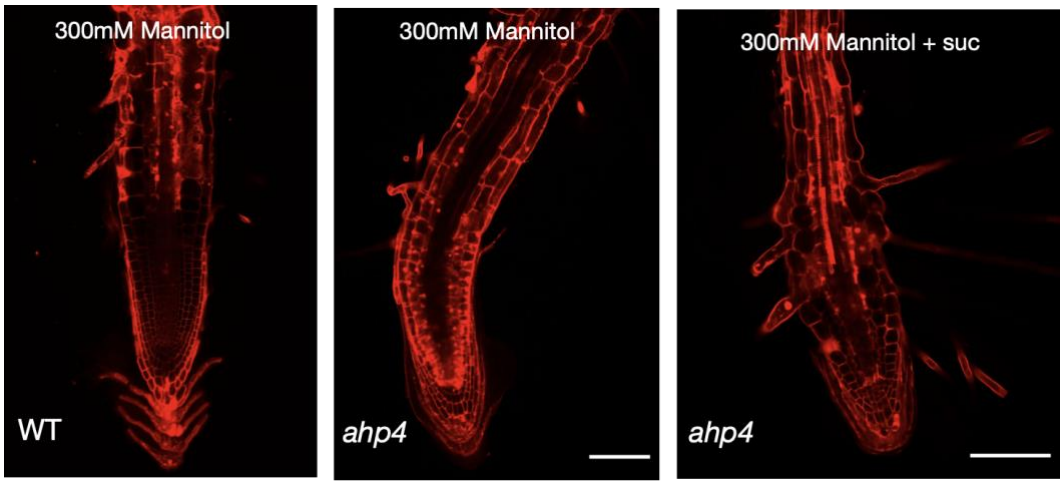


Fig 4.10 Sucrose partly rescues the integrity of the meristematic region of mannitol-grown seedlings in *ahp4* mutants. Confocal laser microscopy images of primary root meristems of plants grown for 7 days on control media and transferred to the indicated media for 7 additional days. Images are taken using the 20x objective and roots were mounted on microscope slides with water. Scale bars: 100µm.

To establish that the difference in the primary root responses observed in the mannitol treatments are due to the inhibition of the phloem and not as a result of the change in osmotic pressure between the internal and external environment of the root, the carboxylflorescein diacetate (CFDA) dye was used to measure the

rate of phloem transport in seedlings grown in control or in water limiting conditions. CFDA is a cell permeable colourless dye used to establish intercellular connection and determine phloem transport. Once in a cell the colourless CFDA dye is digested by endonucleases and becomes impermeable to the cell membranes, therefore its only possible movement is symplastically between cells. When cleaved, the dye is fluorescent and can be seen under laser microscopes (Oparka, Duckett and Fisher, 1994; Oparka and Turgeon, 1999; Ross-Elliott *et al.*, 2017). For the purpose of this experiment, the application of the dye was done to the top of one of the cotyledons while the plants were on the growing medium. The incubation time of the dye was decided to be 45 minutes based on preliminary experiments. During this time the plant was able to take up the dye through phloem all the way to the tip of the root, as shown in the schematic in fig 4.11 A. The root tip was then imaged, and the amount of CF dye quantified. Confocal images of the roots from 7-day old WT or *ahp4-2* seedlings grown on control medium treated with CFDA showed no significant change in the amount of dye that has traveled to the root meristem (Fig 4.11 B and D). However, when these plants were grown on 300 mM mannitol for 24 hours, the amount of dye quantified in the root tips of both the genotypes were less than plants grown in control conditions (Fig 4.11 C and E). This indicates that phloem transport is limited under stress conditions. The *ahp4-2* plants had substantially less accumulation of the dye in their root meristem compared to WT in the 24-hour mannitol treatment (Fig 4.11 C and E). The above indicate that 300 mM mannitol treatment inhibits phloem transport, and that *ahp4-2* plants have significantly reduced phloem flow only under water limiting conditions. This effect precedes the changes in primary root development observed earlier, as these were recorded after 7 days of treatment with 300 mM mannitol (Fig 4.6, 4.9 and 4.10), and in this case the treatment was only 24 hours.

To establish that phloem transport inhibition observed in this case was due to AHP4, its expression was measured in WT seedlings treated with various concentrations of mannitol for 24 hours and compared with plants growing on control conditions (fig 4.11 F). The expression of AHP4 in plants treated with 300 mM mannitol for 24 hours was increased by about 5-fold in this short time span,

indicating that the phloem transport inhibition observed by the CFDA quantifications was due to the absence of AHP4 in *ahp4-2* plants.

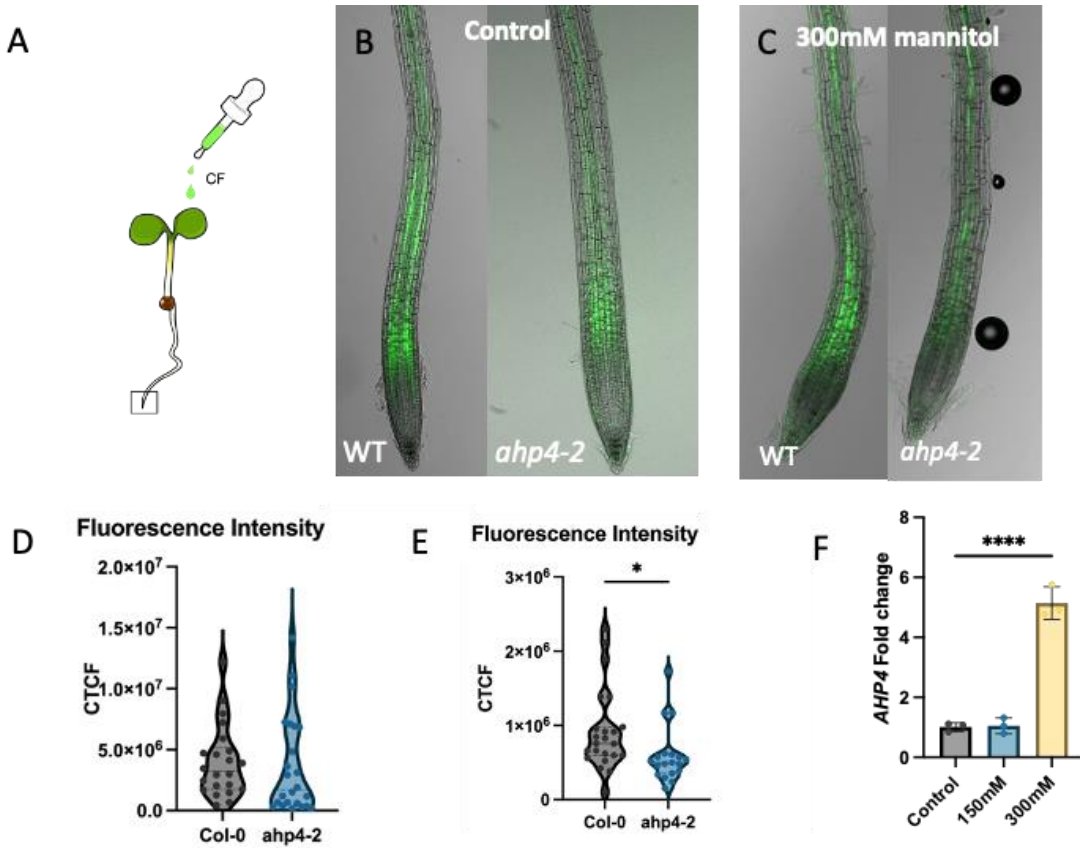


Fig 4.11 Maintenance of phloem transport under osmotic stress is dependent on AHP4. A) Schematic illustrating how CFDA was applied. B and C) Confocal images of the primary root meristem of plants grown on control conditions for 7 days and transferred to the indicated media for 24 hours. The green dye is a phloem permeable stain called carboxylfluorescein diacetate (CFDA) which was applied on one of the cotyledons. The images were taken 45 minutes after the staining event only at the root tip. Plants were selected to have the same root length. D) Quantification of the images obtained from the control treatment shown in A. The quantification was done with ImageJ using the specified area function. The area was specified to start from the end of the elongation zone as determined by the appearance of the first root hair. The values indicated are based on the corrected total cell fluorescence (CTCF) values to exclude any background resulting from dye leakage. This is calculated by: CTCF= Integrated density- (Area of the selected cell x Mean fluorescence of background readings) (developed by Martin Fitzpatrick, University of Birmingham). The statistical test used to determine significance was the unpaired t- test with F test to test variance. (n=22).

E) Quantification of the images obtained from plants treated with 300 mM Mannitol for 24 hours. Values indicate CTCF as before and data collection was done following the same procedure as in C. The statistical test used to determine significance was the unpaired t-test with F test to test variance. (n=20, *P≤ 0.05). F) AHP4 transcript levels in WT plants treated with 300 mM mannitol for 24 hours. Significance was calculated by one-way ANOVA, Brown-Forsythe, and Bartlett's statistical tests. Multiple comparisons to the control were corrected using the Dunnett's test to test the significant differences as compared to AHP4 expression in the control treatment (****P≤ 0.0001).

4.7 Discussion

The results in this chapter indicate that AHP4 is specifically expressed in the phloem companion cells and is able to provide tolerance to water limiting conditions by maintaining a functional phloem flow. Even though such response to water limiting conditions was not observed in the whole-plant level (Fig 4.4), osmotic stress induced the expression of AHP4 as indicated by confocal microscopy and quantification of its transcript levels (Fig 4.6 C and 4.11 F). This induction was consistent with publicly available data showing AHP4 upregulated by a variety of abiotic stresses along with AHP6 in some cases (Singh *et al.*, 2015). The effect of osmotic stress was visible in the roots of *ahp4* plants, which showed inhibition of the primary root growth almost immediately upon contact with the stress agent (Fig 4.7) and this was coupled with longer lateral roots (Fig 4.8). The longer LRs observed in the *ahp4-2* plants under osmotic stress might explain the reason why the effect is not visible at the whole-plant level when plants are subjected to drought. In this scenario the total root biomass may be the same, but the root architecture may be different. Root architecture was not investigated in plants grown on soil and subjected to drought however, if drought had similar effect with osmotic stress, then primary root would have been severely affected in *ahp4* plants under drought and any effect that would otherwise be visible in the shoot was potentially compensated by the increase in lateral roots. Furthermore, the meristematic zone of plants grown under osmotic stress conditions was severely affected for all genotypes but most strikingly in *ahp4-2* plants which had their meristematic zone totally collapsed (Fig 4.10).

The data presented in this chapter shows that the inhibition of primary root observed in *ahp4* plants was most likely caused by the reduced flow of solutes to the root meristem through the phloem. This was evident by the reduced accumulation of the phloem mobile dye CFDA in the meristem in *ahp4* plants (Fig 4. 11 C and D), and by rescuing the primary root inhibition when the growing medium was supplemented with sucrose. A similar response was documented in plants defective in genes belonging to the protein family of annexins (ANN) which are calcium-dependent membrane proteins. Mutations in those genes cause reduction in primary root growth and reduced phloem unloading in the root tip. This is due to the increased accumulation of callose in *ann* roots which restricts sugar transport through plasmodesmata. This phenotype can be rescued by exogenously supplied sucrose as phloem derived sucrose in no longer the primary source for the proper function of the root (Wang *et al.*, 2018). Likewise, *ahp4* plants were found to increase the accumulation of calcium to a larger effect of what was observed in the WT when plants are subjected to drought (Fig 4. 5). Calcium is responsible for callose formation and even though callose deposition was not measured in *ahp4* root tips, this could explain the defects observed in root development in seedlings grown under osmotic pressure.

The effects of AHP4 on drought demonstrated within this chapter indicate that the protein affects phloem transport in a positive way (i.e., enhances phloem transport), and this is especially noticeable under water limiting conditions. This is in contrast to what was observed in the recently published work which documented AHP4 to have a negative role modulating drought (Ha *et al.*, 2022). The authors used the *ahp4-SALK* allele which, even though they claim is a loss-of-function allele, it is not clear that it doesn't produce a truncated protein (Hutchison *et al.*, 2006). For this work, the experiments were conducted using the *ahp4-2* allele and in some cases using three independent alleles (*ahp4-3* and *ahp4-SALK* alleles were included in Fig 4.7). This is because development of the *ahp4-2* allele using the CRISPR-Cas system preceded that of *ahp4-3* allele. However, *ahp4-2* carries a 33-nucleotide deletion at the start of the second exon but has the possibility of

producing a truncated version of the protein which might retain some of the functions, just like the SALK allele. Nevertheless, the primary root inhibition in *ahp4* plants was observed in all the alleles tested, including the *ahp4-SALK* (Fig 4.7) which contradicts the findings of the previous work which indicated that primary root was longer under osmotic stress conditions (Ha *et al.*, 2022).

Based on the information presented above, I propose that AHP4 is essential for maintaining the phloem transport in water limiting conditions to support the continuation of the primary root growth. Most likely, this is done by ensuring the translocation of photosynthates into the root meristem.

Chapter 5: Material and Methods

5.1 Plant Growth

Arabidopsis thaliana seeds were grown predominately on half strength Murashige and Skoog basal medium (½MS, 500mg/L MES, pH 7.8) and supplemented with 0.8% w/v agar. Default growth room conditions were 16 hours light/ 8 hours dark, 22°C. Sterilisation of seeds prior to their growth was done by submersion in 70% ethanol, 0.05% triton solution for 2 min with inversion. The solution was then removed, and the seeds were washed with 95% ethanol and left to dry on filter papers before they are ready to be sowed on relevant media Plates containing sterilized seed were incubated at 4°C, dark cold room for at least 48 hours (and no more than 7 days) prior their transfer in the growth rooms.

5.2 Plant material

The *ahp4* SALK line that was used for this work is the CS860146 (ABRC number) and purchased from Nottingham Arabidopsis Stock Centre (NASC). To establish the homozygosity of the batch, PCR based genotyping was performed based on the instruction of the SALK T-DNA primer tool.

5.3 Preparation of competent cells

Chemically competent cell stocks for transformation were prepared from *Escherichia coli* DH5a strain. This was done by using commercially available competent cells (Max efficiency DH5a Competent Cells, Invitrogen #18258012) as a starting material. Initially, 5 ml LB medium culture was made from single colonies and grown at 37°C, 150 rpm for 16 hrs. From this starter culture, 3 ml were used to inoculate 100ml of LB medium at the same growing conditions to an OD_{600} of 0.4. The 100 ml LB cultures were then transferred into two 50 ml falcon tubes and chilled on ice for 15 min, centrifuged at 3000 rpm for 15 min at 4°C and each resuspended in 50 ml of sterile RF1 solution (100 mM RbCl, 50 mM $MgCl_2$, 30 mM Potassium acetate, 10 mM $CaCl_2$, 15% v/v glycerol, pH 5.8 with acetic acid). The resuspended cells were incubated on ice for 15 min, centrifuged at 3000 rpm for 15

min at 4°C and then each resuspended in 4 ml sterile RF2 solution (10 mM RbCl, 10 mM MOPS, 75 mM $CaCl_2$, 15% glycerol v/v, pH 6.8 with HCl). These were incubated for further 15 min on ice and then aliquots of 50 µl were made before they were flash froze and stored at -80°C.

Electrically competent *Agrobacteria tumefaciens* strain (GV3101) were also used in this work but stocks of them were prepared by Dr John Vaughan-Hirsch.

5.4 Genomic DNA and RNA extraction

The extraction of genomic DNA was done following a slightly modified version of the extraction technique described in Edwards et al., 1991. Briefly, a small amount of tissue was collected in an Eppendorf tube, ground using a disposable pipette tip, 600 µl of Edwards buffer (200 mM Tris, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and further mixed for 5 sec using the vortex. The sample was then centrifuged at maximum speed and 500 µl of the supernatant was collected in a fresh Eppendorf tube. An equal volume of isopropanol was added to the samples and incubated for 2-3 min at room temperature. Subsequently, the pellet was collected by centrifugation at maximum speed and washed with 70% v/v ethanol. The tubes were left to dry completely and then DNA was eluted by the addition of 50 µl water. DNA samples were stored in -20°C.

For the extraction of total RNA from plant tissues, a commercial RNA extraction kit was used at all cases (RNeasy Plant Mini Kit, Qiagen). The extraction procedure was done based on the manufacturer's instructions following the on-column DNase treatment suggestion. The RNA samples were stored in the -80°C for long term storage.

5.5 Synthesis of cDNA and quantitative real-time PCR

A variety of gene expression studies were done using the qRT-PCR technique in this work. To monitor the expression levels of genes, RNA was extracted from the appropriate tissues as described above. The RNA concentration was calculated using the Nanodrop™ (ThermoFisher) and equal amount of RNA for all samples were cDNA synthesised using "RevertAid First Strand cDNA synthesis" (ThermoFisher #K1621) kit, according to the manufacturer's instructions. The real-time quantitative PCR was done using LightCycler FastStart DNA SYBR Green I (Roche). The primers used for qRT-PCR include: E14, E15, qAHP4-endF, qAHP4-endR, ARR1_qPCR_F, ARR1_qPCR_R, ARR2_qPCR_F, ARR2_qPCR_R, ARR3_qPCR_F, ARR3_qPCR_R, ARR4_qPCR_F, ARR4_qPCR_R, ARR9_qPCR_F, ARR9_qPCR_R, ARR10qPCR_F, ARR10qPCR_R, ARR12qPCR_F, ARR12qPCR_R, ARR15_qPCR_F, ARR15_qPCR_R (see primer list for sequences). Analysis of the results was done using the $2^{-\Delta\Delta Ct}$ method in Microsoft Excel (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). To ensure that the resulting data were of high quality the following step were taken into account: 1) The primers used for each reaction were always spanning two exons to ensure no DNA contamination of the sample; 2) Prior of using the primer pairs to record the expression levels of the genes indicated, primer efficiency was established by analysing the expression difference of different concentrations of the same product; 3) The final product was up to 200 bp to ensure the accurate recording of the expression levels; and 3) the melting curves were recorded and visually checked for every reaction and every primer set. A representative of the melting curves visual analysis can be seen in Appendix 5.

5.6 Transcriptomics

The transcriptome of WT and ahp4-2 plants was compared after treatment with and without exogenous CK. Specifically, seven-day old seedlings were germinated and grown on ½ MS media. One the 7th day, the plants were removed from the plates and placed in ½ MS liquid solution with 100 mM 6-Benzylaminopurine (BA) diluted with the help of 0.1% DMSO for 2 hrs or in ½ MS

solution supplemented with 0.1% DMSO as the mock treatment for equal amount of time. This process was repeated five times, one for every biological replicate. At the end of the treatment period, seedlings were carefully placed on ½ MS plates and with a sharp scalpel the roots and shoots of each genotype and treatment type were harvested. To obtain enough RNA on this experiment, material from about 50 roots and about 10 shoots per genotype and treatment were used. The tissues of all five biological replicated were flash frozen in liquid nitrogen prior RNA extraction.

Sequences were sent to Novagene for RNA Sequencing, where the underwent quality control, mRNA library preparation with poly A enrichment and sequencing with Illumina Paired-End 150 bp technology which yielded about 3 Gb raw data per sample. Samples were mapped to the Arabidopsis genome by Novagene and subsequent analysis converted raw counts into fragments per kb mapped reads (fpkm). A cut-off value of < 1 was set to filter out the genes that are very lowly expressed. Gene values that made the cut-off were expressed in as the logarithm of 2-fold change (log2FC) and the threshold for defining DE genes was set to 0.05 (p-value). This analysis was done by Dr Rahul Bhosale.

5.7 Molecular Cloning

The plasmid generation (pKIR1.1_2_sgRNA_Cas9 and pKIR1.1_3_sgRNA_Cas9) used to create the CRISPR lines (*ahp4-2* and *ahp4-3*) was done with pKIR1.1 plasmid using restriction enzyme based cloning method. The hybridised primer (described in section 5.8) and pKIR1.1 plasmid were digested, separately, using *AarI* enzyme (ThermoFisher #ER1581) according to the manufacturer's instructions (1x enzyme buffer, 0.5-1 µg/µl DNA, 0.025 mM oligonucleotide, 0.6 µl *AarI* and water to make the total volume up to 20µl). The reaction was placed in a PCR cycler with the following settings: 16 hrs at 37°C, 20 min at 65°C and 4°C for maintenance. The digested hybridised primer (gRNA) was then inserted in the digested vector using T4 ligase (3U). A fraction of the ligated product was then transformed in chemically competent DH5a cells growing on spectinomycin (50 µl/ml) agar plates. Successful positive colonies were confirmed

by sequencing using the pKIR1.1_seq_F primer (see primer list for sequence) and 50% glycerol stock of the cells was stored in -80°C.

The plasmid used to create the transcriptional reporter lines was generated using the GreenGate system (Lampropoulos et al., 2013). To generate the pAHP4::GFP line, an A-module plasmid was constructed by inserting the promoter region covering an area of 3.25 kbp upstream of the gene's start codon into the pGGA00 (empty vector), called pGGA_AHP4. This was done by isolating the promoter sequence by PCR using Q5 proof reading Taq polymerase and primers with the necessary overhangs AHP4_full_proF and AHP4_full_proR (see primer list for sequence). The promoter region with the overhangs was then digested and ligated using NEB Golden Gate Assembly Kit (#E1601) as per manufacturer's instructions. The same product was used for all GreenGate assembly reactions described in this work. Part of the ligation was used to transformed DH5a cells growing on ampicillin (50 µl/ml) agar plates. Bacterial colonies containing the correct insert were determined by sequencing using primers named: "entry_vector_control_F" and "entry_vector_control_R". The construction of the destination vector (pAHP4::GFP-NLS) was done by digesting and ligating in one pot reaction the following plasmids: pGGA_AHP4, pGGB_003 (B-dummy), pGGC_012 (GFP-NLS), pGGD_002 (D-dummy), pGGE_001 (RBCS terminator), pGGF_007 (Kanamycin plant resistance) and for the backbone the DEAL vector was used which is the original GreenGate destination vector pGGZ003 expressing "tdtomato" chromophore protein as a membrane marker (Kümpers *et al.*, 2022). This ligation product was used to transform DH5a cells growing of spectinomycin (50 µl/ml) agar plates and successful colonies, as determined by sanger sequencing, were subsequently transformed into *Agrobacteria* and Col-0 plants. The sequencing was done using the GreenGate primers designed by Dr Britta Kümpers. Glycerol stocks (50% v/v) of DH5a and *Agrobacteria* cells were stored in -80°C as stocks.

The transcomplementation vectors were constructed taking advantage of the GreenGate system too. The destination vector was assembled by digesting and ligating in one pot reaction using the following plasmids: pAHP6_BK01 (plasmid

containing 1.611 kbp upstream of AHP6 start codon constructed by Dr Britta Kümpers), pGGB_003 (B-dummy), pGGC_AHP4, pGGD_002 (D-dummy), pGGE_001 (RBCS terminator), pGGF_007 (Kanamycin plant resistance) and pGGZ001 for the backbone. The final vector was named pAHP6::AHP4. This assembly was confirmed using sanger sequencing using the following GreenGate primers designed by Dr Britta Kümpers: pAHP6pcontrolR, B-dummy_R, B_dummyF, RBCSt_GGconR, RBCSterminatorjuctionF, KanR_conR and KanR_conF (see Appendix for primer list for sequence). The pGGC_AHP4 vector was assembled by digesting and ligating the AHP4 CDS containing the appropriate overhangs with the C-module vector pGGC000. Primers used to isolate the AHP4 CDS were E30 and E31 (see primer list for sequence). The vector was confirmed by sanger sequencing using entry_vector_control_F and entry_vector_control_R primers.

The assembly of the overexpressing vector was done by GreenGate technology using the following plasmids: pGGA_004 (35S promoter), pGGB_003 (B-dummy), pGGC_AHP4, pGGD_002 (D-dummy), pGGE_001 (RBCS terminator), pGGF_007 (Kanamycin plant resistance) and pGGZ001 for the backbone. The successful vector was confirmed by sanger sequencing using the primers B-dummy_R, B_dummyF, KanR_conR, KanR_conF, D_dummyFnew and the final plasmid was recorded as p35S::AHP4.

The assembly of pAHP4::AHP3 and pAHP4::AHP6 vectors was also done using the GreenGate technology. Initially, the CDS of AHP3 and AHP6 were cloned into pGGC000 vectors creating the pGGC_AHP3 and pGGC_AHP6 vectors respectively. These vectors were confirmed by sanger sequencing using the primers entry_vector_control_F and entry_vector_control_R. For the assembly of the destination vector the following plasmids were used: pGGA_AHP4, pGGB_003 (B-dummy), pGGC_AHP3 or pGGC_AHP6, pGGD_002 (D-dummy), pGGE_001 (RBCS terminator), pGGF_007 (Kanamycin plant resistance) and pGGZ001 for the backbone. A fraction of the ligation product was transformed into chemically competed DH5a cells and successful plasmids were determined by sanger

sequencing using the primers: B-dummy_R, B_dummyF, KanR_conR, KanR_conF, D_dummyFnew.

5.8 Bacteria Transformation

The bacteria transformed in this study are *Escherichia coli (E. coli)* DH5a cells and *Agrobacterium tumefaciens* GV3101.

The transformation of chemically competent cells DH5a was done by the addition of 1 µl of the ligation product in 50 µl plasmid aliquot. The ligation product and the cells were incubated on ice for 30 min for more efficient incorporation of the plasmid into the competent cells. The cells were heat shocked by leaving them in 42°C heat block for 60 sec followed by 2 min on ice. Following the shock, the cells were revived by the addition of prewarmed Lysogeny broth (LB) medium. They were incubated at 37°C for 1 hr, 200 rpm to allow the expression of the resistance gene and then they were plated on the appropriate selection medium. The plates were incubated in the 37°C for 16 hrs and single colonies were tested with colony PCR and sequencing.

For transforming the *Agrobacteria*, 50 µl of electrically competent cells were thawed and incubated with 100 ng of the desired plasmid for 10 minutes on ice. Using sterile and pre-chilled 0.2 cm electroporation cuvettes, the cell/plasmid mixture was electroporated on a single pulse under the setting for agrobacteria cells. The cells were revived using 1 ml LB and transferred to 1.5 ml Eppendorf tubes at 28°C, 200 rpm for 2 hrs. The liquid culture was then spread on double selection LB and 1% w/v agar plates containing 50 µg/ml rifampicin for Agrobacteria selection and the appropriate antibiotic based on the plasmid resistance. Bacteria were incubated at 28°C for 2 days. Successful colonies were determined by colony PCR.

5.9 Plant transformation, crosses, genotyping

Arabidopsis thaliana transformations were done by *Agrobacterium* mediated transformations using the floral dipping method (Clough and Bent, 1998). Briefly, *Agrobacteria* cells containing the plasmid of interest were inoculated firstly on 5 ml starter cultures for 16 hrs at 28°C, 150 rpm and then the entire 5 ml were transferred to 500 ml liquid LB medium cultures to grow at the same conditions overnight. The cells were centrifuged at 4000 rpm for 20 min at 4°C using the ultracentrifuge. Following the centrifugation step, the pellet was resuspended in 500 ml sucrose solution (50 g/l sucrose) and 0.02% v/v of the surfactant SILWET L-77 solution (200 µl/L). This solution was used to dip the flowering plants for 1 min. The entire plant was then wrapped for 2 days in a plastic sleeve. When the sleeve was removed, the plant was left to seed normally. Initially plants were screened for single insertion events by selecting the lines having the ratio 3:1 transformed to WT. Homozygosity was determined on later plant generations when 100% of the plants were resistant to the selection agent used.

The crosses were created by the emasculation of *ahp4-2* plants. The emasculated flowers were left to dry for 2 days and then flowers of pTCSn:GFP Deal, pSUC2:GFP or pDII:Venus plants were touched on the swollen stamen a few times. The matured siliques resulting from the crosses were collected, and seeds were sown to select the seedlings expressing the GFP or the Venus gene using the fluorescent screening microscope. The successful seedlings were left to set seed and seedlings from the next generation were genotyped for the *ahp4-2* allele. This was done by gDNA extraction of a leaf sample and PCR based genotyped was used to separate the heterozygous plants from the WT (383 bp) and the edited *ahp4-2* (350 bp) using the primers AHP4_genF and AHP4_gen R (see primer list for sequence).

5.10 Protein expression, extraction, and purification

Vectors for protein expression in bacteria were assembled by restriction and ligation with T4 DNA ligase (NEB) based on manufacturer's instructions. Specifically,

the CDS of AHP2, AHP3, AHP4, AHP5 and AHP6 was isolated containing BamHI and SalI restriction enzyme overhangs. Primers used to do this were: E16-E17 for AHP2, E18-E19 for AHP3, E20-E21 for AHP5, E24-E25 for AHP4 and E26-E27 for AHP6. The products of these PCRs were digested and ligated into pGEX_6p_1 (GE Healthcare Life Sciences) vectors digested with BamHI and SalI. Success of the cloning was determined by Sanger sequencing using pGEX_GST_F and pGEX_GST_R primers. Vectors for expression of AHP1, SLN-HK and SLN-HK-Rec in bacterial systems were provided by Dr John Vaughan-Hirsch (Vaughan-Hirsch *et al.*, 2021) and Prof Ann West (Fassler and West, 2010).

As part of preliminary sets of experiments to express proteins in the bacteria, 5 ml starter cultures were incubated at 37°C, 150 rpm overnight from glycerol stocks. The following day, 2 ml of the starter culture was transferred to two 50 ml LB/ ampicillin culture grown at 37°C, 150 rpm up to OD_{600} 0.5 (one for the induced sample and one for the uninduced). When the appropriate OD was reached, 0.4 mM IPTG and 2% ethanol was added to one 50 ml liquid culture for the induction of AHP1, AHP2, AHP3, AHP5, SLN1-HK, SLN1-HK-Rec and the empty vector containing only the GST tag; or 0.8 mM IPTG and 2% ethanol (déric Picard-Jean *et al.*, 2018) for AHP4 and AHP6 induction. The second 50 ml bacteria culture was left uninduced as a control. The flasks were incubated in 16°C for 20 hrs to minimise the inclusion body formation around the proteins of interest. The cultures were then centrifuged at 4°C, at 3500 g for 15 min and supernatant was discarded while pellet was snap frozen in liquid nitrogen. The pellet was left to thaw slowly on ice and then resuspended in 1.5 ml (1/10 v/v) of lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1 mM EDTA, 10% glycerol) for 15 min. Lysozyme 50 µg/ml was added, and the cells were disrupted using the "Soniprep" 150 sonicator (four times the settings: ¼ max amplitude 30" ON, 30" OFF). After sonication, 15 µl were taken from the two lysates to be used later for visualisation and 5 µl SDS loading dye (375 mM Tris HCL pH 6.8, 9% SDS, 10% glycerol, 0.03% bromophenol blue, 5% β-mercaptoethanol) was added. These were named: "lysate uninduced" (#1), and "lysate induced" (#2). The remaining lysate samples were centrifuged for 5 min at

maximum speed and 15 µl of the supernatant was collected in a separate tube, 5µl SDS loading dye was added and named as the soluble fraction (#3). Lastly, a small amount of the pellet was diluted to 15 µl of lysis buffer and 5 µl SDS loading dye which represented the insoluble fraction (#4). The samples were then incubated for 5 min to 95°C, centrifuged for 2 min at maximum speed and 10 µl were used to run a 10 % polyacrylamide gel (Appendix 6).

To extract the proteins for purification, their expression was done with the conditions mentioned above for each protein but this time in 250 ml bacteria cultures (LB and ampicillin). At the end of the final incubation, 125 ml of bacteria culture was collected in 50 ml falcon tubes by centrifugation at 4°C, at 3500 g for 15 min. The resulting pellet was snap frozen with liquid nitrogen. One falcon tube was stored as a backup in -80°C and the second was used to purify the expressed protein. The pellet derived from 125 ml liquid cultures was resuspended in 30 µl/ml pre-lysis buffer (50 mM Tris HCl pH 8, 75 mM NaCl). Like previously, bacterial cell walls were digested using 50mg/ml lysozyme for 15 min on ice. To disrupt the cell membrane 10% v/v Triton-X-100 was added and 10 µl/ml protease inhibitors to prevent protein digestion. To maximise the purification efficiency 50 µl of DNase was added (déric Picard-Jean *et al.*, 2018) and therefore the buffer was adapted to be compatible for DNase maximum activity by the addition of the divalent cations Ca^{2+} and Mg^{2} in the form of $MgCl_2$ and $CaCl_2$ to make their concentration 50 mM and 13 mM respectively. The lysate with the DNase was incubated in 37°C for 45 min. Following the DNase treatment, 75 mM NaCl was added to bring the total concentration up to 150 mM, 5 mM DTT and 4 mM EDTA. Cells are completely disrupted, and DNase deactivated by sonication using the "Soniprep" 150 sonicator (four times the settings: ¼ max amplitude 30" ON, 30" OFF) while the sample was kept in an ice bath. After sonication 50 µl was transferred to a new tube from each sample to be used for visualisation as the total protein in the mixture. From the same ssample, 50 µl were transferred in a separate tube to be cleared by centrifugation for visualising the protein levels in the supernatant as the input sample for the GST purification. And lastly, the pellet to indicate how much of the

protein was trapped in the pellet. The pellet was resuspended in a buffer complementing the pre-lysis buffer (13 mM $CaCl_2$, 50 mM $MgCl_2$, 4 mM EDTA, 5 mM DTT, 75 mM NaCl) These samples were then fixed by the addition of 10 µl SDS loading dye, denatured in 95°C for 5 min and kept on ice. The remaining sonicated sample was cleared by centrifugation at 4°C at full speed and the supernatant (input) was transferred in a fresh falcon tube.

All the following purification steps were performed in the 4°C cold room using the Pierce GST Spin Purification Kit (0.2 ml; No. 16106) by ThermoFisher. The agarose resin was equilibrated on column using the equilibration buffer based on the manufacturer's instructions. The resin was then removed from the column and placed on the protein input sample for batch incubation of the resin with the sample. The mixture was incubated with gentle rotation for 1hr in the 4°C cold room. Following this incubation, the mixture of resin and protein extract was passed through the column with successive centrifugations at the recommended by manufacturers speed (0.7 rpm for 2 min). Washes and elution of each sample were performed as per the instructions of the manual. After the elution, the purified protein samples were supplemented with 10% v/v glycerol for cryoprotection, aliquoted and stored in -80C.

5.11 SDS-PAGE and western blotting

A small fraction of the protein samples was mixed with 6X Protein loading buffer (375 mM Tris-HCl pH 7, 9% SDS, 50% glycerol, 0.03% bromophenol blue, 5% β-mercaptoethanol) and boiled to 95°C for 5 minutes for denaturation of the secondary structures. The samples were loaded to acrylamide gel made by 10% resolving gel (1.5 M Tris HCl pH 8.8, 0.4% SDS, 10% acrylamide-bis acrylamide 40%, 0.1% w/v APS, 2 µl/ml TEMED) and 4% stacking gel (0.5 M Tris HCl pH 6.8, 0.4% SDS, 4% acrylamide-bis acrylamide 40%, 0.1% w/v APS, 2 µl/ml TEMED) and were then run at 100V using the Bio-Rad MiniProtean system for separation by electrophoresis. The gels were then washed twice for 5 minutes with distilled water and stained for 1 hour using the Imperial™ protein stain (Thermo Scientific). The

gels were subsequently de-stained using distilled water for 2 hours before the images are taken to visualise the total protein extracted from the samples.

Western blotting was used to determine the presence of AHP4 in the overexpressing lines. Proteins were extracted as described above. For western blotting, the proteins were transferred to 0.4 µm pore size nitrocellulose membrane after the separation by electrophoresis using the semi-dry transfer method. Initially, the gel was washed in distilled water for 5 minutes and then soaked in transfer buffer (48mM Tris-HCl pH 8, 192mM glycine, 20% v/v methanol and 0.0375% w/v SDS) for 5 more minutes. Three Whatman™ paper sheets cut to the size of the gel were pre-soaked in the same transfer buffer and placed over the bottom electrode of the transfer apparatus (anode), overlaid by the nitrocellulose membrane, the gel and three more pre-soaked Whatman™ paper sheets. The layers were straightened by softly rolling with a glass pipette to ensure no air is trapped between them and apparatus was shut securely. The transfer was done for 1 hour at 15 V. To determine the successful transfer and the equal amount of loading the membrane was washed initially with distilled water and stained for 5 minutes with Ponceau Red (1.3 mM ponceau, 0.874 M glacial acetic acid). Subsequently the membrane was washed about 5 times with distilled water to remove the stain and the membrane placed in a clean container with the blocking buffer (5% w/v Marvel dry skimmed milk in PBS buffer supplemented with 0.05% v/v Tween 20-PBS-T) for 1 hour at room temperature or up to 72 hours at 4°C. After blocking, the buffer was removed and the primary antibody (aAHP4 #1374, 1:1000 dilution; Protean Ltd) was added to the same blocking buffer on the membrane for 1 hour incubation in room temperature or up to 72 hours at 4°C. The primary antibody milk solution was then removed, and the membrane was washed 3 times with PBS-TT buffer (PBS supplemented with 0.05% v/v Tween 20 and 0.1% v/v Triton-X-100) for 5 minutes. The secondary antibody (anti-Mouse IgG, AP-conjugated, 1:10000) solution (5% w/v Marvel dry skimmed milk in PBS-T buffer) was then added on the membrane and incubated 1-3 hours at room temperature. The membrane was the washed 3 times using PBS-TT solution for 5 minutes and the

signal was developed by adding a few drops of 1-Step™ NBT/BCIP Substrate Solution (Thermo Fischer #34042) on the membrane at room temperature.

5.12 Phenotyping

5.12.1 Shoot phenotyping

Plants grown in soil for 25 days were used to record the number of inflorescence stems and the length of the primary inflorescence. The size of the seeds was determined by analysing images of the seeds obtained from a stereo microscope. The size of the seeds was recorded in length, measuring the longest side of each seed using FiJi image analysis software.

5.12.2 Drought stress experiment

To examine the biomass difference, and leaf development during drought, a modified protocol of what was described in "Reverse Genetics" book (Ouwerkerk and Meijer, 2011) was used. Following a two-day cold treatment, the plants indented for the drought treatment were germinated on plates grown on short day (8 hours light/ 16 hours dark) growth rooms and then transferred in soil pots growing in the same short-day conditions. Plants were transferred to pots of soil with roughly 100 g of soil in each. The pots were watered by watering the tray leaving the pots to soak overnight. Another 100 g of soil was put in a tray and left in the 60°C oven for three days to dry and record its dry weight. After the overnight soaking, water was removed from the tray and the pots were left for one more day to drain any excess water. The following day, the plants were transplanted in the pots and their weight was recorded daily. The initial weight was the filed capacity weight (=100% water content) which was maintained for the plants in the well-watered trays, however, the pots in drought-treatment trays were allowed to get to the 30% field capacity based on their weight. When all the plants in the drought treatment trays were at 30% field capacity, they were kept for seven days at a constant state. On the following day, the pots in the droughted trays were supplied with water to bring up the field capacity to 70% to recover. They were harvested 14 days later by cutting the shoot and placing it in a paper bag. The fresh weight was recorded for both treatments (well-watered and drought) and the plants were left

in the 70°C for 4 days and their dry weight was recorded. To record the size of the leaves and their number, 4 of those plants per genotype and treatment were randomly selected and before drying they had their leaves placed and pressed flat with glass and imaged using a phone camera. The relevant measurements (area, length) of the leaves were done using Fiji software.

Examination of the differences in the ionome composition was performed using ICP-MS. This was performed by harvesting 150 mg of similar maturity leaf tissue from WT and mutant plants grown on drought and well-watered conditions, using a sharp scalpel and plastic tweezers, rinsed with distilled water, and placed in pre-weighted Pyrex test tubes to be dried at 88°C for overnight. The dry weight of the sample was recorded and then the tissues were digested using 1ml of nitric acid based on the Ionomics facility protocol at the University of Nottingham. The elements analysed by ICP-MS are: K, S, P, Mg, Na, Fe, Zn, Mn, Co, Cu, Ca, Ni, Mo, Sr, Rb, As, Se, Cd, Pb, Li and B.

5.12.3 Osmotic stress experiment

The application of osmotic stress to study root developmental defects was done using ½ MS agar medium supplemented with 0 mM mannitol (mock treatment) or 150 mM and 300 mM mannitol (treatment). Specifically, plants germinated vertically in ½ MS medium for seven days and then transferred to ½ MS media supplemented the indicated mannitol concentration, to continue growing vertically for a period of seven additional days, unless otherwise stated. The same method was followed for investigating the primary root elongation under osmotic stress with the addition of 1% w/v sucrose. The length of the root at the point of transfer was marked by a dot using a black marker. At the end of the treatment period, plates were imaged using the PlateImager robot (Wells *et al.*, 2012).

5.12.4 Cytokinin on root development

For the assessment of root responsiveness in increasing cytokinin concentrations, plants were germinated and grown in ½ MS medium supplemented with 0, 0.01, 0.1, 1 and 10 μM 6- benzylaminopurine (BA) and 0.1% v/v dimethyl

sulfoxide (DMSO). The investigation of the primary root elongation rate under various cytokinin concentrations was done by marking the root length of the plants on the third- and fifth-day post germination (Hutchison *et al.*, 2006). The elongation rate was recorded using the image analysis software Fiji (ImageJ).

5.12.5 Cytokinin on chlorophyll retention

Chlorophyll extraction was performed from plants germinated and grown on ½ MS, 0.8% w/v agar and supplemented with 0, 25, 100 nM BA for 14 days. These were grown on these plates horizontally. Fourteen-day old seedlings were harvested by placing 20-23 mg of fresh plant tissue in 2 ml Eppendorf tubes and snap frozen. The tissue was then lysed for 2 min using the tissue lyser with prechilled handles. Prechilled aqueous acetone solution (80% v/v acetone) was used as a solvent for the extraction of chlorophylls at the ratio of 1ml solvent for every 20 mg of tissue. After the addition of the solvent the samples were placed back in the tissue lyser for 2 min before they were centrifuged at 8000 rpm for 5 min in 4°C. Aliquots of the supernatant were transferred in 200 µl 96-well plates, and the concentration of chlorophyll was determined spectrophotometrically using the Omega reader to record absorbance at A^{664nm} and A^{647nm}. A third absorbance value was recorded for every sample at A^{750nm} which was used as a turbidity correction factor. Total concentration of chlorophylls was calculated using the following equation (Porra, 2002; Ritchie, 2006):

$$Chl^{a+b}(mg/g) = \frac{\{[17.768 * (A^{664nm} - A^{750nm})] + [7.34 * (A^{647nm} - A^{750nm})] * Vsample(ml)\}}{W\ (mg)}$$

5.12.6 Xylem phenotyping

The xylem formation pattern between WT, *ahp4*, *ahp6-1* and the various trans-complementation lines was observed by clearing six-day old seedling. The clearing was performed using acidified methanol (50 % v/v methanol, 10% v/v HCl) for 15 minutes, followed by their incubation in basic ethanol (60% v/v ethanol, 7% w/v NaOH) for 15 more minutes. Then the seedlings were rehydrated using

decreasing concentrations of ethanol solutions (40%, 20% and 10%) for 5 min each time. The roots were then mounted on a microscope slide with 50% glycerol and sealed with clear nail polish to prevent from drying. These were stored in 4°C until the scoring.

5.12.7 Phloem transport assay

Plants used for this experiment were 7 days old. To perform the experiment, each plant was placed on a square microscope slide on top of agar plate. This was done to ensure that the cotyledons were kept away from the medium while roots remained in contact with the medium. The CFDA solution (13mM CFDA, 0.5% (v/v) Adigor, 80% (v/v) acetonitrile) (Knoblauch *et al.*, 2015; Ross-Elliott *et al.*, 2017) was applied on top of one of the cotyledons and plants were placed vertically for 45 minutes. This incubation time was determined based on preliminary experiments to be sufficient for the CFDA to travel to the root tip through phloem. The root tips were then imaged under the confocal laser scanning microscope, and the amount of CFDA dye quantified using Fiji.

The values obtained were corrected towards the background to exclude any dye leakage. This was done by calculating the total cell fluorescence (CTCF) values using the following formula: CTCF= Integrated density- (Area of the selected cell x Mean fluorescence of background readings) (developed by Martin Fitzpatrick, University of Birmingham).

5.13 Microscopy

The experiments involving the GFP transcriptional reporter lines for localisation of AHP4 in various growth conditions were visualised using Leica SP8 or Leica SP5 confocal laser microscopes (CLM). The GFP was visualised with an excitation maxima of 485 nm and emission maxima of 615 nm. The red membrane marker is the tbtomato gene, expressed in membranes and it was visualised with an excitation maxima of 554 nm and emission maxima of 581 nm.

The examination of the meristem size of WT and *ahp4* plants was done using the Leica SP8. Roots of those seedlings were stained using propidium iodide; a cell wall and nuclei acid stain at excitation maxima of 535 nm and emission maxima of 615 nm. Images obtained by the CLMs were analysed using Fiji (ImageJ).

5.14 Phylogenetic analysis

Amino acid sequences of histidine phosphotransfer proteins for *Arabidopsis thaliana*, *Oryza sativa*, *Medicago truncatula*, *Zea mays* and *Solanum lycopersicum* were obtained by BLAST search using the Phytozome database. The amino acid sequence of the closest protein in *Saccharomyces cerevisiae* was obtained by a similar search using the NCBI database. The search for proteins related to AHP4 in the above species was done for each of the *Arabidopsis* AHPs (including AHP6) to include all possible related proteins for the analysis.

This evolutionary analysis involved 35 amino acid sequences and was conducted in MEGA11 software (Stecher, Tamura and Kumar, 2020). Sequences were aligned using Muscle. The evolutionary history was inferred using the Maximum Likelihood method and JTT matrix-based mode, from 500 replicates and is representing the history of the species mentioned above. It was plotted with the S. cerevisiae sequence YPD1 specified as an outgroup.

5.15 Statistical analysis

The statistical analysis for all the data presented in this work was done using the licensed software Prism 9®. Statistical test details and number of replicates for individual experiments are specified in the result chapters under the figure legends. Statistically significant differences are indicated by *P<0.05, **P<0.01, ***P<0.001.

Chapter 6: Final Discussion

Depside the fact that many CK signalling components have been extensively studied and characterised, AHP4 is one of the few proteins that remains a mystery. It is not known to form associations with HKs (Bauer *et al.*, 2013) and there are no indications of its subcellular localisation (Bauer *et al.*, 2013) or its expression patten in the plant (Hradilová and Brzobohatý, 2007). Based on its sequence it is thought to belong in the group of proteins that transfer the phosphorylation signal from the HKs to the nucleus to activate ARRs (i.e., the HPts). However, such evidence of this function does not exist. On the contrary, genetic studies aiming to characterise all the *HPts* proposed that *AHP4* might have an inhibitory action in the CK pathway because in many CK responses it showed an increase in CK sensitivity (Hutchison *et al.*, 2006). However, with the lack of suitable knock-out mutant alleles it was not possible to obtain a clear phenotype. Therefore, the role of AHP4 in CK signalling as well as its function in plant physiology represent a gap of knowledge that this thesis is aiming to shed light to.

In this thesis I have examined the role of AHP4 in CK signalling and its function in plant physiology. To do this a variety of approaches were used and new resources were developed which extended our understanding around the structure of the protein and its function. Specifically, the generation of new and multiple independent alleles (CRISPR *ahp4*) enabled the investigation of several CK responses with greater confidence. Furthermore, for the first time, I showed the expression of AHP4 using GFP transcriptional reporter and through reverse genetics I revealed a function for AHP4 in *Arabidopsis* plants in modulating stress responses. Lastly, I showed that AHP4 is conserved in angiosperms based on phylogenetic analysis and that tertiary conformation of the protein is likely to be reason why it is behaving differently from other HPts of CK signalling.

The role of AHP4 in modulating cytokinin response via the two-component signalling

My first aim was to understand the evolutionary history of AHP4 and determine whether it functioned in cytokinin signalling, and if so, whether it is acting as a positive or a negative regulator.

Previous to my work, phylogenetic analyses based on protein sequence revealed that phylogenetically AHP4 does not cluster with the other Arabidopsis HPts or AHP6 (Vaughan-Hirsch *et al.*, 2021). Instead, Arabidopsis AHP4 clades with the monocot PHPs, which have been shown to negatively influence CK signalling by inducing a variety of *A-ARRs* and *CKXs* resulting in phenotypes affecting shoot morphological traits, like changes in panicle architecture and seed size (Vaughan-Hirsch *et al.*, 2021). Whilst the presence of a His residue at the phospho-accepting location is consistent with the role of AHP4 as a positive regulator of CK, it's close phylogenetic relationship with negative regulators of CK signalling raise the possibility that it might be a negative regulator of CK. This is supported by some genetic evidence which demonstrated that *ahp4* plants increased CK sensitivity in the reduction of LR formation and hypocotyl elongation assays (Hutchison *et al.*, 2006).

The previously published phylogeny was built to explore the evolutionary history of the monocot PHPs, and the primary focus was not to consider AHP4. As such it did not include other sequences from dicots. Therefore, the phylogeny was unable to inform whether AHP4 is conserved throughout dicots, and whether related dicot proteins contained a His at the phospho-receiving residue. To test this, I constructed a gene loss tree. The gene loss tree showed that there is a close *AHP4* relative in every dicot species, suggesting that this locus was strongly evolutionary conserved. The fact that an AHP4 orthologue has been so widely preserved suggests a strong functional conservation, re-enforcing the idea that AHP4 is worthy of study. Furthermore, the fact that these loci has been so widely retained suggests that it may have an important function unique from the other AHPs. As

the HPts have been shown to act redundantly (Hutchison *et al.*, 2006), this is incompatible with such strong selection on a single locus.

To explore this further, I then expanded the existing phylogeny of HPts (both PHPs and AHPs) to include additional dicot species along with monocots. Here the AHP4 orthologues of *Medigaco, Aradidopsis* and tomato all cladded with the monocot PHPs (Fig 2.1). Sequence analysis showed that all of these contained the conserved His residue. Collectively, these results show that AHP4 is unlikely to be a pseudogene or quirk of *Arabidopsis*, and instead is a strongly conserved gene. The strong selective pressure suggests that it has a role distinct from either the AHPs or the negative regulator of cytokinin AHP6. The phylogeny alone cannot tell us whether AHP4 acts as a positive regulator of CK signalling, a negative regulator or has acquired a role independent of CK signalling. To address this, I explored AHP4 structurally, biochemically, and genetically.

The inhibition of CK signalling resulting from AHP6 and OsPHPs is based on their inability to accept the phosphate as they both lack the necessary His residue, thus they are unable to participate in the phosphorelay. Structurally, AHP4 contains the His residue in the conserved region of the protein that would in theory allow for phosphorylation to occur. While this is considered as an absolute requirement for participation in the phosphorelay, the His residue needs to be at a position which is accessible to both AHKs and ARRs. In fact, structural predictions of all the HPts, presented in this thesis, indicate that the His residue on AHP4 has different orientation on the tertiary structure of the protein (Fig 2.3). The His residue on AHP4 aligns at the same position as the HPts indicating it is potentially accessible for phosphorylation (i.e., His is not positioned at a region which could be blocked by the folding of the protein). However, the change in His orientation seen on AHP4 suggests that it might not be optimally positioned for the phosphorylation to happen. If AHP4 cannot accept the phosphate, then like AHP6, it could act as a negative regulator of phosphorelay via competitive inhibition.

Prediction modelling of the protein structure suggest AHP4 might be a negative regulator like AHP6 but instead of lacking the His residue all together, it has the His in such orientation making it suboptimal for phosphotransfer. To investigate whether the protein interacts positively or negatively (i.e., accepting the phosphate or not accepting it respectively) in the TCS, *in-vitro* phosphotransfer assays are needed, similar to what was done for AHP6 and OsPHPs when they were examined for their phosphor-accepting ability (Mähönen *et al.*, 2006; Vaughan-Hirsch *et al.*, 2021). All *Arabidopsis* HPt and PHP were expressed in bacteria cells and successfully purified to high quality (Fig 2.5 and 2.6) but due to time constrains the phosphotranfer assay was not completed. Completion of this experiment would provide key evidence to the role of AHP4 in CK signalling and therefore it is essential. Since all the materials needed for the completion of this assay are ready, it is only a matter of time.

Identification of the actual role of AHP4 in CK signalling is hard to predict without having the data from the phosphotransfer assays. However, a possible scenario is that AHP4 might be a protein with a conditional action. In other words, AHP4 might not participate in the phosphorelay like the other HPts because of the orientation of the His residue normally. This would mean that at optimal conditions the protein is inactive. But under certain conditions, such that alter the biochemical environment within the cell (i.e., temperature or pH change) AHP4 structure might change to a degree where the orientation of the His shifts back to optimal position (i.e., the His orientation of HPts). Such scenario is possible. For example, in my later work I show that AHP4 affects phloem transport (Fig 4.11), and phloem transport efficiency is dependent on the pH which changes in drought stress conditions (Giaquinta, 1977; Wilkinson and Davies, 1997; Geilfus, 2017).

I then explored the role of AHP4 genetically, by looking at CK related phenotypes and CK sensitivity in an *ahp4* mutant. To do this, I first had to generate new independent *ahp4* alleles using the CRISPR-Cas technology enable the investigation of a number of these CK responses in both the root and the shoot with greater confidence (Chapter 3, Section 3.4). As CK regulates many aspects of

plant growth and development, I selected root inhibition response, chlorophyll retention, seed size, and inflorescence physiology as good readouts to measure CK response in *ahp4* plants because CK perception is well implicated in regulating those responses. Furthermore, transcriptomic analysis between WT and *ahp4* plants performed to supplement the physiological responses to CK. Through the comparison of differentially expressed genes in *ahp4* plants, I would be able to link AHP4 to its function and determine its role in CK signalling pathway.

Phenotypically, *ahp4* plants had subtle changes at several exogenously supplied CK concentrations (Fig 3.5-3.7), while *ahp6* plants responded more prominently to CK. These changes were small and hard to interpret. I later showed that AHP4 was expressed only in the phloem cells, and as such looking for changes in CK sensitivity at the whole plant level was too broad. The same is probably true for the differences not seen at the transcriptome analysis of *ahp4* plants regarding CK responses. Even though transcriptomic comparisons can show the slightest change in the transcriptome pool, in this case it was not possible to do so because the expression of *AHP4*, being so restricted, is probably resulting in changes seen only in a small subset of cells. By using the whole organ as tissue, any changes we would have seen had we taken a more specified area (i.e., root sections in maturation and elongation zones) they were masked.

To explore whether like AHP6, AHP4 could be a negative regulator of cytokinin I explored the phylogenetic relationship of these two proteins in dicot species. To do so it was important to first establish whether AHP6 is conserved in dicot species using the gene loss trees. This analysis indicated that AHP6 is conserved among the dicots in a similar manner that AHP4 is. Furthermore, phylogenetic analysis including some of the dicot species indicated that AHP4 and AHP4-like proteins clade away from the AHP6 and AHP6-like proteins of those dicot plant species (Fig 2.1). Collectively, from these two analyses it is fair to conclude that like most components of the CK signalling pathway, both AHP6 and AHP4 are conserved in angiosperms suggesting a conserved function of those proteins but this function is not necessarily the same for the two proteins.

To establish whether AHP4 and AHP6 have redundant function, CK responses on several assays, mentioned above, were also performed for *ahp4 ahp6* plants (Fig 3.5-3.7). The redundancy of the two proteins could have been the reason as to why differences were not observed in *ahp4* plants for any of the CK responses tested. However, such differenced in CK responses were not detected in *ahp4 ahp6* plants either. Additionally, AHP4 protein cannot substitute the function of AHP6, as demonstrated by the inability of ectopically expressed AHP4 driven by AHP6 promoter to rescue the fragmented protoxylem formation in the roots of *ahp6* plants (Fig 2.8).

Taking into consideration the limited AHP4 expression in the phloem, together with the results of these assays, it can be inferred that AHP4 and AHP6 do not act redundantly in modulating CK responses. Additionally, these assays suggest minimal role of AHP4 in the CK signalling cascade. But because AHP4 has such a limited expression pattern it is difficult to know the true effect of its disruption in the mutant plants just by looking global CK responses.

Overall, I have shown that AHP4 forms a functionally conserved clade of genes. Through observing the predicted structure, I present a possible mechanism of how the protein might act differently from the AHPs. At the same time, through its inability to trans-complement AHP6, I show that it unlikely to act as a strong negative regulator of CK signalling the same manner as AHP6. Additionally, genetic studies show that it doesn't act redundantly with AHP6. These suggest that AHP4, just like the OsPHPs, might be acting through a different mechanism to inhibit CK signalling. However, all the assays done to investigate CK responses, including the transcriptomics experiment did not show any involvement of AHP4 in modulating CK signalling responses. This can mean two things. First possibility is as mentioned before, i.e., the expression of AHP4 is restricted to so few cells that any effect is diluted by the whole organ investigations. Lastly, the second possibility is that AHP4 has no role in CK signalling and that all AHP4-like proteins in dicots have diverged from the HPts of CK signalling so much so to retain a function not indented from participating in the TCS.

To obtain a resolution to the question whether AHP4 has any role in the TCS, the phosphotransfer assays would play an instrumental role. Aside from this assay however, strengthening these findings would also be the investigation whether the TCS reporter lines (TCS:GFP) show disruption changes in their expression patterns when expressed in *ahp4* backgrounds. In fact, such lines have been developed and future experiments are in place. This would enable the monitoring of CK signalling (through tracking the TCS::GFP) specifically in the phloem, where AHP4 is expressed. By doing so, I will be able to measure the precise effect on CK signalling as a result of *AHP4* disruption and therefore determine whether the protein is affecting CK signalling and how (negatively or positively). This method ensures higher resolution than any of the physiological responses to CK measured so far, including the transcriptomics experiments because it is possible to localise the signal of TCS and measure it only in the affected cells (i.e., phloem companion cells).

The role of AHP4 in modulates stress responses in the root

Phylogenetic analysis and gene loss genes suggest that AHP4 is conserved in angiosperms. This means that they most likely retain a function. Whether this function is associated with TCS system remains to be determined. However, in this thesis I present for the first time a function for the AHP4 which has not been documented before about any of the HPts.

Transcripts of *AHP4* are expressed in the phloem companion cells (Fig. 4.2 and 4.3). Based on previously published work, meta-analysis of the microarray data found that AHP4 is the only protein in its family upregulated by conditions causing abiotic stress (Singh *et al.*, 2015). Indeed, I showed that transcriptionally *AHP4* is induced when plants are subjected to high osmotic environment (Fig 4.6 and 4.11F). I propose that AHP4 indirectly maintains cell division at the growth tip under drought conditions. I observed that in the absence of AHP4 (i.e., in *ahp4* plants) the primary root stops growing upon contact to high osmotic environments (Fig 4.7) and that the root meristem terminates in those seedlings (Fig 4.10). I also

demonstrated that the termination of the root meristem is because of disruption in the phloem transport. External application of sucrose rescued the *ahp4* root tip phenotype, and phloem transport was inhibited in *ahp4* plants under osmotic stress (Fig 4.9-4.11). Together these suggest that AHP4 is important for the maintenance of primary root growth when water availability is limited by inducing the flow of photosynthetic assimilates in the root tip. This ensures the adaptability of the plant to drought stress by preserving the primary root growth to continue its downward journey reaching deeper soil levels where there is possibly more water available.

Additionally, the changes observed in LR development in *ahp4* plants grown under high osmotic conditions (i.e., these plants had longer LRs; Fig. 4.8) supports the idea that AHP4 promotes drought tolerance by modulating changes in root architecture. In fact, in maize field trials, *ZmHP4* (orthologue to *AHP4*) found to be an important QTL in regulating LR length under water stress. While, just like in the case of AHP4 in *Arabidopsis*, maize *ZmHP4* had no effect under well-watered conditions (Schneider *et al.*, 2020). This, along with the phylogenetic analyses, supports the idea that AHP4 has a conserved function between angiosperms.

Interestingly, the above field trial looked at roots dug out of the soil and therefore only a fraction of the root system investigated. In *Arabidopsis*, AHP4 influenced primary root growth under water limiting conditions. However, primary roots of maize reach to deeper layers in the soil and were not considered in the study mentioned above (Schneider *et al.*, 2020). Therefore, it is hard to know if ZmHP4 functions the same way as in *Arabidopsis*, but indications from the effect it has on LR length in high osmotic environments suggest that indeed the function is conserved.

In fact, experiments of drought performed for this project showed no effects on biomass change in *ahp4* plants (Fig 4.4). However, such changes would be expected to be observable if the root growth is affected. Just like it is affected when plants grow on high osmotic stress (Fig 4.7). Primary root growth of plants was not examined here as it is difficult to separate *Arabidopsis* roots from the soil

due to them being fragile. However, assuming drought had the same effect on primary root growth as osmotic stress, then LRs (being longer in *ahp4* plants) would have taken over and cover the space primary root would otherwise occupy in the pot. Moreover, for the drought experiment presented here, plants grew in small soil pots. This is another possible reason as to why there was no effect in the biomass in *ahp4* plants. If the effect depends on the depth which the primary root can reach, then in the small pots such effect would not be noticeable as the root reaches the bottom rapidly. To investigate this better, perhaps the plants should be allowed to grow on bigger pots with enough space to travel deeper and the watering should be done from the bottom.

Future work

The work presented here for the completion of this thesis indicate that AHP4 is important to maintain primary root growth in water limiting conditions and this is done by enabling phloem transport. This is a novel function to AHP4, and it has not been shown for any of the other Arabidopsis HPts. While the HPts negatively regulate drought (Nishiyama *et al.*, 2013), their effect on root development through phloem transport remains to be determined. Investigation of such process will firstly inform whether phloem transport is affected by disruptions of CK signalling components. And secondly, the comparison of the effect (if any) will enable an inference on the role of AHP4 in CK signalling pathway. More specifically, if HPts have a negative effect in phloem transport during stress and AHP4 has a positive role, then these proteins are acting antagonistically to regulate phloem transport. This could also imply an antagonistic action in the TCS.

Additional validation of the role AHP4 in TCS would be to investigate the phloem transport with other CK mutants like *CKX* or *IPT* genes. If for example *CKX* mutants exhibit similar defects concerning the transport of sugars in the root tip via phloem, then it would mean that reduced CK signalling is correlated with the phenotype. Extending this, it could be hypothesised that AHP4 (having the same effect on phloem transport) acts as an inhibitor of CK signalling.

Strengthening the hypothesis of AHP4 being a negative regulator of the CK signalling is the outcome of the phosphotransfer assays mentioned above, as well as investigation whether the TCS reporter lines (TCS:GFP) show disruption in their expression patterns when expressed in *ahp4* backgrounds.

I showed in this project that AHP4 cannot substitute the function of AHP6 in modulating the protoxylem development in *ahp6* plants (Fig 2.8). While this shows that AHP4 has a different mechanism of action, it does not indicate whether is negatively affecting CK signalling. Therefore, to investigate whether the disruptions observed in phloem transport are due to negative regulation of CK signalling, AHP6 was expressed ectopically driven by the AHP4 promoter (pAHP4::AHP6) in *ahp4* background. Just like with the TCS:GFP in *ahp4*, these lines have been developed too.

Recently published work identified the rice PHPs as inhibitors in CK signalling (Vaughan-Hirsch *et al.*, 2021). These are proteins closely related to AHP4 (Fig 2.1) and therefore it would be interesting to investigate how these proteins respond to stress. This would firstly suggest a conserved function between AHP4 in dicot and PHPs in monocot species. Secondly, with rice being a valuable economic crop, the identification of genes enabling drought stress tolerance can benefit its yield.

Further investigation on the function of AHP4 includes its relationship with ABA signalling. HPts negatively regulate drought stress both in ABA-dependent and ABA-independent manner (Nishiyama *et al.*, 2013). It would be interesting to see whether AHP4 function is dependent or not by ABA signalling. This could be a point of crosstalk between CK and ABA. Additionally, yeast-two-hybrid screening can reveal interactions of AHP4. Through the identification of protein targets the protein function would be expanded. Lastly, since AHP4 is likely to be involved in the CK signalling cascade which functions through a phosphorelay, an investigation of the phosphoproteomic profile of *ahp4* plants and/or plants overexpressing AHP4

will enable the identification of proteins requiring AHP4 for their post translational activation. This can further our understanding into the processes AHP4 is involved in.

Overall, the ever-changing environmental conditions along with the rising demand in food production, made necessary the adaptability of the plants we cultivate as crops. Abiotic stresses on plants are one of the important contributors to yield losses worldwide. Therefore, efforts to reduce those losses through the production of resilient plants are essential.

Among the many challenges the modern agricultural industry is facing is the limited water availability. Understanding the mechanisms which enable the plants to better adapt to abiotic stresses such as drought and water availability, is of profound importance for promoting the increases in yield without depleting the limited water resources. In this work, I have demonstrated that phloem transport is inhibited in water limiting conditions and that this significantly impacts the primary root growth. Furthermore, there is a direct connection of positive influence of AHP4 and the maintenance of phloem transport under stress conditions. CKs are transported through the vasculature (including phloem) around the plant. While there is no clear indication of AHP4 action in the CK signalling pathway, in this thesis I have presented several evidence to show that it ensures in the maintenance of the phloem transport in water limiting conditions. This, in turn, has positive effects on the viability of the primary root, by enabling the continuation of its development under these stress-inducing conditions. Learning how to harness the molecular mechanisms to sustain sufficient phloem transport when plants are under stress can contribute to more resilient plants. This resilience will come from the direct benefits the proper phloem transport and translocation of sugars have on the root development as demonstrated in this work. Moreover, the benefits of a healthy or enhanced phloem transportation system under stress conditions can have other indirect effects such as enhancing or maintaining mycchoryzal associations and preservation of the root microbiome which provide many benefits to the plant health and yield, especially under challenging conditions.

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Appendix:

Appendix 1: List of primers and their corresponding sequence used in this thesis:

AHP4_full_proF	GGCGGGTCTCCACATTAATGGAAGAAACTTGGAAAA
AHP4_full_proR	TTTTGGTCTCCATGTGTGGAGGTGAGAGGGTGTTGTG
entry_vector_control_F	GTGGAATTGTGAGCGGATAAC
entry_vector_control_R	GTTTTCCCAGTCACGACGTT
AHP4_gen_F	GCAGTCCCTCTTTGATCAGG
AHP4_gen_R	CGGCTGGATAGTTACATGCA
gRNA_1F	ATTGATCATTTGATTTCAATCGGC
gRNA_1R	AAACGCCGATTGAAATCAAATGAT
gRNA_2F	ATTGTATACTTCAAAGATTCAGCT
gRNA_2R	AAACAGCTGAATCTTTGAAGTATA
gRNA_3F	ATTGATATCTCGACGAACAATTCA
gRNA_3R	AAACTGAATTGTTCGTCGAGATAT
pGGZ003conR	GATTTCCGATCCCCGGAATT
pAHP6pcontrolR	GGCACGTATAAGAAGCCTCT
B-dummy_R	GGTACCAGTCGACTGAATAC
B_dummyF	GTATTCAGTCGACTGGTACC
RBCSt_GGconR	TTCTGGTGTGTGCGCAATGA
KanR_conR	CTGACGTATGTGCTTAGCTC
KanR_conF	TTGCCGGTCTTGCGATGATT
RBCSterminatorjuctionF	CCTTGTCAGATTCTAATCATTGC
E30	ACCAGGTCTCGGGCTGCATGCAGAGGCAAGTGGCACT CATCA
E31	AACAGGTCTCTCTGATTACTTGGGCCTACGTGCTGTCTC
D_dummyFnew	CAGGTGGATCCTAGATAACC
pGEX_GST_F	CCAATGTGCCTGGATGCGTTCCC
pGEX_GST_R	GGGCGACACGGAAATGTTGAATA
E16	ACGGGATCCATGGACGCTCTCATTGCTCA
E17	TTAGTCGACTTAGTTAATATCCACTTGAGGAAC
E18	TTAGGATCCATGGACACACTCATTGCTCAGTTA
E19	ACTGTCGACTTATATATCCACTTGAGGGATTC
E20	TAGGGATCCATGAACACCATCGTCGTTGCTC
E21	TTAGTCGACCTAATTTATATCCACTTGAGGA
E24	ACGGGATCCATGCAGAGGCAAGTGGCACTC
E25	ACTGTCGACTTACTTGGGCCTACGTGCTGT
E26	TTAGGATCCATGTTGGGGTTGGGTGTGGACC
E27	ACCGTCGACTTACATTGGATATCTGACTCCT
AHP4_genF	GCAGTCCCTCTTTGATCAGG
AHP4_gen R	CGGCTGGATAGTTACATGCA
SALK	
SALK	
SALK	
E14	TTGACCAAGCTTTGGAAAGAG
E15	CACTTTACTTGCCCCAATGC

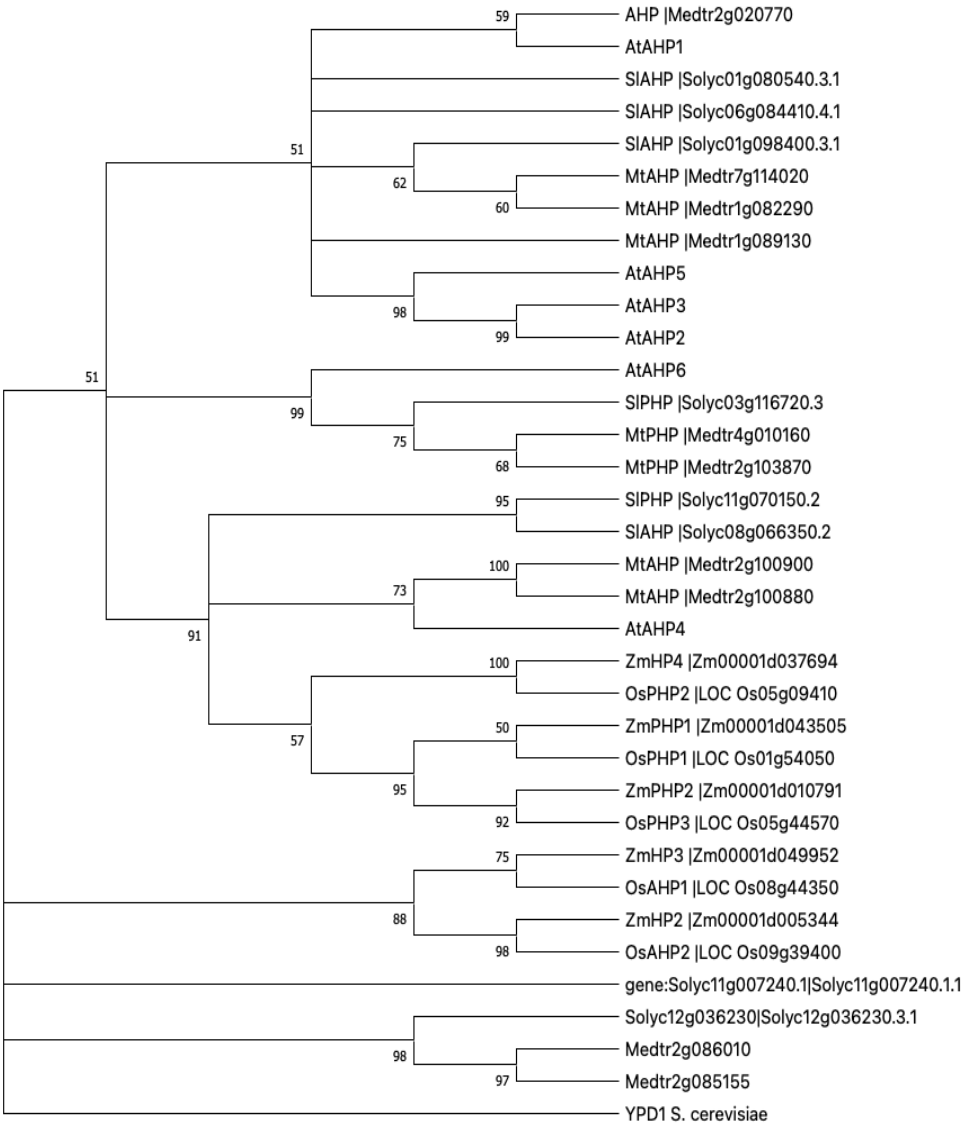
ARR3_qPCR_F	TTGCTTCGAATCACATCCTGC
ARR3_qPCR_R	CGAATTCGACGGCGGCTTTGT
ARR4_qPCR_F	GTCATCGAGAGATTGCTTCGT
ARR4_qPCR_R	ACGCCATCCACTATCTACCG
ARR9_qPCR_F	ACGTTCCTGCAAGAATCAGC
ARR9_qPCR_R	CAGCCAATCTTACTGGTTTCAA
ARR15_qPCR_F	GAGAACATACAACCTCGTATAGAACAA
ARR15_qPCR_R	GCTAATTTCACCGGTTTTAGCA
AHP4_gen_F2	CAAGTGGCACTCATCAAGCA
qAHP4-endF	ACGAGCATTGGGGCAAGTAA
qAHP4-endR	TGCTGGAAAGTCCTCAAGCA
ARR1qPCR_F	TGTTAATCAGCTCGGCGTTG
ARR1qPCR_R	CTCGGGTTAGACCAGGAACA
ARR10qPCR_F	TTCAGCGCTGCCAATATCAC
ARR10qPCR_R	TCAACAACTCCAATGCGGTC
ARR12qPCR_F	TGTTTGGACTGTTGAGCTGC
ARR12qPCR_R	AGGCATAGCCTTCTCATATCCC
ARR2qPCR_F	AGAGAGGATGCTTATGACTTGTCT
ARR2qPCR_R	GAAGCAGAGACAATGCGCTC

Appendix 2: List of gRNAs used in this thesis to generate CRISPR-Cas mutant plants. The top table indicates the sequence on the AHP4 gene next to PAM sites along with the specificity and efficiency of the gRNA binding score. Table below shows the gRNA sequence with the appropriate overhangs for AarI enzyme.

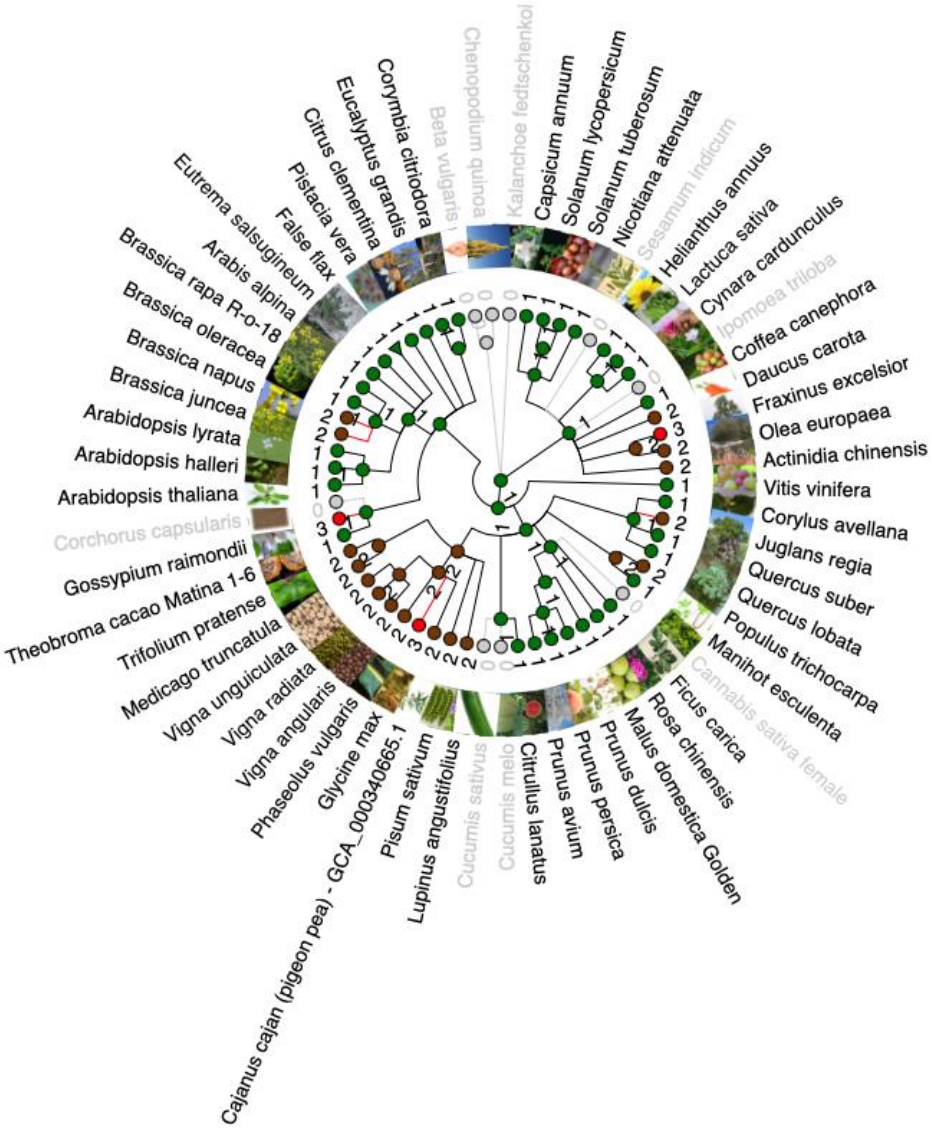
Name	Position	Strand	Sequence	PAM	Specificity Score	Efficiency Score
AHP4_3	5554530	1	ATATCTCGACGAACAATTCA	TGG	95.6229914	45.21850095
AHP4_2	5554610	1	TATACTTCAAAGATTCAGCT	CGG	96.039843	55.22678942
AHP4_1	5554741	1	ATCATTTGATTTCAATCGGC	TGG	99.0582879	41.10834749

Primers

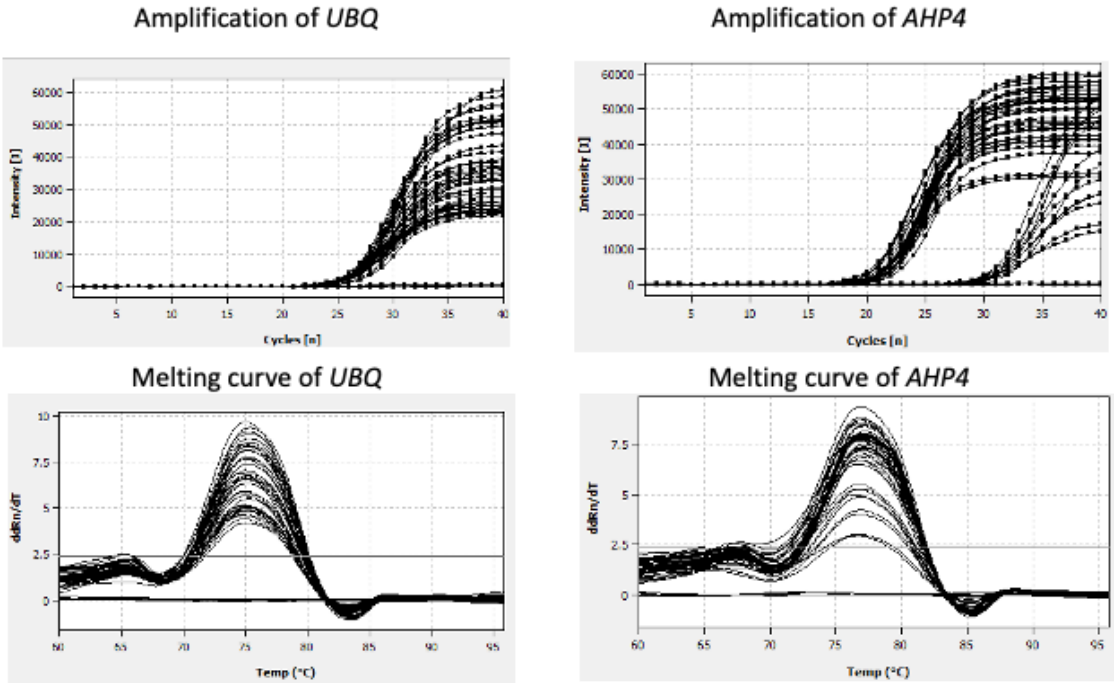
AHP4_1_AarI_F	ATTGATCATTTGATTTCAATCGGC
AHP4_1_R	AAACGCCGATTGAAATCAAATGAT
AHP4_2_AarI_F	ATTGTATACTTCAAAGATTCAGCT
AHP4_2_R	AAACAGCTGAATCTTTGAAGTATA
AHP4_3_AarI_F	ATTGATATCTCGACGAACAATTCA
AHP4_3_R	AAACTGAATTGTTCGTCGAGATAT



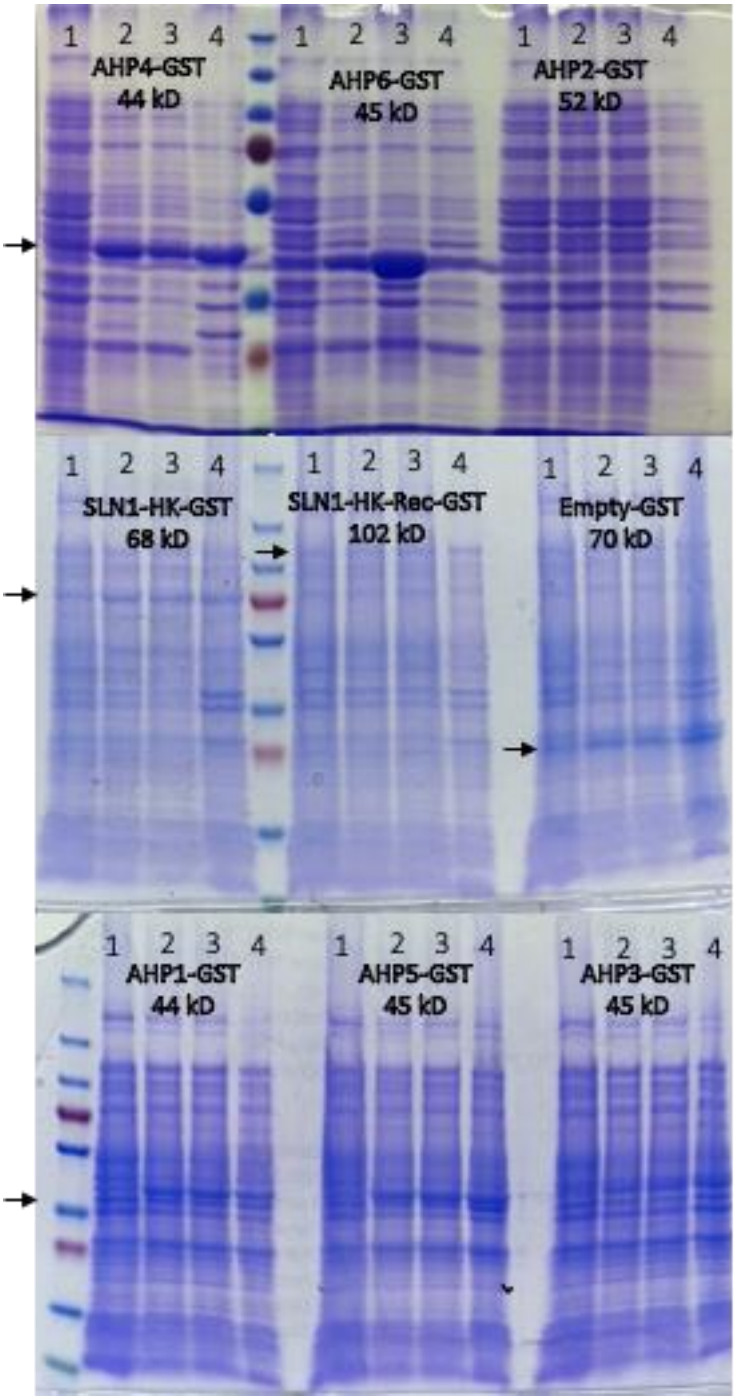
Appendix 3: Phylogenetic tree revealing that AHP4 forms a clade of proteins in dicots species distinct from the monocot PHPs and known HPts. This rooted phylogenetic tree shows the relationship of HPt and PHP proteins from *Medicago truncatula* (green), *Solanum lycopersicum* (pink), *Arabidopsis thaliana* (black), *Zea mays* (yellow), *Oryza sativa* (blue) and *Saccharomyces cerevisiae* (black) as determined by their aminoacid structure. This phylogenetic tree was contracted using the Mega software and numbers on branches represent bootstrap values. Any branches with bootstrap values less than 50 were collapsed to increase confidence of the branches.



Appendix 4: AHP6 is conserved in all dicot species. Gene loss tree demonstrating that AHP6 is conserved in virtually all dicot species. Numbers on the junction of the branches demonstrate the number of homologous gene members for each species. This tree was constructed using *EnsemblPlants* website.



Appendix 5: Amplification and melting curves of *UBQ* and *AHP4* from qRT-PCR. The *UBQ* was used as a reference gene to monitor the relative expression of *AHP4* in WT and overexpressing lines.



Appendix 6: Preliminary experiments to express proteins in the bacteria as described in chapter 5. Proteins which failed to induce in the soluble fraction where solubilised and refoleded as described in chapter 5 and shown in chapter 2. 1: lysate uninduced, 2: lysate induced, 3: soluble fraction, 4: insoluble fraction.